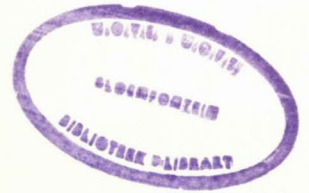


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**A PHYLOGENETIC STUDY
OF SOME SOUTH
AFRICAN
REPRESENTATIVES OF
THE TRIBE ARUNDINEAE**

Rouvay Roodt

**Dissertation presented in order to qualify for the
degree *Magister Scientiae* in the Faculty of
Science (Department of Botany and Genetics:
Division Genetics) at the University of the Orange
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20 May 1999

Supervisor: Prof. J.J. Spies

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To Martin, with love

**“In my opinion, the climax of
flowering-plant evolution is
represented by the grasses,
which, in addition, are the most
useful to man of all families”**

— G. LEDYARD STEBBINS, 1974

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Abbreviations

A	Adenine
AFLP	Amplified Fragment Length Polymorphism
APS	Ammonium Persulphate
BLFU	Geo Potts Herbarium, Department of Botany and Genetics, University of the Orange Free State, Bloemfontein
bp	Basepair
BgII	<i>Bacillus grobigi</i> II
BS	Bootstrap Monophyly Index
C	Cytosine
°C	Degrees Centigrade
CI	Consistency Index
CTAB	Hexadecyl-Trimethyl-Ammonium Bromide
D	Genetic Distance
DAF	DNA Amplification Fingerprinting
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	(Ethylenediamine) Tetra-Acetic Acid
Ethanol	Ethylalcohol
F	Coefficient of Similarity
Fig.	Figure
G	Guanine
g.	Gravitational Force
g	Gram
HCl	Hydrochloric Acid
HI	Homoplasy Index
Hinfl	<i>Haemophilus influenzae</i> RF I
ITS	Internal Transcribed Spacer Region
JMI	Jackknife Monophyly Index
kb	Kilobase

M	Molar
MAAP	Multiple Arbitrary Amplicon Profiling
min.	Minute
mg	Milligram
MgCl₂	Magnesium Chloride
ml	Milliliter
mM	Millimolar
mm	Millimeter
mmol	Millimoles
m/m	Mass per Mass
m/v	Mass per Volume
n	Gametic Chromosome Number
2n	Somatic Chromosome Number
NaCl	Sodium Chloride
nrDNA	Nuclear Ribosomal Deoxyribonucleic Acid
PAUP	Phylogenetic Analysis Using Parsimony
PCR	Polymerase Chain Reaction
pg	Picogram
pp	Pages
pmol	Picomoles
RAPD	Random Amplified Polymorphic DNA
<i>rbcL</i>	Ribulose -1,5- bisphosphate carboxylase large subunit
RC	Rescaled Consistency Index
rDNA	Ribosomal Deoxyribonucleic Acid
RI	Retention Index
RFLP	Restriction Fragment Length Polymorphism
<i>rpoC2</i>	Chloroplast gene coding for RNA polymerase II, β subunit
rRNA	Ribosomal Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
sec.	Seconds
SNL	Signal to Noise
sp.	Species

subsp.	Subspecies
T	Thymine
TAE	Tris-Acetic Acid-EDTA
Taq DNA pol	<i>Thermus aquaticus</i> DNA Polymerase
TEMED	N,N,N',N'- Tetramethyl-Ethylenediamine
Tris	2-Amino-2-(Hydroxymethyl)-1,3-Propanediol
u	Units
μl	Microlitre
UV	Ultraviolet
V	Volt
v/v	Volume per Volume
x	Basic Chromosome Number
%	Percentage

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To my Lord God, for without my faith and His love, nothing would have been possible!!!!!!

CHAPTER 1

INTRODUCTION

1.1 General introduction

The grasses are the most important family on earth, in numbers of individuals, biomass, area covered, diversity of habitats and value to man. Over 30% of the land area of the earth is covered by natural grasslands and savannah vegetation, dominated by grasses (Walter 1979).

According to Watson and Dallwitz (1989) there are about 770 genera and 9700 species of grasses in the world. Although Poaceae is only the fifth largest plant family, in number of species (Watson & Dallwitz 1989), it is ecologically the most dominant and economically by far the most important family in the world (Clayton 1978). The grasses' value to the human race is incalculable, as they effect and support virtually every facet of human existence (Pohl 1978). They provide all the cereal crops, most of the world's sugar and grazing for domestic and wild animals, as well as bamboos, canes and reeds (Clayton 1978). The major part of the land area devoted to crops, is occupied by the great cereals: maize, wheat and rice, with, in marginal climates, smaller tracts devoted to oats, barley, rye and millets (Gibbs Russell *et al.* 1990).

The grasses had apparently begun to diversify before oceans separated the continents. The subfamilies and tribes are fairly uniformly distributed across the continents in broad climatic bands, but the genera, which are of more recent origin, tend to be restricted to a single continent (Clayton 1983).

In southern Africa, the grasses include 194 genera and 967 species and infraspecific taxa, of which 115 are naturalised and 847 are indigenous, including 329 endemic taxa (Gibbs Russell 1985). In the southern African flora, grasses rank second in number of genera and seventh in number of species (Gibbs Russell 1985).

1.2 The subfamily Arundinoideae

Grasses have been classified into five major subfamilies: Arundinoideae Tateoka, Bambusoideae Asch. & Graebn., Chloridoideae Rouy, Panicoideae A. Br., and Pooideae

(Watson *et al.* 1985), with a sixth smaller subfamily, Centothecoideae Soderstrom, sometimes segregated from the Bambusoideae (Clayton & Renvoize 1986).

Arundinoideae is an ancient and somewhat heterogeneous assemblage (Gould 1968). This subfamily is the least sharply defined and specialised of all the grass subfamilies, and lacks reliable diagnostic features. Many features that are taxonomically discriminating in the other subfamilies, vary in this group and, consequently, there is no clearly defined central core group and the subfamily is probably polyphyletic (Ellis 1987). The heterogeneity within the subfamily results from the inclusion of genera (and tribes) that do not fit well in other well-defined subfamilies (Renvoize 1981).

Arundinoideae are typically non-kranz grasses with slender microhairs, cuneate lodicules and arundinoid embryos. Their origin is obscure, links with Bambusoideae and Pooideae being no more than speculative, but they are thought to represent the basic stock from which the tropical savannah grasses evolved (Clayton & Renvoize 1986).

Arundinoideae appear to be descendants of pioneer grasses, for although adapted structurally to open habits, they show little specialisation in their spikelet structure. They are widely distributed, but do not show any physiological adaptations as a group, to specific environments and have mostly retained the apparently primitive C₃ photosynthetic pathway (Renvoize 1981). Grasses of this subfamily are widespread in the world, but the majority is distributed throughout the Southern Hemisphere (Gould 1968). A reason for this could be the climatic isolation from the continuous landmasses to the north (Goldblatt 1978). Arundinoideae have evolved a number of strategies that enable them to spread and survive (Philipson 1978; Connor 1979). Most of the Arundinoideae species are perennial, only a few annuals having evolved (Conert 1987).

As noted by Kellogg and Campbell (1987), there is no single character that unites the subfamily Arundinoideae, or even a large subset of it. When the subfamily was described, Tateoka (1957) identified no less than 17 tribes.

Three competing tribal classifications are acknowledged today. These are the classifications of Clayton and Renvoize (1986), Conert (1987) and Watson (1990).

Following earlier studies by Renvoize (1981, 1986), Clayton and Renvoize (1986) proposed four tribes: Arundineae Dumort., Aristideae C.E.Hubb., Thysanolaeneae C.E. Hubb. and Micraireae Pilger. The last two tribes are monogeneric. The tribe Arundineae is the largest (Table 1.1), and includes genera which are considered by others to belong to Danthonieae Zotov (Barker 1995a). Arundineae are defined by embryo features, non-kranz leaf anatomy (including the presence of slender microhairs) and a generally simple spikelet

Table 1.1 The genera of Arundinoideae (Poaceae) according to Clayton and Renvoize (1986). Generic names followed by A, C and D are included, respectively, in Arundineae, Cortaderieae and Danthoneiae by Conert (1987). *Rytidosperma sensu* Clayton and Renvoize (1986) include *Rytidosperma sensu stricto*, *Karoochloa* and *Merxmullera*, the first two of which are placed in Danthoneiae by Conert (1987), and the latter in Cortaderieae. Conert did not take unmarked genera into consideration.

ARUNDINEAE		
<i>Alloochaete</i> C	<i>Dregeochloa</i> D	<i>Phragmites</i> A
<i>Amphipogon</i>	<i>Elytrophorus</i>	<i>Piptophyllum</i>
<i>Anisopogon</i>	<i>Gynerium</i> A	<i>Plinthanthesis</i> D
<i>Arundo</i> A	<i>Hakonechloa</i> A	<i>Prionanthium</i>
<i>Centropodia</i> D	<i>Lamprothyrsus</i> C	<i>Pseudopentameris</i> D
<i>Chaetobromus</i> D	<i>Leptagrostis</i>	<i>Pyrrhanthera</i> D
<i>Chionochloa</i> C	<i>Monachather</i>	<i>Rytidosperma</i> C, D
<i>Cortaderia</i> C	<i>Molinia</i> A	<i>Schismus</i>
<i>Crinipes</i>	<i>Nematopoa</i>	<i>Spartochloa</i>
<i>Danthonia</i> D	<i>Notochloe</i>	<i>Styppeiochloa</i>
<i>Danthonidium</i>	<i>Pentameris</i>	<i>Tribolium</i>
<i>Dichaetaria</i>	<i>Pentaschistis</i> D	<i>Urochlaena</i>
<i>Diplopogon</i>	<i>Phaenanthoecium</i> D	<i>Zenkeria</i>
THYSANOLAENEAE		
<i>Thysanolaena</i> A		
MICRAIREAE		
<i>Micraira</i>		
ARISTIDEAE		
<i>Aristida</i>	<i>Sartidia</i>	<i>Stipagrostis</i>

structure (Clayton & Renvoize 1986).

This classification of Arundinoideae is still based on spikelet morphology, but as many convergences occur, it is necessary to draw on other characters as well, such as the habitat of the plants, features of the leaf sheaths, leaf anatomy, ecology, chorology, breeding systems and cytotaxonomy (Conert 1987).

The following tribal definition is based on the basis of breeding systems, and Conert (1987) outlined three tribes: Arundineae, Danthonieae and Cortaderieae (Zotov) Conert (Table 1.1). The latter tribe is unique in this classification and is considered by Conert (1987) to be the youngest and the most modern in the subfamily.

Zotov (1963) described Cortaderieae and classified the tribe as being phylogenetically between Arundineae and Danthonieae. The second tribe, Danthonieae, comprises many genera, most of which are of Gondwanean distribution. The third tribe is considered to be very ancient and, in support of this, Conert cites the small number of species in each genus in this group, and the fact that so many are pandemic (Conert 1987).

In 1976, Watson and Clifford placed representatives of the subfamily Arundinoideae in four informal groups: Aristideae, Stipeae Dumort., 'arundinoids' and 'danthonoids'. In a recent review of the classification of the family, Watson (1990) recognised 11 tribes within the subfamily Arundinoideae: Stipeae, Steyermarkochloae Davidse & Ellis, Nardeae Koch, Lygeae Lang, Arundineae, Danthonieae, Spartochloae Tateoka, Cyperochloae Tateoka, Micraireae, Aristideae and Eriachne Ohwi.

The classification of Watson divides the subfamily into numerous smaller tribes on the basis of phenetic similarity (Watson 1990).

Barker (1995a) used data from chloroplast gene sequences, *rpoC2* and *rbcL*, to elucidate relationships among the genera and tribal lineages of the subfamily Arundinoideae. The variable grass-specific region within the *rpoC2* gene, was used to show relationships between genera and tribes, and the more conserved *rbcL* gene was used to determine the tribal and subfamilial relationships of the major lineages in the family (Barker 1995a). Owing to the interdependence of the plastid data sets the analysis of the combined data sets was recommended (De Queiroz 1993). Recognition, in the past, by some taxonomists (eg. Watson 1990) of Danthonieae and Arundineae as separate tribes, was supported by both the *rpoC2* and *rbcL* phylogenies (Barker 1995a). The relationships of the tribe Aristideae to Danthonieae and Chloridoideae remained unresolved. On the basis of the various analyses of the molecular data, both separately and in combination, the subfamily Arundinoideae was shown to be polyphyletic. None of the previous

classification systems corresponded with the results found by Barker (1995a). He proposed a new classification in which Panicoideae and Bambusoideae would not be changed from the classification of Clayton and Renvoize (1986). Pooideae would include the Stipeae, to which the previously danthonoid genus *Anisopogon* was tentatively added. New subfamilies and tribes and changes to existing subfamilies that would be required, were as follows (changes given in bold) (Barker 1995a):

Subfamily Centothecoideae (emend)

Tribe Centotheceae

Tribe Thysanolaeneae (tribe nov.)

Subfamily Chloridoideae (emend)

Tribe Pappophoreae

Tribe Orcuttieae

Tribe Eragrostideae

Tribe Leptureae

Tribe Cynodonteae

Tribe Centropodieae (trib. nov.)

Subfamily Aristidoideae (subfam. nov.)

Tribe Aristideae

Subfamily Danthonioideae (subfam. nov.)

Tribe Danthonieae (emend)

Subfamily Arundinoideae (emend)

Tribe Arundineae (emend)

Tribe Phragmiteae (trib. nov.)

In this study the classification of Clayton and Renvoize (1986) will be used. This classification provides a broad definition for the tribe Arundineae, which encompasses most of the genera in the subfamily (Fig. 1.1). Many of the genera included in this tribe were previously placed in the tribe Danthonieae, but as no convincing boundaries could be drawn between the danthonoid genera and the reedlike grasses *Arundo* L., *Phragmites* Adans. and *Cortaderia* Stapf, they have been amalgamated into a single tribe (Renvoize 1981).

This classification is, as noted earlier, based on embryological features, non-kranz leaf anatomy (including the presence of slender microhairs) and a generally simple spikelet structure and is, therefore, a broadly anatomical and morphological classification (Clayton

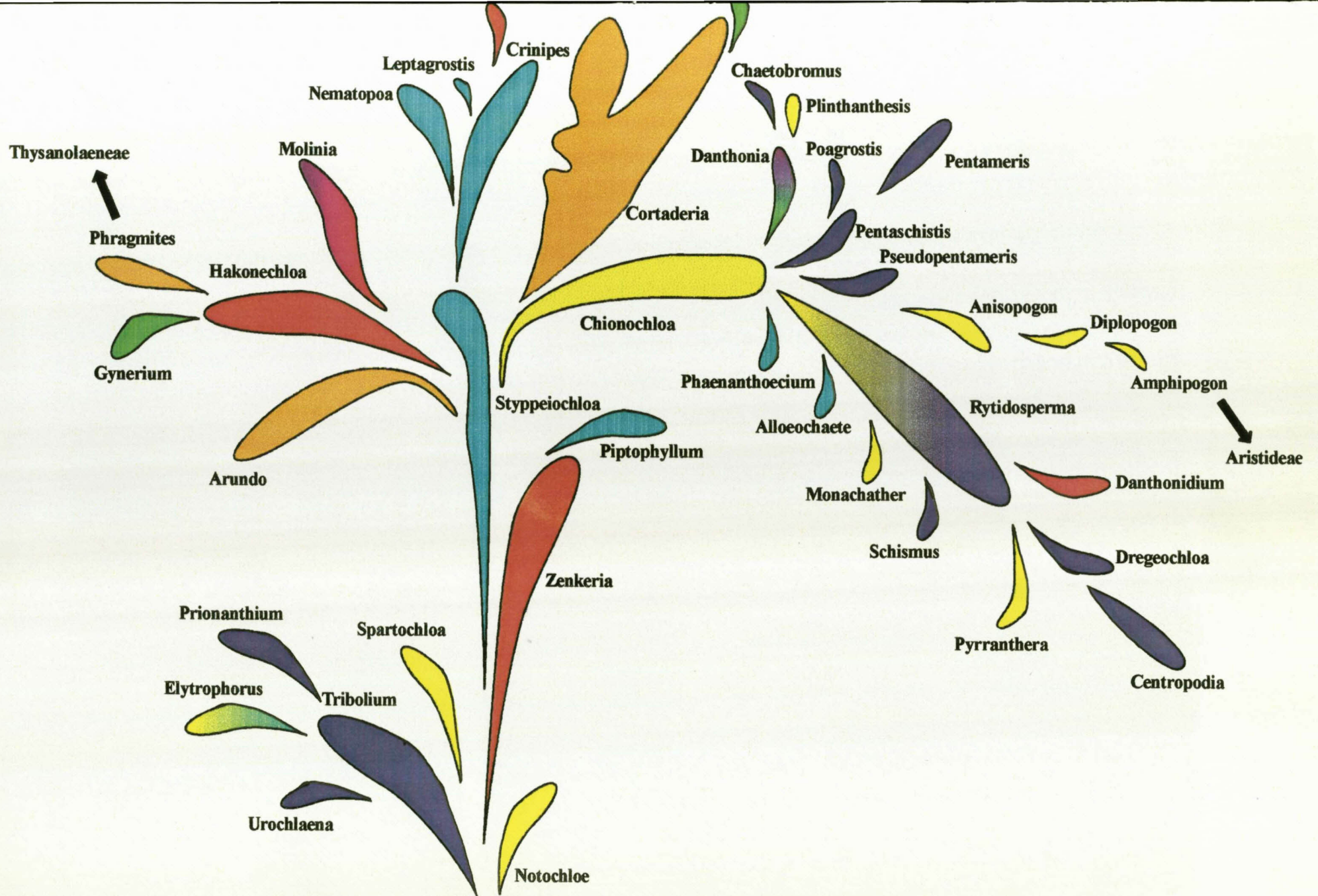


Figure 1.1 Diagram of the relationships in Arundineae (Clayton & Renvoize 1986). South Africa ■ Australia and New Zealand ■ Asia ■ South America ■
 Africa ■ Cosmopolitan ■ Europe ■

& Renvoize 1986). This tribe is otherwise difficult to characterise for it is heterogeneous with numerous isolated or weakly linked genera, whose relationships are highly conjectural. It is also difficult to categorise any of the features as primitive or advanced, and hence to infer the direction evolution has taken (Clayton & Renvoize 1986). However, this classification does not correspond with criteria such as geographical distribution (Fig. 1.1), chromosome numbers (Fig. 1.2), and anatomical features (Fig. 1.3) found in these genera.

1.3 South African representatives of the subfamily Arundinoideae

The southern and south-western parts of the Cape Province of South Africa possess a distinct floristic region, the Cape flora (Good 1964; Taylor 1978). Goldblatt (1978) delimited the geographical area of this flora and called it the Cape floristic region. The major vegetation type here is fynbos.

Bond and Goldblatt (1984) listed almost 200 species of the family Poaceae for this region. Of these 200 species almost all the endemic species belong to the subfamily Arundinoideae (Linder & Ellis 1990a). Arundinoids have a wide range in habits, from annuals to reeds like *Phragmites* (Renvoize 1981). Therefore, it is not unexpected that arundinoids have developed specialised habitats to cope with the Cape fynbos and the variety of niches in the Cape vegetation, with various structural and morphological adaptations which allow them to survive (Linder & Ellis 1990a).

South African representatives of the tribe, some of which will be investigated in this study, are the following:

1.3.1 *Arundo* L.

Arundo (Spanish Reed or Giant Reed) is a pandemic genus. This is a robust perennial (mostly reeds with long canes) with a creeping, knotty rhizome. The leaf blades are linear lanceolate, up to 70 mm wide, expanded and rounded at the base tapering towards a long fine point, glabrous, smooth or glaucous. The inflorescences are paniculate (plumose) and open. The panicle is 30-60 cm long, contracted, dense, silkily haired and cream-coloured or brown (Chippindall 1955; Gibbs Russell *et al.* 1990).

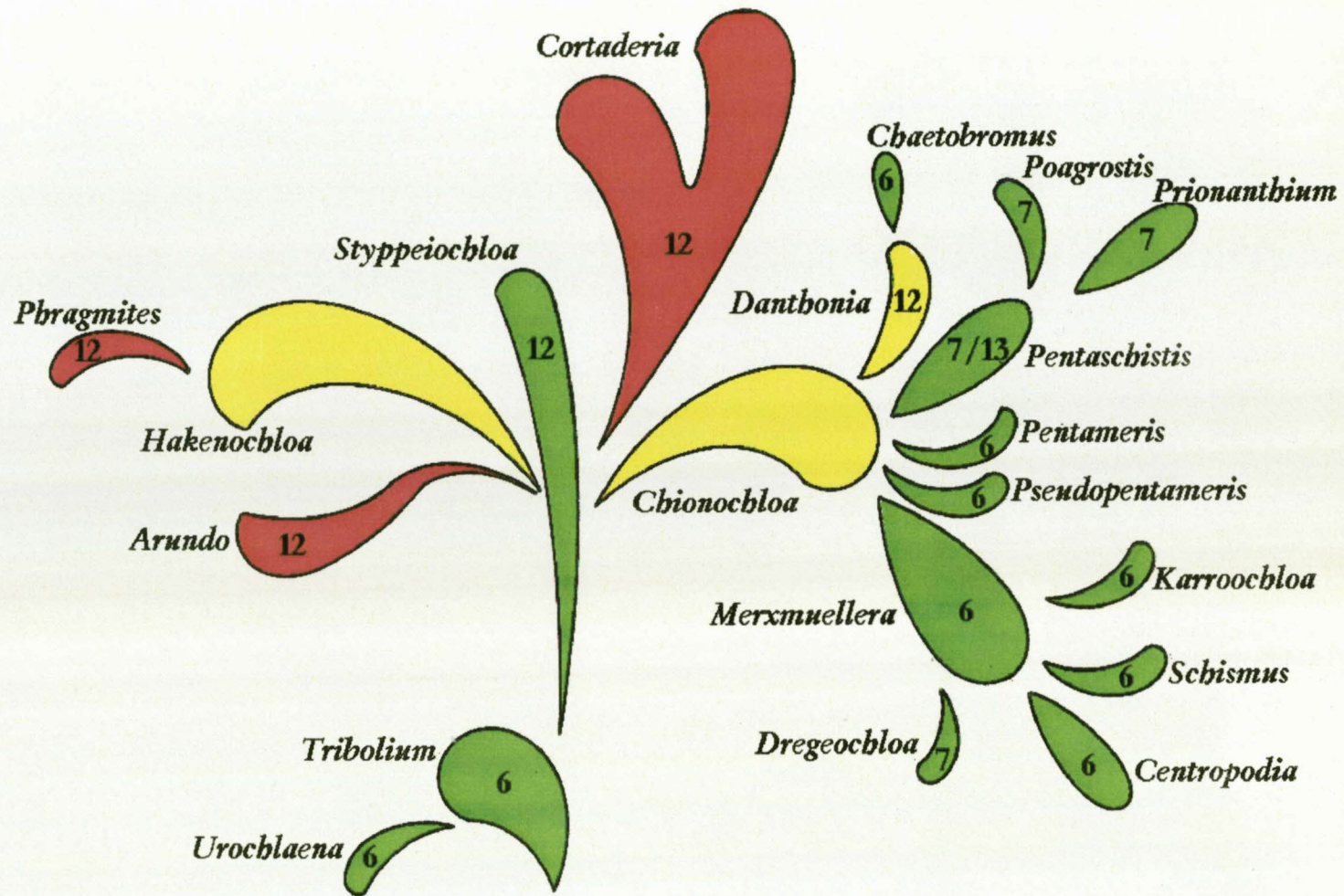


Figure 2.1 Suggested relationships among the South African Arundinoideae, and related genera in the subfamily based on basic chromosome numbers (Modified by Spies from Clayton & Renvoize 1986). ■ South Africa ■ Worldwide distribution ■ Australia and New Zealand

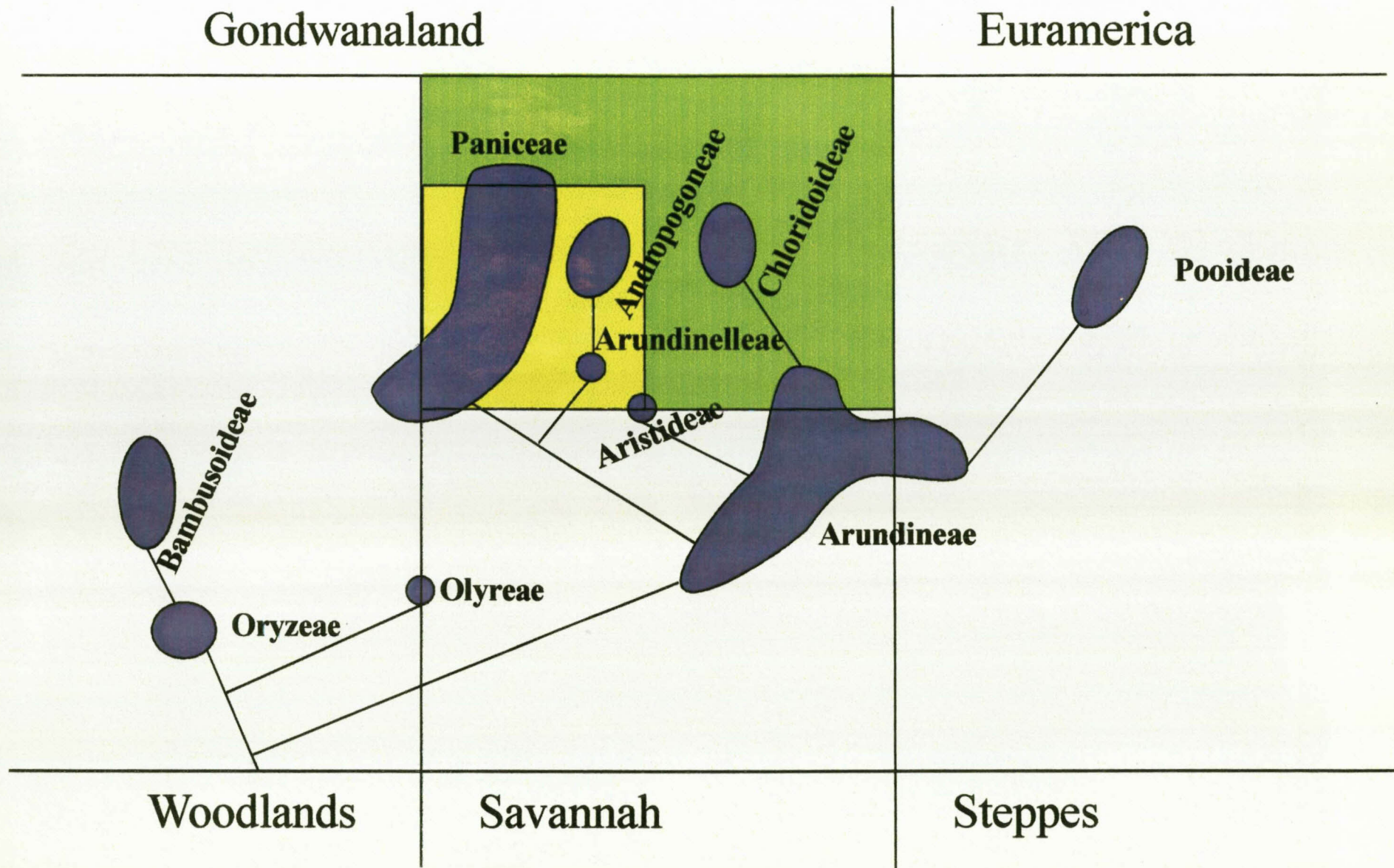


Figure 1.3 Suggested relationships among the major groups of grasses, based on anatomical features (Clayton 1981).

□ C3 photosynthesis ■ C4 photosynthesis (MS type) ■ C4 photosynthesis (PS type)

This is an introduced reed occurring in cultivation or as a stray from cultivation. *Arundo* grows on riverbanks and in other wet places (Chippindall 1955). In Europe the culms are used for making the reeds of musical instruments. *Arundo donax* L. var. *versicolor* (Miller) Stokes is especially popular in South African gardens and parks and is frequently cultivated for ornamental purposes (Chippindall 1955).

1.3.2 *Centropodia* Reichenb.

The genus *Centropodia* consists of four species of which two are indigenous to South Africa. This genus consists of plants that are annual or perennial with glaucous stems and leaves. The leaf blades are linear lanceolate and flat or rolled (convolute). The inflorescences are paniculate and contracted (Gibbs Russell *et al.* 1990).

The distribution of this genus includes Namibia and Angola (Cope 1983).

Centropodia was originally described as belonging to the genus *Danthonia* D.C. In 1934 Nevski separated it from *Danthonia* as *Asthenatherum* Nevski. The most meaningful diagnostic characteristic, which encouraged the first separation, is the presence of radiate chlorenchyma that no other *Danthonia* species possesses and constitutes the most important justification for the separation (Ellis 1984). However, in the meantime a cryptic reference, in a listing of generic names by Reichenbach [*Conspectus Regni Vegetabilis*: 221a (1828)], had gone unnoticed. Thus, the neglected but valid name *Centropodia* Reichenbach predates *Asthenatherum* and, consequently, replaced it, with the combinations *Centropodia forskalii* (Vahl) T.A.Cope, *C. fragilis* (Giunet & Sauvage) T.A.Cope, *C. glauca* (Nees) T.A.Cope and *C. mossamedensis* (Rendle) T.A.Cope made (Cope 1983).

1.3.3 *Chaetobromus* Nees

Chaetobromus is a small genus in which four closely allied species was described by Ellis (1988b). Clayton and Renvoize (1986) recognised only three specific names. These were *C. dregeanus* Nees, *C. involubrates* (Schrad.) Nees and *C. schraderi* Stapf. The name *C. schlechteri* Stapf, the fourth species that had been described, has fallen into disuse (Smook & Gibbs Russell 1985). Chippindall (1955) considered it to be indistinct from *C. dregeanus*. Morphological merging and the existence of intermediates rendered the separation of these species difficult (Spies *et al.* 1990). In 1998, Verboom and Linder described *Chaetobromus* as the monotypic genus *C. involubrates*, in which three subspecies are acknowledged. The subspecies are *C. involubrates* (Schrad.) Nees subsp.

involucrates, *C. involucrates* (Schrad.) Nees subsp. *sericeus* (Nees) Verboom and *C. involucrates* (Schrad.) Nees subsp. *dregeanus* (Nees) Verboom.

Chaetobromus species are perennials, sometimes stoloniferous or with culms rooting from the lower nodes, long-rhizomatous, caespitose or decumbent. The leaf blades are usually expanded, more rarely rolled or folded, tapering either shortly or longly to an obtuse rounded apex. The inflorescences are paniculate (rarely racemose, indepauperate plants), usually contracted, sometimes scanty, and consisting of a few spikelets (Chippindall 1955; Gibbs Russell *et al.* 1990).

Chaetobromus is indigenous to southern Africa with the centre of distribution in the Western Cape, Namaqualand and southern Namibia (Ellis 1988b). According to Ellis (1988b) *Chaetobromus* appears to possess an excellent potential as a fodder grass, and with correct management, this grass could help considerably in enhancing the range quality and carrying capacity of the Succulent Karoo.

1.3.4 Cortaderia Stapf

Cortaderia is a perennial genus, caespitose (mostly large, tussocky) with the leaf blades disarticulating from the sheaths (the sheaths disintegrating or rolling). The inflorescences are paniculate or open (Gibbs Russell *et al.* 1990).

Cortaderia selloana (Schult.) Asch. & Graebn. (Pampas grass) is a graceful, perennial dioecious reed. This species is a native from South America and widely cultivated for ornamental purposes in warm climates. This is a well-known plant in South African parks and gardens (Chippindall 1955).

1.3.5 Dregeochloa Conert

The genus *Dregeochloa* was described to accommodate the species *Danthonia pumila* Nees (= *Dregeochloa pumila*) and a later described species *D. calviniensis* Conert (Conert 1966). These two species have certain distinct characteristic spikelet morphology, leaf anatomy and particularly the structure of the mature karyopsis, which indicates that this genus occupies a unique and somewhat isolated position in Danthonieae (Ellis 1977). The genus is perennial, long stoloniferous (sometimes), or caespitose (with short, often creeping rhizomes). The leaf blades are linear, or ovate-lanceolate to ovate, to 3 mm wide, usually folded and not disarticulating. The inflorescences are single racemes, paniculate (of

4-12 spikelets, rarely a reduced, contracted panicle) or contracted (Gibbs Russell *et al.* 1990).

The species *D. pumila* is confined to a small area of Namibia and the northern extreme of the Northern Cape. The second species, *D. calviniensis*, has only been found in the Calvinia region (Conert 1971).

Plants of this genus were previously assigned to the genus *Danthonia* (Chippindall 1955). In addition, these species exhibit characteristic leaf anatomy, which tends to confirm their being placed together in a separate genus, but throws little light on the phylogenetic position of the genus (Ellis 1977). The observations made by Ellis (1977) based solely on leaf anatomy, confirm that these two species closely resemble each other. Their structure is unique amongst Danthonieae and they show little anatomical resemblance to any other South African members of this tribe (Ellis 1977).

1.3.6 *Elytrophorus* P.Beauv.

Elytrophorus species are glabrous, water-loving annuals with culms much branched at the base. The leaf blades are expanded, often overlapping the inflorescence, linear or flat. The inflorescences are false spikes with dense, globose or cylindrical clusters of spikelets on a central reduced axis, the whole forming an interrupted or uninterrupted spike-like panicle (Chippindall 1955; Gibbs Russell *et al.* 1990).

Two species of *Elytrophorus* are present in southern Africa, *E. spicatus* (Willd.) A. Camus and *E. globularis* Hack. This is a genus of unusual small grasses found in tropical Africa, India to south China and Australia, with the centre of distribution apparently in tropical Africa. This genus is, therefore, restricted to the warm tropical areas of the Old World, surrounding the Indian Ocean (Ellis 1986b).

Both species occur in southern Africa, where they are restricted to the tropical northern part of the region. They are water-loving plants and are found exclusively on the edges of rainwater pans, ponds, depressions and in rice fields, particularly on the periphery of these shallow water bodies when moist mud is exposed as the water evaporates and recedes (Ellis 1986b).

The classification of *Elytrophorus* has been the subject of many debates. Some authors consider it as belonging to Chloridoideae, and in 1955, Chippindall placed it in the tribe Eragrostideae of this subfamily. Jacques Felix (1962) isolated the genus in a separate tribe, Elytrophoreae, belonging to his series Arundinoideae. The classification upheld by

most authors is either with *Elytrophorus* assigned to Arundinoideae in the tribe Danthoneae (Clayton 1970; Loxton 1976) or to the tribe Arundineae (Renvoize 1981).

1.3.7 *Karroochloa* Conert & Türpe

Karroochloa consist of four species, two perennials and two annuals. The species are caespitose. The leaf blades are linear, up to 2 mm wide, flat, folded or rolled, and not disarticulating. The inflorescences are paniculate and contracted (10-60 mm long) and more or less ovoid (Gibbs Russell *et al.* 1990).

The perennials *K. curva* (Nees) Conert & Türpe and *K. purpurea* (L.f.) Conert & Türpe are adapted to specific environments (Conert 1971). *Karroochloa curva* grows on the lower levels of the south-western Cape Mountains, never exceeding 600 meters above sealevel. *Karroochloa purpurea* occurs in mountainous habitats at altitudes of between 2000 and 2300 meters. However, the two annuals, *K. schismoides* (Stapf ex Conert) Conert & Türpe and *K. tenella* (Nees) Conert & Türpe, are widely distributed (Conert 1971).

This genus was previously grouped with *Danthonia* but described by Conert and Türpe (1969) as the new genus *Karroochloa*.

1.3.8 *Merxmuellera* Conert

This genus consists of perennials that are caespitose. The leaf blades are linear, 4-15 mm wide and nearly always rolled. The inflorescence is a single raceme up to 60 mm long (rarely in *M. disticha*) or paniculate and contracted (narrow, occasionally spike-like; usually longer than 60 mm, in contrast with *Karroochloa*) (Gibbs Russell *et al.* 1990).

At present 20 species are recognised in the genus *Merxmuellera*, two of which are only known from the mountains of Madagascar (Barker 1994).

This is the largest and most interesting group amongst the species previously lumped into *Danthonia*. Several characteristics, for example many morphological and anatomical features, have now shown convincingly that *Merxmuellera* is distinct from *Danthonia*, and that, as a matter of fact, it is not even related to it (Conert 1971).

1.3.9 *Pentameris* P.Beauv.

Pentameris is a genus of nine species endemic to the south-western regions of the Cape Province (Barker 1993). The plants are tufted perennials, often robust and with woolly bases. The leaf blades are linear to linear-lanceolate, hard and rigid or wiry, often

strongly curled and usually tightly rolled at an early stage. The inflorescences are paniculate, open or constricted (sometimes scanty) and non-digitate (branching sometimes trichotomous) (Chippindall 1955; Gibbs Russell *et al.* 1990).

The genus *Pentameris* occurs in the winter rainfall region of the Cape Province, South Africa, where it is restricted to soils derived from Table Mountain sand stone or the shale bands associated with this geology (Barker 1993). Consequently, it may be considered an endemic genus of the Cape flora (Goldblatt 1978).

Only one species is mentioned under the generic description: *Pentameris thuarii* Beauv. This single species was placed in *Danthonia* by a number of early taxonomists (Nees 1841; Steudel 1855; Durand & Schinz 1895), whereas others retained it in the genus *Pentameris* (Roemer & Schultes 1817; Kunth 1833, 1835). Stapf (1900) expanded the genus to include four other taxa. Gibbs Russell *et al.* (1985) lists these five taxa as *P. dregeana* Stapf, *P. longiglumes* (Nees) Stapf, *P. macrocalycina* (Steud.) Schweick., *P. obtusifolia* (Hochst.) Schweick. and *P. thuarii* Beauv.

1.3.10 *Pentaschistis* (Nees) Spach.

The genus *Pentaschistis*, with its 68 species (Linder & Ellis 1990b) is one of the largest genera in the tribe Danthonieae. It is endemic to Africa with the greatest number of species (58) endemic, or at least indigenous, to South Africa (Linder & Ellis 1990b).

Pentaschistis species are perennials, more rarely annuals, and usually caespitose, of widely varying habitat. The leaves are linear to lanceolate or filliform, often with few to many tubular, stalked or saucer-shaped glands, especially on the veins and margins, rolled (usually) or flat. The inflorescences are panicles (the branches often have glands), open, contracted or spike-like, rarely a raceme (Chippindall 1955; Gibbs Russell *et al.* 1990).

In South Africa most species are restricted to the western and southern coastal regions with a few species in the Drakensberg. Most of the species in this genus are endemic to the Cape floristic region, especially the Fynbos region. There is a marked concentration of species in the winter rainfall areas of the Cape, with a few species being important constituents of mountain grassland (Chippindall 1955).

There are difficulties with the generic delimitation of *Pentaschistis* from *Merxmuellera* and *Pentameris* (Chippindall 1955; Conert 1987; Linder & Ellis 1990b). Linder and Ellis (1990b) classified *Pentaschistis* into six groupings according to their morphological and leaf anatomy. A core group of species is common to both the

morphological and anatomical groupings, illustrating the complementary aspects of these two data sets and, therefore, enhancing the applicability of these groupings. However, these groupings are purely for classification and do not have any phylogenetic significance (Linder & Ellis 1990b).

1.3.11 *Phragmites* Adans.

Phragmites is a pandemic genus with two species occurring in southern Africa, *Phragmites australis* Trin. and *P. mauritanus* Kunth (Barker 1994). *Phragmites australis* (= *P. communis*), the Common Reed or "fluitjiesriet", is the most widely distributed flowering plant in the world (Chippindall 1955).

Phragmites species are robust, aquatic or semi-aquatic perennial reeds with creeping rhizomes. The leaf blades are linear-lanceolate to lanceolate, expanded or rolled in from the margins (convolute). The inflorescences are paniculate. The mature panicle is open (200-600 mm long, plumose and the fertile lemmas are surrounded by long, white silky hairs) or contracted (Chippindall 1955; Gibbs Russell *et al.* 1990).

Phragmites australis is almost cosmopolitan in distribution, growing on riverbanks and in other wet places, except in Polynesia, New Zealand and the oceanic islands. In South Africa it is widely distributed almost throughout the country. *Phragmites mauritanus* has not been recorded as being from the Cape and is more tropical in distribution (Chippindall 1955).

1.3.12 *Prionanthium* Desv.

The genus *Prionanthium* is a small genus of three species, all endemic to the southwestern Cape Province. It includes small, ephemeral or annual plants, which are exceedingly difficult to locate (Ellis 1989). *Prionanthium* is seen as one of the rarest grass genera of southern Africa (Davidse 1988), with one of the species being listed as endangered (Hall & Veldhuis 1985). The genus contains three morphologically distinct species: *Prionanthium dentatum* (L.f.) Henr. (= *P. rigidum* Desv.), *P. ecklonii* (Nees) Stapf and *P. pholioroides* Stapf (Ellis 1989).

Prionanthium species are tufted annuals with leaf blades expanded at first, but soon rolled and tapering to a rounded apex. The inflorescences are single spikes or single spike-like racemes, contracted (30-80 mm long, the axis curved beside each spikelet) (Chippindall 1955; Gibbs Russell *et al.* 1990).

Most authors now agree with the placement of this genus in Arundinoideae, usually in the tribe Arundineae (= Danthonieae) (Ellis 1989). Following Hubbard's (1948) formal recognition of the tribe Danthonieae, Chippindall (1955) was the first to explicitly and exclusively associate *Prionanthium* with arundinoid genera in the modern sense of Clayton and Renvoize (1986).

The relationship of *Prionanthium* to other arundinoid genera has only been explicitly discussed by Clayton and Renvoize (1986), who consider it to be one of the primitive arundinoid genera, along with *Tribolium*, *Urochlaena* Nees, *Elytrophorus*, *Spartochloa* C.E.Hubb., *Notochloe* Domin., *Zenkeria* Trin., *Pitophyllum* C.E.Hubb. and *Styppeiochloa*.

1.3.13 *Pseudopentameris* Conert

Pseudopentameris was described so as to accommodate two species previously placed in *Danthonia*, namely *Pseudopentameris macrantha* (Schrad.) Conert and *P. brachyphylla* (Stapf) Conert (Conert 1971). This new genus was characterised by unusually large spikelets which, although similar to both the spikelets in *Pentameris* and *Danthonia*, differed in having many-nerved glumes. The fruit of these two species also set them apart from the other two genera (Ellis 1985a). In 1995, Barker confirmed the inclusion of the species *Pentameris obtusifolia* into the genus *Pseudopentameris* as *P. obtusifolia* (Hochst) N.P.Barker and described the new species *P. caespitosa* N.P.Barker.

Pseudopentameris species are perennial, caespitose, scandent or sometimes branched. The leaf blades are linear, 25-500 mm long, soft or rigid, open and flat to rolled and rigid. The inflorescences are paniculate, lanceolate and somewhat contracted (Barker 1995b). *Pseudopentameris macrantha* and *P. brachyphylla* are easily distinguished from each other by the pronounced rolling or circling of the lower leaves of *P. brachyphylla* (Ellis 1985a).

Pseudopentameris species are confined to the south-western Cape (Ellis 1985a). *Pseudopentameris brachyphylla* is very rare and occurs mainly in the Hottentots Holland range, along with *P. obtusifolia*. These two species sometimes occurs together with *P. macrantha*. The latter is particularly common in the Cape peninsula, as is the species *P. caespitosa* (Ellis 1985a; Barker 1995b).

De Wet (1956) considered *Pseudopentameris* to be closely related to both *Danthonia* and *Pentameris*, but Conert (1971) is of the opinion that the genus occupies an

isolated position with no obvious relationships to other danthonoid grasses. Renvoize (1981), who includes *Pseudopentameris* in the peripheral genera of Arundineae, confirms this.

1.3.14 *Schismus* P.Beauv.

Schismus species are tufted annuals or weak perennials, caespitose (rarely) or decumbent (low). The leaf blades are linear to linear-lanceolate expanded or rolled, setaceous or glabrous. The inflorescences are contracted or spike-like panicles (Chippindall 1955; Gibbs Russell *et al.* 1990).

The type species, *S. barbatus* (Loefl. ex L.) Thell., grows in southern Africa as well as the Mediterranean region, ranging from the Canary Islands, southern France and Morocco, to the Nile delta in the south and from Arabia to the Caucasus in the north. The closely related *S. arabicus* ranges from the Himalayas to Greece in one direction and from Pakistan to the Nile delta in the other direction (Conert 1971).

Three more species, all perennials, are endemic to South Africa, where they have adapted to extreme environmental conditions. Only the annual *Schismus* species was able to occupy a wide area in South Africa and to migrate from there to the north of the continent along the western coast (Conert 1971; Conert & Türpe 1974).

Although only one of the five species of this genus was originally described as *Danthonia* the whole genus was later removed from *Danthonia* (Conert 1971; Conert & Türpe 1974). The genus is of special importance in connection with some related taxa that were also originally placed in *Danthonia*, namely species on which Conert along with Türpe (1969) based the genus *Karroochloa*.

1.3.15 *Styppeiochloa* de Winter

Styppeiochloa gynoglossa is a monotypic genus from southern Africa. It was first described as *Crinipes gynoglossa* by Goossens (1934) (de Winter 1966). After a reassessment of the generic delimitation in this group was undertaken by Hubbard, the South African species was excluded from *Crinipes* and described as a distinct genus (de Winter 1966).

The species is perennial and densely caespitose (the hard, fibrous basal sheaths forming tough, fire-resistant mats). The leaf blades are linear, to 1 mm wide, setaceous and

rolled (convolute). The inflorescences are paniculate, contracted (scanty, the spikelets appressed to the panicle branches) (Gibbs Russell *et al.* 1990).

The species occurs all along the Drakensberg escarpment from Natal northwards through Swaziland to Mpumalanga (= Eastern Transvaal) and in the eastern districts of Zimbabwe (= Rhodesia) (de Winter 1966). This is one of the few species of South African Arundinoideae absent from the Cape floristic region.

An investigation of the leaf anatomy of *S. gynoglossa* has shown the genus not to belong in Eragrostideae, but it is more closely allied to *Danthonia* and its allies in Danthoneiae (de Winter 1966). So the cripinoid grasses were initially placed in Eragrostideae, presumably because of similarities in the spikelet and lemma structure, but later moved by Jacques Felix (1962) to Arundinoideae.

1.3.16 *Tribolium* Desv.

The genus *Tribolium* is endemic to the winter rainfall region of South Africa. This genus includes variable perennials or annuals, sometimes tufted, long-stoloniferous or long-rhizomatous, with the leaf blades expanded at first, but soon rolled, simple or branched culms and narrow leaf blades. The inflorescences are spikes or spike-like panicles or racemes (Chippindall 1955; Gibbs Russell *et al.* 1990).

This is a typical Cape grass genus (Linder & Davidse 1997). It is largely restricted to the Fynbos and Succulent Karoo biomes (Rutherford & Westfall 1986) with some populations of few, often widespread species, occurring marginally in neighbouring biomes. *Tribolium* is a temperate grass genus (Linder 1989) typical of the grasses of the Cape floristic region.

Tribolium has been divided into a number of different species by different authors: nine (Spies *et al.* 1992), twelve (Visser & Spies 1994c, d, e) and ten (Linder & Davidse 1997), with the genus comprising of three sections according to Visser and Spies (1994c, d, e): *Acutiflorae*, *Tribolium* and *Uniolae*.

Traditionally, this representative of the tribe Arundineae has been divided into two separate genera, i.e., *Lasiochloa* Kunth and *Plagiochloa* Adamson & Sprague (Chippindall 1955). Renvoize (1985) has recently united the two genera under an earlier name *Tribolium*. Clayton and Renvoize (1986) consider *Tribolium* to be an outlier in Arundineae, with at least superficial similarities to genera in Eragrostideae.

Of particular interest in the genus is the distinctive species *T. pusillum* (Nees) H.P.Linder & Davidse. The species is a small annual, characterised by a dense spike-like panicle, embraced by the uppermost leaf sheath (Ellis 1988a). The leaf blades are linear, flat or rolled. The inflorescences are paniculate and deciduous in their entirety as tumbleweeds (Gibbs Russell *et al.* 1990). At maturity the entire inflorescence breaks off, including the peduncle and the uppermost leaf sheath, the sheath of which is expanded and flared out as a wing at maturity (Chippindall 1955).

Tribolium pusillum is endemic to the western mountain Karoo and Succulent Karoo of the Vanrhynsdorp, Nieuwoudtville and Clanwilliam districts of the Cape Province of South Africa (Acocks 1988).

Due to the unusual manner of dispersion of the inflorescences, this species was originally described as the monotypic genus, *Urochlaena* Nees. This genus was initially placed in Eragrostideae (Chippindall 1955), but its relationship is now considered to lie with Arundinoideae. Loxton (1976) and Watson *et al.* (1986) included *Urochlaena* in Danthoneiae and Clayton and Renvoize (1986) place it in Arundineae, in which the tribe Danthoneiae is included. In 1997, Linder and Davidse incorporated the genus into the genus *Tribolium* as the species *T. pusillum* in the section *Tribolium*.

1.4 Cytogenetics

Besides providing fundamental information for the improvement of grass species by breeding, cytogenetical investigations have been initiated to serve as an adjunct to morphological data in studies of the taxonomy and phylogeny of the Poaceae (Pienaar 1955).

A great stimulus to cytogenetical investigations of the grasses, was provided by the growing appreciation of the importance of forage plants. The meiotic behaviour within species and in interspecific and intergeneric hybrids can be investigated, as well as the origin of polyploidy, cytogenetics of polyploids, inheritance and linkage relations (Pienaar 1955).

Any data, which shows differences from species to species, are of taxonomic significance, and thus constitutes part of the evidence that may be used by taxonomists (Stace 1980). Cytogenetics includes studies dealing with observations of chromosomal pairing or meiotic behaviour. Cytotaxonomy refers to the use of these characteristics and others, such as chromosome number and chromosome morphology, as data for classification (Jones & Luchsinger 1987).

Despite certain limitations, cytological investigations are an aid in establishing systematic and phylogenetic relationships among many species and genera and, are of great value when used in conjunction with morphological, geographical and ecological studies. Of special significance to the cytological data are the chromosome numbers, their shape and size (Pienaar 1955).

The value of cytotaxonomic data depends greatly on the group or category under consideration. For more than 70 years, cytogenetics has been an element of great importance in the evaluation of relationships, and in the deduction of phylogenetic sequences, in angiosperms (Raven 1975).

Cytogenetically, the grasses engage in a diversity of behaviour that raises many problems for those attempting to divide them into discrete species. Some 80% of them have a polyploid chromosome number (Clayton 1978), and the occurrence of polyhaploidy has also been demonstrated. Apomictic swarms are not unusual and over 2000 hybrids have been recorded, of which 200 are fertile (Clayton 1978).

In 1931 the first important work on grass cytogenetics appeared on a study done by the Russian cytogeneticist Avdulov. He indicated that the classification of grasses based on the size and number of their chromosomes is very similar to the classification based on histology and anatomy. Both these classification systems are equally different from the classical system based on inflorescence characteristics (Stebbins 1956). Stebbins (1956) suggested that the realignment of the tribes and genera, as proposed by Avdulov (1931), is supported by basically all the characteristics studied and reflect genetic and evolutionary relationships better than the traditional system. Furthermore, this approach revealed a major division between tropical and temperate grasses (Renvoize 1980).

The primary chromosome number for Arundinoideae has been considered to be $x = 12$ (Clayton & Renvoize 1986). However, it is more likely that this is a secondary base number derived by polyploidy, since a number of arundinoid genera are now known with $n = 6$:

1. *Centropodia* (Du Plessis & Spies 1988).
2. *Chaetobromus* (Du Plessis & Spies 1988; Spies & Du Plessis 1988; Spies *et al.* 1990).
3. *Karoochloa* [(as *Danthonia*, De Wet 1954a, 1960); Du Plessis & Spies 1988; Spies & Du Plessis 1988].
4. *Merxmuellera* [(as *Danthonia*, De Wet 1954a, 1960); Du Plessis & Spies 1988; Spies & Du Plessis 1988].

5. *Pentameris* (Barker 1993).
6. *Pseudopentameris* (Barker 1995b).
7. *Schismus* (numerous reports, for example Faruqi & Quirash 1979; Du Plessis & Spies 1988; Spies & Du Plessis 1988).
8. *Tribolium* [(Spies *et al.* 1992; Visser & Spies 1994c, d, e), not $x = 7$ as incorrectly reported by De Wet (1960). (As *Urochlaena*, Spies & Du Plessis 1988; Visser & Spies 1994c, d, e)].

Stebbins (1956) as well as Hunziker and Stebbins (1987) consider $x = 6$ to be the basic chromosome number for Arundinoideae. A less common base number in the subfamily is $x = 7$:

1. *Dregeochloa* (Du Plessis & Spies 1988; Spies & Du Plessis 1988).
2. *Merxmullera* (Du Plessis & Spies 1988; Spies & Du Plessis 1988).
3. *Pentaschistis* (Davidse *et al.* 1986; Du Plessis & Spies 1988; Du Plessis & Spies 1992; Klopper *et al.* 1998; Spies & Du Plessis 1988; Spies *et al.* 1994a).
4. *Prionanthium* (Davidse 1988; Du Plessis & Spies 1988; Spies & Du Plessis 1988).

A basic chromosome number of $x = 13$ also occurs in *Pentaschistis* (Hedberg 1952, 1957; Spies & Du Plessis 1988; Du Plessis & Spies 1992; Klopper *et al.* 1998).

Species delimitation of grasses is difficult, because two processes have blurred many infraspecific boundaries: hybridisation and chromosome doubling or polyploidy (Stebbins 1956). According to Stebbins (1985), more than 80% of the grass taxa have undergone polyploidy sometime during their evolutionary history.

In order to explain the high frequency of polyploidy in the Poaceae and other plant groups, Stebbins (1985) proposed his "secondary contact hypothesis". According to this hypothesis, taxa with "patchy" distributions would offer frequent opportunities for secondary contact and hybridisation between differentiated diploid populations. Following hybridisation, highly adapted gene combinations could be generated. These gene combinations could have been buffered and maintained largely by the effects of polyploidy in the favouring of tetrasomic inheritance and preferential pairing of homologous chromosomes, as opposed to homoeologous chromosomes (Stebbins 1985). Polyploidy can occur in four kinds of numerical series (Stebbins 1985):

1. Multiples of the original low basic chromosome number.
2. Multiples of the secondary basic chromosome number derived from the original numbers by an earlier cycle of polyploidy.
3. Multiples of basic chromosome numbers, which are the lowest in the genus, but were derived from that of a pre-existing genus by a cycle of polyploidy in the remote past.
4. Basic chromosome numbers derived through aneuploidy from secondary basic chromosome numbers (De Wet 1987).

Accessory or B-chromosomes are relatively common in the Poaceae. Grasses with B-chromosomes tend to show an accumulation mechanism in the male, but not the female side (Jones 1975; Murray 1979). The most common accumulation mechanism in the Poaceae is directed nondisjunction at the first pollen grain mitosis (Jones & Rees 1982). Sometimes B-chromosomes are known to influence and regulate the amount of genetic variability within populations, by affecting chiasma frequency and homeologous chromosome associations. B-chromosomes may also affect chiasma formation by altering their distribution. The effects of B-chromosomes upon the distribution of chiasmata could have, in some cases, adaptive significance, especially in some cases of new polyploids (Hunziker & Stebbins 1987).

1.5 Molecular studies

Plant systematists infer relationships among plant groups from a wide variety of biological criteria. These criteria include morphological similarities at both the gross, anatomical and ultrastructural levels, and similarities in respect of plant secondary metabolites, isozymes, and other protein systems (Clegg & Durbin 1990).

In the past decade, a number of workers have used DNA analyses as a basis for systematic studies (for example, Palmer 1987; Ritland & Clegg 1987; Bremer 1988; Clegg & Durbin 1990; Doyle 1993). These investigations strongly support the use of molecular methods (DNA studies) in biosystematic research (Doyle 1993). Molecular studies include the following:

1. Restriction fragment length polymorphism (RFLP) analysis (Danna *et al.* 1973).
2. The sequencing of portions of the DNA molecule (Schuler & Zielinski 1989).

3. Random amplified polymorphic DNA (RAPD) analysis (Williams *et al.* 1990).
4. DNA amplification fingerprinting (DAF) analysis (Caetano-Anollés *et al.* 1991a).
5. Amplified fragment length polymorphism (AFLP) analysis (Vos *et al.* 1995).

DNA amplification fingerprinting and DNA sequencing are molecular studies, which will be used in this study to clarify relationships and phylogeny in Arundinoideae.

1.5.1 DNA Amplification Fingerprinting (DAF)

The degree of relationship between individual organisms can be determined by the degree of how their DNA corresponds. This similarity in DNA can be measured by the variation in length or sequence of DNA segments. However, the identification of these molecular markers requires prior knowledge of DNA sequence, cloned and characterised probes and experimental manipulation (Caetano-Anollés 1993).

A technique that circumvents this problem, that is simple and independent of the amount and the quality of DNA, is the generation of multiple arbitrary amplicon profiling (MAAP) markers (Caetano-Anollés 1994).

In this study MAAP markers have been used to study genetic diversity and phylogenetic and taxonomical relationships. This has been successfully done on, for example, broccoli and cauliflower (Hu & Quiros 1991); *Brassica* (Demeke *et al.* 1992); peanuts (Halward *et al.* 1992); banana (Kaemmar *et al.* 1992); wheat (Vierling & Nguyen 1992); Bermudagrass (Caetano-Anollés *et al.* 1995), to name but a few.

Three MAAP techniques, random amplified polymorphic DNA (RAPD) analysis (Williams *et al.* 1990), arbitrarily primed PCR (AP-PCR) (Welsch & McClelland 1990) and DNA amplification fingerprinting (DAF) (Caetano-Anollés *et al.* 1991a), generate DNA profiles of varying complexity primarily defined by the sequence of the arbitrary primer used to direct amplification.

A fourth technique, selective restriction fragment amplification (SRFA) (Vos *et al.* 1995), also known as AFLP analysis, uses DNA digestion with one or more restriction endonucleases, cassette ligation and PCR amplification to generate multi-banded profiles. These techniques can be successfully used in plant breeding, general fingerprinting, population biology, taxonomy and molecular systematics (Caetano-Anollés 1994).

The nucleotide scanning technique, DAF, uses very short primers, optimally 7-8 bases. Polyacrylamide gel electrophoresis and silver staining usually resolve fragments generated by DAF's, which allows detection of DNA at about 1pg/ μ l (Prabhu & Gresshoff 1994). In general, DAF procedures generate scoreable polymorphisms in the molecular size range 100-800 bp. However, fragments at higher molecular weight (up to 1 800 bp) are also scoreable (Gresshoff 1995).

Some additional tailoring strategies known to increase the generation of polymorphic DNA in DAF analysis are:

1. Amplification with more than one primer (multiplex DAF) (Caetano-Anollés *et al.* 1991a).
2. Endonuclease digestion of template DNA (tecDAF) and amplification products (CAPS) (Caetano-Anollés *et al.* 1993).
3. Arbitrary mini-hairpin oligonucleotide primers (Caetano-Anollés & Gresshoff 1994b).

These tailoring strategies have been useful in those cases where polymorphisms are to be detected between organisms that are closely related, such as near isogenic lines (NILS), or spontaneous or induced mutants (Caetano-Anollés *et al.* 1995).

The amplification of anonymous genomes with arbitrary oligodeoxyribonucleotides has proved a versatile and universal method for detecting polymorphisms for genetic mapping, phylogenetic analysis, population biology and general fingerprinting applications (reviewed in Caetano-Anollés 1993, 1994).

This technique has not, as yet, been used in the fingerprinting of the subfamily Arundinoideae, or even members of the subfamily.

1.5.2 DNA Sequencing

One of the most important technologies that have emerged in molecular biology is that of rapid DNA sequencing (Schuler & Zielinski 1989). Sequencing had, in the past, not been frequently used for systematic purposes in plants, largely because of the time, effort and expense involved (Crawford 1990a).

Until recently most plant systematists reserved DNA sequencing for phylogenetic analysis of taxa too divergent to be easily interpreted by restriction mapping. With recent advances in polymerase chain reaction (PCR) technology, however, DNA sequencing is now inexpensive enough and also easy to use for phylogenetic studies at all taxonomic

levels (Baldwin *et al.* 1995). The primary challenge to using nucleotide characters for lower level phylogenetic studies is the identification of easily amplified and relatively rapidly evolving, but unambiguously alignable, DNA regions that can provide sufficient, suitable variation within a short sequence segment (Baldwin *et al.* 1995).

Different sequence information is available for taxonomic use, for example:

1. Ribosomal RNA sequences placed *Oryza* at the base of a panicoid clade and *Arundinaria* Michaux (Bambusoideae) as a sister to the rest of the family (Hamby & Zimmer 1988, 1992).
2. Sequences of a portion of *rpoC2*, the chloroplast gene for the β subunit of RNA polymerase II, placed *Oryza* with *Ehrharta* Thunb., in accordance with morphological cladograms (Cummings *et al.* 1994).
3. Sequences of a portion of *rpoC2* and *rbcL* helped elucidate some relationship among 73 grass species from all currently recognised subfamilies (Barker 1995a; Barker *et al.* 1995 and in press).
4. Data of the *rbcL* sequences placed *Oryza* as the sister taxon to all other grasses (Chase *et al.* 1993).
5. Variation in the rRNA genes is a useful indicator of genetic diversity in *Eragrostis teff* (Zucc.) Trotter germplasma (Pillay 1997).
6. *ITS* sequence data, along with morphology, provided more resolution than either technique by itself in determining the phylogenetic relationships in *Asarum* (Aristolochiaceae) (Kelly 1998).
7. The entire *ITS* region was used to generate the first phylogeny of *Rubus* based on a large, molecular data set (Alice & Campbell 1999).

The genomic region that has attracted increased attention among those interested in applying nuclear DNA sequencing analysis to lower level phylogenetic questions is the internal transcribed spacer (*ITS*) regions of the 18-26S nuclear ribosomal DNA (nrDNA). This region includes three components: the 5.8S subunit, an evolutionary conserved sequence, and the two spacer regions flanking the gene, i.e. *ITS1* and *ITS2* (Baldwin *et al.* 1995) (Fig. 1.4).

The tandem structure and extremely high copy number of nrDNA (Rogers & Bendich 1987) make it especially easy to detect or clone in the laboratory. More importantly, considerable research indicates that this gene family undergoes rapid concerted evolution (Arnheim *et al.* 1980; Arnheim 1983; Zimmer *et al.* 1980) within and

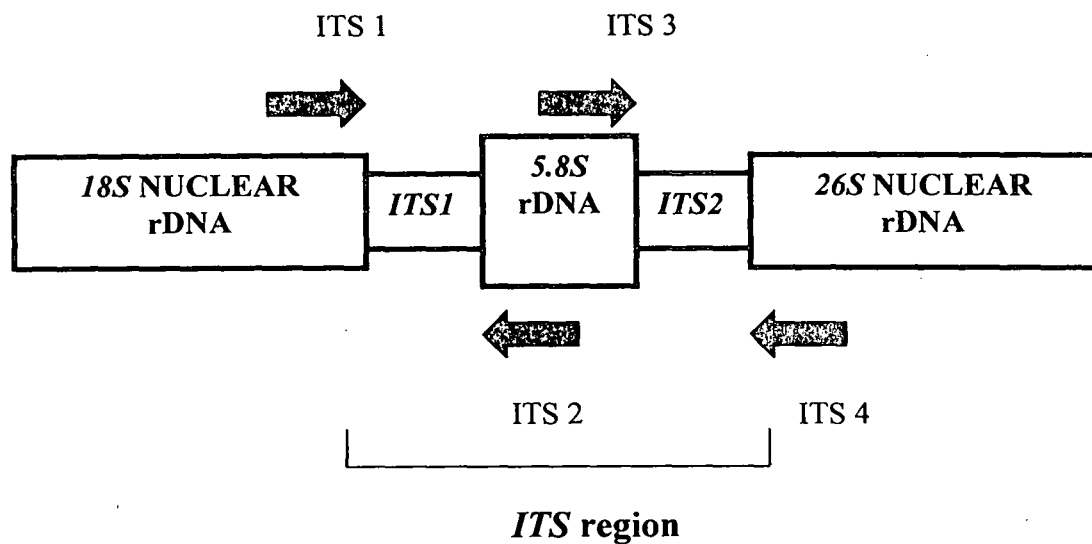


Figure 1.4 Schematic representation of the internal transcribed spacer regions of nuclear ribosomal DNA.

even between loci (Arnheim *et al.* 1980; Arnheim 1983), promoting its usefulness in phylogenetic reconstruction (Sanderson & Doyle 1992).

The reason for this is the effects of paralogous genes (similar genes by means of gene duplication) on phylogeny reconstruction. Duplication of genes followed by divergence usually leads to greater similarity between some members of a multigene family across species, than within the multigene family of the same species (Doyle *et al.* 1992). Therefore the need arises to identify orthologous genes (similar genes derived by speciation), to identify organismal phylogeny and not gene phylogeny. The problem of mixing orthologous and paralogous genes seems to be overcome by the process of concerted evolution, where the members of a multigene family are “homogenised” (Doyle *et al.* 1992). Concerted evolution can produce situations in which the genes in a single species are more closely related to one another than any genes from another species. The 18S – 25S ribosomal RNA cistron, is an example of such a large but homogeneous multigene family in most plants (Doyle *et al.* 1992).

The genes encoding nuclear ribosomal DNA offer several advantages for systematic studies. Their presence, in many copies per genome, minimises the amount of plant material needed. The simplicity of the methods for isolating nrDNA is likewise a

significant consideration for the practising plant systematist, because many individual plants can be examined (Schaal & Learn 1988). The highly conserved nature of the genes encoding *18S* and *25S* nrDNA as contrasted to the highly variable nontranscribed spacer region means that nrDNA can be employed at a wide variety of taxonomic levels (Jorgensen & Cluster 1988).

Although the results of relatively few studies are presently available in which ribosomal genes for systematic and phylogenetic studies were employed, those that have been done attest to their value and potential. These factors combined with the relative simplicity of the method suggest that an ever-increasing number of studies will incorporate length data from nrDNA (Crawford 1990b).

The *ITS* region of the subfamily Arundinoideae has recently been used to investigate the phylogeny of this group (Hsiao *et al.* 1998a). Studies done so far proof this technique to be very useful in the determination of phylogenetic relationships, especially for a difficult group such as the Arundinoideae. The results from the previous study, as well as those obtained in this study, will be used to investigate the relationships within the South African members of the tribe.

1.6 Phylogeny

The broad goals of systematics are phylogenetic reconstruction and elucidation of the evolutionary processes that generate biological diversity. Recent advances in analytical techniques have improved our ability to reconstruct plant phylogeny (Soltis *et al.* 1992), when phylogeny is the evolutionary history of an organism or taxonomic group (Häckal 1866). The species living today are the end products of a long history of evolutionary diversification. The unique pattern of common descent and relationships embodied in that history provide the basis for constructing species phylogenies.

Procedures for constructing phylogenetic hypotheses have been greatly developed by the discipline of phylogenetic systematics, or cladistics (Hennig 1966), which is presently dominating the field of systematics (Hull 1989). In cladistics only shared derived characters (synapomorphies) are used as evidence to support hypotheses about phylogenetic relationships. Similarities due to the retention of primitive characters (symplesiomorphies) are, thus, ignored because in determining relationships, they are uninformative (Miyamoto & Cracraft 1991).

It is the ultimate goal of this study to utilise various techniques to determine the phylogenetic relationships between the South African representatives of the tribe Arundineae. This will be done by implementing cladistic methods for the phylogeny reconstruction.

1.7 Aim of the study

Of the five major subfamilies recognised by the most recent classifications, Arundinoideae is generally considered the most complicated and taxonomically problematic and has been retained only because its members show slightly more overall similarity with each other than with members of any other groups (Watson & Clifford 1976).

There are many views existing around the tribal classification of the subfamily Arundinoideae and many researchers have tried to answer these questions. By further examination of the phylogeny of Arundinoideae, we hope to find answers concerning the tribal classification best suited and if needs be a new tribal classification. It should, however, be kept in mind that this is a genetic study and not a taxonomical investigation.

The three methods being used in this study are cytogenetics, DAF analysis and sequencing of the *ITS* region of the nrDNA. Each of these can provide adequate data for obtaining phylogenetic relationships and, in combination, hope to prove the means by which the phylogeny and classification of Arundinoideae can be reassessed.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Voucher herbarium specimens were collected in the veld and stored in the Geo Potts Herbarium Bloemfontein (BLFU). The plants collected are listed in Table 2.1.

DNA Molecular Marker VI (pBR328 DNA cleaved with a mixture of BgII and HinfI) [Boehringer Mannheim Cat. no. 1062590], *Thermus aquaticus* (Taq) Super Therm DNA polymerase with 10X Buffer (Southern Life Biotechnology LPI-801, LPI-455), DAF primers (Boehringer Mannheim), sequencing primers (DNAgency Cat no. GK 071101, GK 071102) and the Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham Life Sciences, product number US 79765) were used during this study. All other chemicals used during the study were of either analytical or electrophoretic grade.

Data on some of the *ITS* sequences were obtained from Genbank. These taxa were (accession numbers indicated in brackets): *Arundo donax* (AF019809), *Centropodia glauca* (AF019861), *Cortaderia selloana* (AF019812), *Dregeochloa pumila* (AF019853), *Karoochloa purpurea* (AF019874), *Merxmuellera dura* (AF019872), *M. macowanii* (AF019863), *M. rangei* (AF019862), *M. setacea* (AF019867), *M. stricta* (AF019871), *Pentameris macrocalycina* (AF019864), *Pentaschistis aspera* (AF019865), *Phragmites australis* (AF019810), *Prionanthium ecklonii* (AF019866) and *Schismus barbatus* (AF019873).

In this study the Genbank specimens will be referred to without any voucher numbers.

Table 2.1 List of localities and voucher herbarium numbers of specimens investigated in this study. ☼ indicates chromosome studies only, ✱ indicates molecular studies only (DAF analysis and/or DNA sequencing) and ✖ indicates both cytogenetic and molecular studies. Grid references are presented using the degree reference system (Edwards & Leistner 1971).

Arundo

A. donax L.

FREE STATE.—2926 (Bloemfontein): UOFS campus (-AA), *Spies* 6574 ✱.

Centropodia

C. glauca (Nees) T.A.Cope

NORTHERN CAPE.—2816 (Oranjemund): 27 km east of Alexanderbay (-DA), *Spies* 5694 ☼.

Chaetobromus

C. involucrates (Schrad.) Nees subsp. *dregeanus* (Nees) Verboom.

WESTERN CAPE.—3118 (Vanrhynsdorp): 17 km from Doring Bay to Lamberts Bay (-CD), *Spies* 5976 ✱. 3320 (Montagu): Burger's Pass (-CC), *Spies* 6237 ✱.

NORTHERN CAPE.—2917 (Springbok): 78 km from Steinkopf to Port Nolloth (-BA), *Spies* 5691 ☼.

Cortaderia

C. selloana (Schult.) Asch. & Graebn.

FREE STATE.—2926 (Bloemfontein): UOFS campus (-AA), *Spies* 6573 ✱.

Ehrharta

E. capensis Thunb.

WESTERN CAPE.—3218 (Clanwilliam): Versveld Pass (-DC), *Spies* 6095 ✱.

E. villosa Schult. f. var. *villosa*

WESTERN CAPE.—3118 (Vanrhynsdorp): Gifberg (-CB), *Spies 6299* ❄.

Karroochloa

K. purpurea (L.f.) Conert & Türpe

WESTERN CAPE.—3319 (Worcester): FM tower at Matroosberg (-BC), *Spies 6244* ❄. 3320 (Montagu): 48 km from Montagu to Touwsriver (-CD), *Spies 6241* ❄.

EASTERN CAPE.—3027 (Lady Grey): 18 km from Barkly East to Lady Grey (-CD), *Spies 4748* ❄. 3126 (Queenstown): Penhoek Pass (-BC), *Spies 2473* ❄, *Spies 2477* ❄.

K. tenella (Nees) Conert & Türpe

NORTHERN CAPE.—3119 (Calvinia): 35 km from Vanrhynsdorp to Nieuwoudtville (-AC), *Spies 4350* ❄; Vanrhyns Pass (-AC), *Spies 6290* ❄.

Merxmuellera

M. arundinacea (Berg.) Conert

WESTERN CAPE.—3118 (Vanrhynsdorp): Gifberg (-DC), *Spies 4322* ❄. 3218 (Clanwilliam): Pakhuis Pass (-BB), *Spies 6257* ❄.

M. cincta (Nees) Conert

EASTERN CAPE.—3424 (Humansdorp): 16 km from Humansdorp to Cape St. Frances (-BB), *Spies 3504* ❄.

M. decora (Nees) Conert

WESTERN CAPE.—3420 (Bredasdorp): 8 km south from Ouplaas to De Hoop nature reserve (-AD), *Spies 3465* ❄.

M. disticha (Nees) Conert

EASTERN CAPE.—3125 (Steynsburg): 29 km from Steynsburg to Oviston (-BD), *Spies 6140* ❄.

M. dura (Stapf) Conert

NORTHERN CAPE.—3119 (Calvinia): 15 km from Nieuwoudtville to Clanwilliam (-AC), *Spies* 5307 ☼; 113 km from Clanwilliam to Nieuwoudtville (-AC), *Spies* 6285 ☼.

M. stricta (Schrad.) Conert

NORTHERN CAPE.—3119 (Calvinia): Vanrhyns Pass (-AC), *Spies* 6288 ✱. 3220 (Sutherland): 15 km from Sutherland to Matjiesfontein (-BC), *Spies* 3140 ☼.

WESTERN CAPE.—3419 (Caledon): Shaw's Pass (-AD), *Spies* 6227 ☼.

EASTERN CAPE.—3323 (Willowmore): 9 km from Uniondale to Willowmore (-CA), *Spies* 6145 ☼.

Pentameris

P. longiglumes (Nees) Stapf

WESTERN CAPE.—3219 (Wuppertal): 37 km from Clanwilliam to Cedarberg (-BB), *Spies* 6072 ☼. 3419 (Caledon): MTN tower on scenic route, Hermanus (-AC), *Spies* 6225 ☼.

EASTERN CAPE.—3323 (Willowmore): 13 km from Uniondale to Oudtshoorn (Potjiesberg Pass) (-CA), *Spies* 6154 ☼.

P. macrocalycina (Steud.) Schweik.

WESTERN CAPE.—3219 (Wuppertal): Uitkyk Pass (-AC), *Spies* 6316 ☼. 3319 (Worcester): Franschoek Pass, 19 km to Franschoek from turnoff on Villiersdorp-Grabouw road (-CC), *Spies* 3644 ☼. 3419 (McGregor): Galgeberg (-BA), *Spies* 6235 ☼.

P. oreophila N.P.Barker

WESTERN CAPE.—3322 (Oudtshoorn): Swartberg Pass (-AC), *Spies* 6166 ✱.

P. thuarii Beauv.

WESTERN CAPE.—3322 (Oudtshoorn): Montagu Pass (-CD), *Spies* 6160 ✱.

Pentaschistis

P. aristifolia Schweik.

WESTERN CAPE.—3119 (Calvinia): In Vanrhyns Pass (-AC), *Spies* 6295 ☼.

P. rupestris (Nees) Stapf

WESTERN CAPE.—3219 (Wuppertal): In Uitkyk Pass (-AC), *Spies* 6308 ☼.

Phragmites

P. australis (Cav.) Steud.

FREE STATE.—2926 (Bloemfontein): UOFS campus (-AA), *Spies* 6574 ☼.

Prionanthium

P. dentatum (L.f.) Henr.

NORTHERN CAPE.—3119 (Calvinia): 118 km from Clanwilliam to Nieuwoudtville (-AC), *Spies* 6047 ☼, 6286 ☼.

P. ecklonii (Nees) Stapf

WESTERN CAPE.—3218 (Clanwilliam): 12 km from turn off from Clanwilliam to Citrusdal (-BD), *Spies* 6061 ☼, *Spies* 6254 ☼.

P. pholioroides Stapf

WESTERN CAPE.—3318 (Cape Town): 3 km east from Mamre road (-BC), *Spies* 6101 ☼, *Spies* 6252 ☼. 3420 (Bredasdorp): 3 km north of De Hoop nature reserve (-CA), *Spies* 6213 ☼.

Pseudopentameris

P. macrantha (Schrad.) Conert

WESTERN CAPE.—3318 (Cape Town): Tafelberg (-AB), *Spies* 3431 ☼.

Schismus

S. barbatus (Loefl. ex L.) Thell.

FREE STATE.—2925 (Jagersfontein): Petrusberg (-AB), *Spies* 6596 ☼.

NORTHERN CAPE.—2917 (Springbok): 14 km from Steinkopf to Port Nolloth (Aninaus Pass) (-CB), *Spies 6353* ☼.

WESTERN CAPE.—3219 (Wuppertal): 44 km from Clanwilliam to Calvinia (-AA), *Davidse 34033* ☼.

EASTERN CAPE.—3323 (Willowmore): 13 km from Uniondale to Oudtshoorn (Potjiesberg Pass) (-CA), *Spies 6155* ☼.

S. scaberrimus Nees

WESTERN CAPE.—3122 (Victoria West): 55 km from Loxton to Fraserburg (-BC), *Spies 4660* ☼.

Styppeiochloa

S. gynoglossa (Goossens) De Winter

MPUMALANGA.—2430 (Pilgrim's Rest): 3 km from Graskop to Bosbokrand (-DD), *Saayman 79* ☼. 2530 (Lydenburg): In the Steenkampsberge, 6 km from Goede Hoop to Roosenekal (-AA), *Spies 1485* ☼.

SWAZILAND.—2631 (Mbabane): Moimba beacon, 16 km from Mbabane to Oshoek (-AD), *Spies 2642* ☼.

Tribolium

T. acutiflorum (Nees) Renvoize

NORTHERN CAPE.—3119 (Calvinia): Vanrhyns Pass (-AC), *Spies 6291* ☼.

WESTERN CAPE.—3319 (Worcester): 5 km from Gouda to Porterville (-AC), *Spies 3866* ☼.

T. brachystachyum (Nees) Renvoize

WESTERN CAPE.—3319 (Worcester): Dutoitskloof Pass (-CA), *Spies 6249* ☼.

T. echinatum (Thunb.) Renvoize

WESTERN CAPE.—3218 (Clanwilliam) 13 km from Clanwilliam to Citrusdal (-BD), *Spies 6255* ☼. 3219 (Wuppertal): 6 km from Algeria to Citrusdal (On the top of Nieuwoudts Pass) (-AC), *Spies 6084* ☼.

T. hispidum (Thunb.) Renvoize

NORTHERN CAPE.—3018 (Kamiesberg): 8 km from Kamieskroon to Leliehoek (-AC), *Spies 5967* ☼.

WESTERN CAPE.—3319 (Worcester): On the top of Dutoitskloof Pass (-CA), *Spies 6106* ☼. 3320 (Montagu): Burger's Pass (-CC), *Spies 6240* ☼.

EASTERN CAPE.—3424 (Humansdorp): 30 km from Humansdorp to Knysna (-AA), *Spies 3509* ☼.

T. obtusifolium (Nees) Renvoize

WESTERN CAPE.—3219 (Wuppertal): 6 km from Algeria to Citrusdal (On the top of Nieuwoudts Pass) (-AC), *Spies 6085* ☼. 3319 (Worcester): FM tower at Matroosberg (-BC), *Spies 6245* ☼.

T. pusillum (Nees) H.P.Linder & Davidse

WESTERN CAPE.—3118 (Vanrhynsdorp): 2 km from Vanrhynsdorp to Gifberg (-DA), *Spies 6296* ☼. 3218 (Clanwilliam): 2 km from Clanwilliam to Nieuwoudtville (-BB), *Spies 6256* ☼; 7 km from Clanwilliam in Pakhuis Pass (-BB), *Davidse 34033* ☼.

T. uniolae (L.f.) Renvoize

WESTERN CAPE.—3218 (Clanwilliam): 21 km from Clanwilliam to Nieuwoudtville (-BB), *Spies 6025* ☼; in Versveld Pass (-DC), *Spies 6096* ☼. 3322 (Oudtshoorn): Robinson Pass (-CC), *Spies 6181* ☼. 3420 (Bredasdorp): 500m north from De Hoop reserve (-CA), *Spies 6201* ☼, *Spies 6203* ☼.

T. utriculosum (Nees) Renvoize

NORTHERN CAPE.—2917 (Springbok): 7 km from Steinkopf to Port Nolloth (-BA), *Spies 5892* ☼.

2.2 Methods

2.2.1 Cytogenetics

2.2.1.1 Meiotic analysis

Young inflorescences were fixed in Carnoy's fixative [ethanol: chloroform: acetic acid - 6:3:1] (Carnoy 1886). The fixative was replaced by 70% (v/v) ethanol 24-48 hours after fixation. Anthers of the inflorescences were squashed in 2% (m/v) aceto-carmin (Darlington & La Cour 1976) on a microscope slide. Contrast between cytoplasm and chromosomes was enhanced by adding a droplet of 45% (v/v) acetic acid, saturated with iron acetate, to the stain immediately before making the squash (Thomas 1940) and then gently heating the slide over a spirit flame. Squashes were made according to Darlington and La Cour's (1976) method. The slides were made permanent by freezing them with liquid carbon dioxide (Bowen 1956), followed by dehydration in ethanol and mounting in Euparal.

Whenever possible, at least twenty cells of each of diakinesis, metaphase I, anaphase I and telophase I were examined in each specimen. The haploid chromosome numbers, the presence of B chromosomes as well as the percentage rod and ring bivalents and multivalents were recorded. In the case of metaphase I, anaphase I and telophase I the number of chromosomal abnormalities (univalents, chromosome laggards and micronuclei) were recorded as well (Appendix A).

2.2.1.2 Microphotography

Microphotography was done using a Nikon Microphot-FXA photomicroscope, with Pan-F 35-mm (ASA 50) black and white films. The films were developed for twelve minutes in Agfa Rodinol film developer, then rinsed in water for approximately 5 minutes. After fixation in Ilford rapid fixer for 10 minutes, the films were rinsed in running water for 20 minutes. The films were then left to dry overnight.

Ilfospeed developer was used to develop the photographs and development was then stopped in water to which some acetic acid was added. The photographs were fixed with Ilford Hypam fixative whereafter the photographs were rinsed in water for 5 minutes

and left face up, to dry. Ilford Multigrade IV RC DE LUXE paper was used for the photographs.

Microphotographs depicting meiotic stages, abnormalities or certain behavioural trends during meiosis were mounted on herbarium sheets and are stored in the Geo Potts Herbarium, Bloemfontein. Selections of these photographs, which depict certain of these phenomena the best, are included in this thesis.

2.2.2 Molecular studies

The leaves of the different specimens were collected in the veld and stored in a saturated sodium chloride and hexadecyl trimethyl ammonium bromide (CTAB) solution (Rogstad 1992).

2.2.2.1 DNA extraction

The CTAB method (Rogstad 1992) was used to extract DNA from ± 0.5 g of leaf material. The leaves were rinsed with distilled water and blotted with paper before the extractions were carried out in eppendorf tubes. The material was ground to a fine powder in liquid nitrogen. The frozen tissue was then immediately incubated at 65°C for one hour, in 600 μ l of CTAB extraction buffer [1% (m/v) CTAB, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, (pH 8.0), 0.7 M NaCl to which 1% (m/v) 2-mercapto-ethanol had been added just before use]. After one hour 600 μ l of chloroform:iso-amylalcohol (24:1) was added, mixed thoroughly and the resultant mixture centrifuged for five minutes at 3 000 g. The supernatant was transferred to a clean tube, and to this 600 μ l of cold (-20°C) absolute ethanol, containing 3 M sodium acetate (25:1) was added to precipitate the DNA. After one hour of incubation at 4°C, the mixture was centrifuged at 7 000 g. for eight minutes. The supernatant was discarded and the DNA pellet washed twice with 70% (v/v) ethanol containing 10 mM ammonium acetate. After decanting the ethanol and evaporating any excess ethanol left, the DNA was dissolved in sterilised, distilled water (20-50 μ l, depending on the size of the pellet).

2.2.2.2 Taguchi optimisation

The PCR based DAF reactions were optimized according to a modified Taguchi method (Cobb & Clarkson 1994). With this optimisation, the optimal conditions for four

reaction variables can be achieved, by using only nine reactions. The number of experimental reactions (E) required (nine), can be calculated by using the equation $E = 2k + 1$, where k is the number of factors to be tested (four) (Cobb & Clarkson 1994). These variables are the primer, magnesium chloride, DNA and dNTP concentrations. In this optimisation, three concentrations of each reaction component is varied in an orthogonal array. With this Taguchi method, the product yield for each reaction is used to estimate the effects that the individual components have on the amplification products (Cobb & Clarkson 1994). This yield can be calculated by using quadratic loss functions, which are referred to as signal to noise (SNL) ratio's by Taguchi (Taguchi & Wu 1980; Taguchi 1986):

$SNL = -10 \log [1/n \sum 1/y^2]$, where SNL is the signal to noise ratio, y is the yield for each amplification reaction and n is the number of levels. For each of the four reaction components the largest SNL value represents the optimal condition.

2.2.2.3 DNA amplification fingerprinting (DAF)

For this molecular amplification technique eleven primers were used. These are eight bp in length and their GC contents varied from 50% to 75%.

DAF₁ - 5' AACGGGTG 3'

DAF₂ - 5' GTAACGCC 3'

DAF₃ - 5' GAGGGTGG 3'

DAF₄ - 5' CCTCGTGG 3'

DAF₅ - 5' GGAACGCC 3'

DAF₆ - 5' GTTACGCC 3'

DAF₇ - 5' CTGGACTA 3'

DAF₈ - 5' GTAACGCC 3'

DAF₉ - 5' GTRACTGCC 3'

DAF₁₁ - 5' CCTGCTGG 3'

DAF₁₂ - 5' CAGCTCGG 3'

Amplification was done in a Techne Genius thermocycler with denaturation for 60 sec. at 95°C, followed by 40 amplification cycles of 10 sec. at 94°C, 15 sec. at 30°C and 75 sec. at 72°C. The reactions were cooled to 4°C.

Repeatability is very important in PCR procedures. This is due to the sensitivity of the system and, therefore, each reaction was duplicated.

2.2.2.3.1 Gel electrophoresis

DNA amplification products were separated by polyacrylamide gel electrophoresis. Separation was in 1-mm thick slab gels of 8% acrylamide and 10 M urea. Inclusion of urea in the gels (at least 1.6 M) gave superior fragment resolution. The ratio of acrylamide to the crosslinker bisacrylamide was 20:1. Gels were prepared in a total volume of 50 ml which consists of 20 ml acrylamide-bisacrylamide solution, 24.5 ml water, 7.5 g urea, 500 µl 10% APS (Ammonium persulphate) and 5 ml 10X TAE. Prior to gel casting 20 µl TEMED (N, N', N'-Tetramethyl ethylene diamine) was added.

Gels and running buffer were prepared in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Samples were loaded in 5 µl of 10 M urea and 2 µl of bromophenol blue in double distilled water. Usually 5 µl of the amplification reaction (diluted 1 in 5) was loaded. Electrophoresis was at 150 V for two hours.

2.2.2.3.2 Silver staining

The photochemical derived silver staining method (Goldman & Merrill 1982; Blum *et al.* 1987), modified to decrease background staining and increase sensitivity (Bassam *et al.* 1991; Caetano-Anollés & Gresshoff 1994a), was used to visualize the DNA amplification products.

Briefly the procedure is as follows:

1. Fixation in 7.5% (v/v) acetic acid for 30 minutes.
2. Rinsing three times with water for 2 minutes each.
3. Impregnation with a silver solution [0.1% (m/v) silver nitrate (AgNO_3) + 0.056% (v/v) formaldehyde (HCOH)] for 30 minutes.
4. Brief rinsing with water for 5 – 20 seconds.

5. Developing in an alkaline solution [3% (m/v) sodium carbonate (Na_2CO_3), 0.056% (v/v) HCOH and 0.0002% (m/v) sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$)] until required fragment intensities are reached.
6. Image development was stopped in 7.5% (v/v) acetic acid.

2.2.2.3.3 Image documentation

Gel images were saved for future reference by scanning images into the Molecular Analyst Software Plus program (Anonymous 1995) and by photography. The photographs were taken with a TLC camera (shutterspeed 60, F-stop 4.5 - 5). A 13-mm extension tube was used to obtain the right enlargement. Film and photograph development was done in a similar way to the development of meiotic photographs (See section 2.2.1.2).

2.2.2.3.4 Data analysis

The analysis of the amplification products were done with the Molecular Analyst Fingerprinting Plus program, as well as manually. The following criteria were considered:

1. Number of fragments
2. Repeatability of the reaction

The Molecular Analyst computer program involves three steps:

1. Conversion. In this section all of the amplification images are labeled with the corresponding species names.
2. Normalisation. During this process the gel images are normalised, by aligning the molecular markers on each gel to a standard.
3. Analysis. In this section the amplification products are analysed and the amount of fragments present per gel are graphically indicated on the gel. Different gels can be combined and with the comparative quantification option these combined gels are analysed as a unit. A position tolerance of 10 and band position of 10 was used to adjust the sensitivity of the system.

Graphical representations of each of the analysed primers were created in this way. These representations were checked manually and scored for absence (0) or presence (1) of fragments.

2.2.2.3.5 Consistency test

Fragment sharing analyses were carried out for the DAF data, by the pairwise comparison of the samples according to the consistency formula of Nei and Li (1979).

$$F = 2(X_{1,2}) / (X_1 + X_2),$$

where $X_{1,2}$ is the number of shared fragments with similar molecular weights, X_1 is the total number of DAF fragments in the one reaction, X_2 is the total number of DAF fragments in the other reaction and F is the coefficient of similarity (Nei 1987). An F value of one will indicate that the samples are identical, or fully repeatable, and lower values will indicate a lesser correspondence.

Genetic distances can be calculated by using the Nei-formula (Nei 1987):

$$D = -\ln(F),$$

where D is the genetic distance among the different samples.

2.2.2.4 Sequencing

2.2.2.4.1 ITS fragment amplification

Genomic DNA was used to amplify the DNA region between the 18S and 5.8S nrDNA genes (the *ITS1* region), as well as between the 5.8S and 26S nrDNA genes (the *ITS2* region), with the polymerase chain reaction. A small portion of the 5.8S gene was amplified in both cases as well, due to the annealing sites of the primers. The primers used for the PCR were ITS_L and ITS_2 (for *ITS1*) and ITS_3 and ITS_4 (*ITS2*) (White *et al.* 1990).

ITS_L 5'- TCGTAACAAGGTTTCCGTAGGTG-3'

ITS_2 5'- GCTGCGTTCTTCATCGATCG-3'

ITS_3 5'- GCATCGATGAAGAACGCAGC-3'

ITS_4 5'- TCCTCCGCTTATTGATATGC-3'

The PCR reactions were performed in a total volume of 50 μ l. The reactions were optimised according to Taguchi (2.2.2.2).

The reactions were briefly centrifuged and placed in the Perkin Elmer GeneAmp PCR system 9600. An initial denaturation step at 94°C was followed by 40 amplification cycles, each consisting of 30 sec. at 94°C, 30 sec. at 50°C and 90 sec. at 72°C (Baldwin 1992).

The amplification products were separated on 1% (m/v) agarose gels with 1X TAE running buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), intercalated with ethidium bromide and visualised by illumination with ultraviolet (UV) light.

2.2.2.4.2 Sequencing

Sequencing reactions were carried out by using the system based on Sanger's dideoxynucleotide method (1977).

For each template to be sequenced the following were combined:

Sequence reagent pre-mix	8 μ l
Primer (50 pmol)	1 μ l
DNA template	<u>11 μl</u>
Total volume	20 μ l

These reactions were placed in the Perkin Elmer thermal cycler with an initial denaturation step at 94°C for 1 min, followed by 25 amplification cycles, each consisting of 94°C for 30 sec., 50°C for 15 sec. and 60°C for four min.

After amplification 7 μ l of 7.5 M of ammonium acetate was added to each reaction, as well as 2.5 volumes (\pm 68 μ l) of 100% (v/v) ethanol (-20°C). These reactions were mixed and placed on ice for at least 15 min. Each sample was then centrifuged for 15 minutes at 10 000 g., whereafter the supernatant was discarded and 250-500 μ l of 70% (v/v) ethanol (-20°C) was added to wash the pellet. The mixtures were centrifuged briefly, and after the supernatant was drawn off the pellets were vacuum dried for three to five minutes. The pellets were stored in this dry state at -20°C, till they were loaded on the gel. Prior to gel loading each pellet was resuspended in 4 μ l of formamide loading buffer, and then heated to 100°C for 2-5 minutes to denature. An amount of 1.5-2 μ l of the samples was then loaded on a 6% polyacrylamide gel and separated for 4-6 hours on a ABI PrismTM 377 fluorescent sequencing system.

2.2.2.4.3 Sequence alignment

The *ITS_L - ITS₂* and *ITS₃ - ITS₄* sequence combinations were aligned for each specimen, using the Sequence Navigator software (Applied Biosystems Inc., a Division of the Perkin Elmer Corporation) for Apple Macintosh. The sequences were aligned using the comparative alignment option with a mismatch penalty of 5, gap penalty of 4 and gap

extend penalty of 3. The *ITS1* and *ITS2* sequences of each specimen were then aligned using CLUSTALW (Thompson *et al.* 1994) and MALIGN (Wheeler & Gladstein 1994). Final alignment was visually inspected and manually optimised for phylogenetic analysis.

2.2.3. Phylogenetic Analyses

2.2.3.1 PAUP (Phylogenetic analysis using parsimony) analysis

Data were analysed with the computer program PAUP (version 3.1) by converting each data set (e.g. DAF fragment patterns or aligned sequences) into a datamatrix.

PAUP uses the principle of maximum parsimony, which searches for minimum length cladograms. HEURISTIC searches using RANDOM (200 replications) stepwise addition of taxa, followed by TBR (tree bisection-reconnection) branch swapping (STEEPEST DESCENT and MULPARS in effect) were used to find the most parsimonious cladograms. Topological constraints were not enforced and branches of zero length were collapsed to yield polytomies.

Searches were conducted to find multiple islands of equally parsimonious trees (Maddison 1991). This was done according to methods outlined in Olmstead and Palmer (1994).

Heuristic search options explores many trees but gives no guarantee that the trees found will in fact be the shortest for the data set (Kellogg & Watson 1993). The branch and bound and exhaustive search options were not considered due to the time consuming nature of these search options. Exhaustive searches are guaranteed to find the shortest trees, but become computationally prohibited if there are more than 11 taxa in the data set. The branch and bound algorithm, also guaranteed to find the shortest trees, is more efficient, but only for up to 30 taxa (Swofford 1993).

When dealing with DNA sequencing data each nucleotide position was scored as a uniformly weighted character, with gaps scored as missing data.

Sets of equally parsimonious trees were summarised using Strict, Semistrict (combinable component) and Adams consensus trees. Multistate taxa were treated as uncertain. Uninformative characters were ignored and all characters were unordered (Fitch optimisation) with a weight of one. Furthermore, characters were mapped on the consensus

cladograms using ACCTRAN (Accelerated Transformation) (Swofford & Maddison 1987), which prefers reversals to parallelisms (homoplasy) when both optimisations are equally parsimonious.

Statistics for evaluating and comparing the trees generated, were also created. The two reported here are the CI (consistency index), which divides the minimum number of changes of characters on a tree by the actual number of changes (Farris 1989a, b; Kluge & Farris 1969), and the retention index (RI), which corrects for the actual distribution of character states in the data matrix by subtracting both the minimum number of changes and the actual number of changes from the maximum number of changes possible (Farris 1989a, b). Both these indices are measures of homoplasy and can be applied to individual characters or to entire trees. When these statistics are used to describe trees, only the phylogenetic informative characters are included.

All uninformative characters were excluded from the data matrices, due to the fact that these characters will inflate CI values by adding both one unit to the numerator and denominator in the calculations. These are both invariant (characters in which a single state is possessed by all groups under consideration), as well as uninformative [characters in which only one of the included taxa possesses a particular derived state (autapomorphy)] characters (Sanderson & Donoghue 1989).

By stepwisely increasing the length of the cladogram, with the Strict cladogram option, decay indices were obtained (Bremer 1988; Donoghue *et al.* 1992). Bootstrap values were calculated from 200 replicates (Felsenstein 1985), by using the general HEURISTIC search with TBR branch swapping and CLOSEST ADDITION sequence of taxa (STEEPEST DESCENT and MULPARS in effect). Bootstrapping phylogenies is a means of estimating the robustness of phylogeny reconstruction to sampling error (Sanderson & Doyle 1992). Hillis and Bull (1993) showed that bootstrapping provides a very conservative test of the accuracy of the cladogram, but that the absolute values may not be very meaningful.

Where applicable some characters were excluded from the matrix where low CI:RI ratios was observed. Successive weighting (Farris 1969) was also applied to the characters to determine the effect that larger weights for certain less homoplasious characters would have on the parsimony of the phylogeny.

2.2.3.2 HENNIG86

Data were also analysed with the HENNIG86 software package (Farris 1988). This computer program uses Wagner optimisation (all characters ordered as default setting). Mhennig and branch swapping options were used to search for the most parsimonious tree.

The implicit enumeration option was not used due to the time consuming nature of this option, especially when dealing with large data sets. Where more than one equally parsimonious tree was obtained the nelsen command was used to obtain a consensus tree. The default character options in HENNIG86 are unitary weight (1), ordered (+) and active ([]). This was changed to unordered when sequences were analysed. Successive weighting and the exclusion of certain characters on the basis of low CI to RI ratio's were done in a similar manner as with PAUP to investigate the effect on the parsimony of the phylogeny.

In the Random Cladistics software package (Siddall 1994) the datamatrices were firstly subjected to the jackknife monophyly index. This is based on Lanyon's (1985) jackknife. This monophyly index calculates the frequency of each monophyletic group and the values indicate the proportion of most equally parsimonious trees in a jackknife replicate that support the monophyly of a group.

In the HEYJOE option of the Random Cladistics software package, bootstrap monophyly indices were calculated from 100 replicates.

Trees computed by PAUP and HENNIG 86, were adjusted with the TREEVIEW computer software program (Page 1996).

CHAPTER 3

CYTOTAXONOMY

3.1 Introduction

The tribe Arundineae forms the major portion of the grass subfamily Arundinoideae, with approximately 41 genera and 300 species (Gibbs Russell *et al.* 1985). The basic chromosome number of this tribe was considered to be $x = 12$ (Clayton & Renvoize 1986). This is certainly a secondarily derived number by polyploidy from $x = 6$ (Hunziker & Stebbins 1987; Davidse 1988), since nine of the 41 genera, endemic to South Africa, are known to have basic numbers of $x = 6$. Additional to the five genera listed by Davidse (1988), four more diploids were added: *Centropodia* (Du Plessis & Spies 1988), *Chaetobromus* (Spies & Du Plessis 1988; Du Plessis & Spies 1988; Spies *et al.* 1990), *Pentameris* (Barker 1993) and *Urochlaena* [= *Tribolium*] (Spies & Du Plessis 1988; Visser & Spies 1994c, d, e).

Previous cytogenetic studies indicated that this tribe has three basic chromosome numbers i.e. $x = 6, 7$ and 13 (Spies *et al.* 1990). Only four genera, namely *Dregeochloa*, *Merxmuellera*, *Pentaschistis* and *Prionanthium*, share the basic chromosome number of seven. Davidse *et al.* (1986) suggested that $x = 7$ is a primitive number, as in the genus *Pentaschistis*, and that $x = 13$ was secondarily derived through an aneuploid reduction from $x = 14$. The basic chromosome number of 13 appears to be predominantly geographical in distribution, with the majority of species having this basic chromosome number, namely *Pentaschistis borussica* (K.Schum.) Pilg., *P. pictigluma* var. *mannii* (C.E.Hubb.) S.M.Phillips, *P. pictigluma* var. *minor* (Ballard & C.E.Hubb.) S.M.Phillips, *P. pictigluma* (Steud.) Pilg. and *P. trisetoides* (Hochst. ex Steud.) Pilg., occurring in the eastern mountain ranges of Africa (Du Plessis & Spies 1992). The exception is *P. eriostoma* (Nees) Stapf, which occurs only in the Western Cape.

The major aim of this study is to determine whether cytogenetic information can contribute to an increased knowledge of the phylogenetic relationships among the South African Arundineae, particularly by investigating representatives of the groups having different basic chromosome numbers.

3.2 Chromosome studies in the Arundineae

Various studies were conducted on the Arundinoideae, and then especially on the Arundineae. Most of the chromosome numbers listed in Table 3.1 were gathered from the summary listings of Ornduff (1967, 1968, 1969), Federov (1969), Moore (1970, 1971, 1972, 1973, 1974, 1977), Goldblatt (1981, 1983, 1985, 1988) and Goldblatt and Johnson (1990, 1991, 1994, 1996, 1998).

3.3 Results and Discussion

3.3.1 Basic chromosome numbers

The original basic chromosome number of the taxon studied is of great importance in the application of any chromosomal information at family level (Raven 1975). The basic chromosome number (x), represents the lowest gametic chromosome number in a taxon (Rieger *et al.* 1976).

Meiotic analysis of the different genera in this study, again confirmed a haploid chromosome number of six for most genera in the tribe Arundineae:

- *Chaetobromus involucretes* subsp. *dregeanus* (Spies 5691), Fig. 3.1A-C.
- *Karoochloa purpurea* (Spies 2477), Fig. 3.1D-F.
- *Pseudopentameris macrantha* (Spies 3431), Fig. 3.1G-H.
- *Schismus barbatus* (Davidse 34033 & Spies 6596), Fig. 3.2D-G.
- *Tribolium pusillum* (Davidse 34022), Fig. 3.2A-C.

Multiples of six were also observed:

- *Centropodia glauca* (Spies 5706), Fig. 3.3A ($n = 3x = 18$; hexaploid).
- *Karoochloa purpurea* (Spies 2473), Fig. 3.3B, C ($n = 2x = 12+2-5B$; tetraploid).
- *Merxmuellera cincta* (Nees) Conert (Spies 3504), Fig. 3.3D ($n = 3x = 18$; hexaploid);
M. decora (Nees) Conert (Spies 3465), Fig. 3.3E, F ($n = 4x = 24$; octaploid);
M. stricta (Schrad.) Conert (Spies 6288), Fig. 3.4A-C ($n = 2x = 12$; tetraploid);
M. stricta (Spies 3140), Fig. 3.4D-F ($n = 4x = 24$; octaploid).

Table 3.1 Published chromosome numbers of some of the studied genera. Gametic chromosome numbers (n) refers to meiotic studies and somatic chromosome numbers (2n) refers to mitotic studies. Species names indicated in brackets represents the name under which a particular species was published in that citing. Unpublished chromosome numbers from this study done by myself are indicated with *; done by Henriette du Plessis indicated with *hdp; done by Francisca Holder with *fh and done by Paula van Rooyen with *pvr.

Species	n	2n	Reference
<i>Arundo donax</i>	12		Sanhi & Bir 1985
	32		Mehra 1982
	32-36		Kalia 1978
	35		Mehra & Kalia 1975
	36		Mehra 1982
		60	Bochantseva 1972
		~60	Larsen 1963
		72	Fernandes & Queiros 1969; Pizzolongo 1962
		100	Devesa <i>et al.</i> 1991
		~100	Avdulov 1931
	110	Hunter 1934; Delay 1947; Heiser & Whittaker 1948; Fernandes & Queiros 1969; Pizzolongo 1962	
<i>A. plinii</i>		72	Fernandes & Queiros 1969
<i>Centropodia forskalii</i>		24	Sokolovskaya & Probatova 1978
<i>C. glauca</i>	18		*
		24	De Wet 1954a
	24		Spies & Du Plessis 1988

<i>C. involubrates</i>	6		Spies <i>et al.</i> 1990
	12		Du Plessis & Spies 1988; Spies <i>et al.</i> 1990
	18		Spies <i>et al.</i> 1990
<i>Chaetobromus involubrates</i> subsp. <i>dregeanus</i> (= <i>C. dregeanus</i>)	18		Spies <i>et al.</i> 1990
	27		Du Plessis & Spies 1988; Spies <i>et al.</i> 1990
	36		Spies & Du Plessis 1988; Spies <i>et al.</i> 1990
<i>C. involubrates</i> subsp. <i>involubrates</i> (= <i>C. schaderi</i>)	6		Spies <i>et al.</i> 1990
	18		Spies <i>et al.</i> 1990
	24		Du Plessis & Spies 1988; Spies <i>et al.</i> 1990
<i>Chaetobromus</i> species	18		Spies <i>et al.</i> 1990
<i>Cortaderia argentea</i>		70	Kihara <i>et al.</i> 1931
<i>C. atacamensis</i>		108	Beuzenberg in C 1965
<i>C. dioica</i>		76	Parodi 1946
<i>C. fulvida</i>		90	Hair & Beuzenberg 1966
<i>C. richardii</i>		90	Hair & Beuzenberg 1966
<i>C. selloana</i>	36		Mehra & Sharma 1975; Kalia 1978
		72	Muniyamma <i>et al.</i> 1976
		72 + 1B	Avdulov 1931
		76	Hunter 1934
		108	Beuzenberg in C 1965
<i>C. toetoe</i>		90	Hair & Beuzenberg 1966
<i>Dregeochloa pumila</i>	21		Du Plessis & Spies 1988; Spies & Du Plessis 1988
<i>Elytrophorus spicatus</i>	12		Mehra & Kalia 1975; Kalia 1978; Mehra 1982
		26	Veyret 1958

<i>Karroochloa curva</i>	6		Malan, personal communication
		12	De Wet 1954a
<i>K. purpurea</i>	6		*hdp; Malan, personal communication
	12		Spies & Du Plessis 1986b
	12+2-5B		*hdp
		24	Stebbins (Myers 1947); De Wet 1954a
<i>K. schismoides</i>	6		Malan, personal communication
	12		Du Plessis & Spies 1988
<i>K. tenella</i>	6		Spies & Du Plessis 1988; Malan, personal communication
		24	De Wet 1960
<i>Merxmuellera arundinacea</i>		12	De Wet 1960
	12		Malan, personal communication
<i>M. cincta</i>	12		Malan, personal communication
	18		*hdp
	24		Malan, personal communication
<i>M. cincta</i> subsp. <i>sericea</i>	18		Barker 1999
<i>M. decora</i>	18		Malan, personal communication
	24		*hdp
<i>M. disticha</i>		12	De Wet 1954a
	12		Malan, personal communication
<i>M. drakenbergensis</i>	24		Malan, personal communication
<i>M. dura</i>	12		Malan, personal communication
	28		Spies & Du Plessis 1988
<i>M. lupulina</i>	24		Malan, personal communication
<i>M. macowanii</i>	12		De Wet 1960; Malan, personal communication
<i>M. rangei</i>	12		Du Plessis, personal communication

	18		Du Plessis & Spies 1988
<i>M. stricta</i>	12		*; Malan, personal communication
		36	De Wet 1954a
	~51/2		*hdp
<i>P. distichophylla</i>		36	Barker 1993
<i>P. macrocalycina</i>	21		*
<i>P. oreophila</i>	7		*
<i>P. thuarii</i>	6		Barker 1993
	7		*; *hdp
<i>Pentaschistis aff. patula</i>	14		Spies <i>et al.</i> 1994a
<i>P. aff. patula</i> (= <i>airoides</i>)	28		Spies & Du Plessis 1988
<i>P. airoides</i>	7		Spies & Du Plessis 1988; Spies <i>et al.</i> 1994a
	7+0-2B		Spies <i>et al.</i> 1994a
	14		Spies & Du Plessis 1988; Spies <i>et al.</i> 1994a
<i>P. airoides</i> (= <i>capillaris</i>)	14		Du Plessis & Spies 1988
<i>P. airoides</i> subsp. <i>airoides</i>	7		Klopper <i>et al.</i> 1998
	21		Klopper <i>et al.</i> 1998
<i>P. airoides</i> subsp. <i>jugorum</i>	14		Klopper <i>et al.</i> 1998
<i>P. argentea</i>	21		Du Plessis & Spies 1992
<i>P. aristidoides</i>	7		Klopper <i>et al.</i> 1998
		14	De Wet 1960
<i>P. aristifolia</i>	14		Spies & Du Plessis 1988; Du Plessis & Spies 1988
<i>P. aurea</i> subsp. <i>aurea</i>	7		Du Plessis & Spies 1992
<i>P. barbata</i> (= <i>angulata</i>)	42, ~90/2		Du Plessis & Spies 1988
<i>P. barbata</i> (= <i>rupestris</i>)	14		Du Plessis & Spies 1988

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<i>P. borussica</i>		26	Tateoka 1965 ; Hedberg 1957; Hedberg & Hedberg 1977
		39	Hedberg & Hedberg 1977
<i>P. capensis</i>	7+0-2B		*pvr
<i>P. capillaris</i>	7		Du Plessis & Spies 1992; Klopper <i>et al.</i> 1998
	7+2B		Du Plessis & Spies 1992
<i>P. cirrhulosa</i>	7		Klopper <i>et al.</i> 1998
<i>P. cirrhulosa</i> (= <i>patuliflora</i>)	14+0-2B		Du Plessis & Spies 1988
<i>P. colorata</i>	14		Klopper <i>et al.</i> 1998
<i>P. curvifolia</i>	7		Du Plessis & Spies 1992; Klopper <i>et al.</i> 1998
	7+0-4B		Du Plessis & Spies 1992; *pvr
<i>P. densifolia</i>	7		Klopper <i>et al.</i> 1998
<i>P. elegans</i>	49		*pvr
<i>P. eriostoma</i>	13		Du Plessis & Spies 1992
	13+0-3B		Du Plessis & Spies 1992; Klopper <i>et al.</i> 1998
	26		Spies & Du Plessis 1988; Klopper <i>et al.</i> 1998
	26+0-2B		Du Plessis & Spies 1992; Klopper <i>et al.</i> 1998
	39		*fh
	39+0-4B		Klopper <i>et al.</i> 1998
	26		*fh
	~90/2		Du Plessis & Spies 1992
<i>P. lima</i>	42		Klopper <i>et al.</i> 1998
<i>P. malouinensis</i>	7		Spies & Du Plessis 1988
<i>P. natalensis</i>	7		Davidse <i>et al.</i> 1986; Du Plessis & Spies 1992
		28	Tateoka 1965

<i>P. pallescens</i>	35		*fh
<i>P. pallida</i>	7,14		Klopper <i>et al.</i> 1998
	7+0-3B		Klopper <i>et al.</i> 1998
		14	De Wet 1954a
<i>P. pallida</i> form A	7		Du Plessis & Spies 1992
	14		Du Plessis & Spies 1992
<i>P. pallida</i> form B	7		Du Plessis & Spies 1992
	7+1-2B		Du Plessis & Spies 1992
<i>P. pallida</i> form E (= <i>angustifolia</i>)	14		Du Plessis & Spies 1988
<i>P. pallida</i> form F	21		Du Plessis & Spies 1992
<i>P. pappilosa</i>	7		Du Plessis & Spies 1992
	7+4B		Du Plessis & Spies 1992
<i>P. patula</i>	7		Spies <i>et al.</i> 1994a
	7+0-3B		Spies <i>et al.</i> 1994a
<i>P. perrieri</i>		28	Tateoka 1965
		56	Morton 1993
<i>P. pictigluma</i>		26	Hedberg & Hedberg 1977
<i>P. pictigluma</i> var. <i>minor</i> (= <i>minor</i>)		26	Hedberg & Hedberg 1977
		52	Hedberg 1957; Tateoka 1965b
<i>P. pictigluma</i> var. <i>mannii</i> (= <i>mannii</i>)		26	Hedberg & Hedberg 1977
		±40	Hedberg 1952, 1957
		52	Hedberg & Hedberg 1977
<i>P. rigidissima</i>	7		*pvr
	7+0-2B		Klopper <i>et al.</i> 1998
	21		Klopper <i>et al.</i> 1998
<i>P. rupestris</i>	28+0-1B		Klopper <i>et al.</i> 1998
<i>Pentaschistis</i> species	7+0-2B		Klopper <i>et al.</i> 1998
<i>P. thunbergii</i>		14	De Wet 1954a
		52	Hedberg 1957

<i>P. tomentella</i>	7		Du Plessis & Spies 1988; Klopper <i>et al.</i> 1998; *pvr
	7+0-2B		Du Plessis & Spies 1992; *pvr
	14		Spies & Du Plessis 1988; Klopper <i>et al.</i> 1998
	14+2B		Du Plessis & Spies 1988
<i>P. tortuosa</i>	7		Du Plessis & Spies 1992
	14		*pvr
<i>P. trisetata</i>	7		Du Plessis & Spies 1992; Klopper <i>et al.</i> 1998
<i>P. trisetoides</i>		26	Hedberg & Hedberg 1977
<i>P. viscidula</i>	14+0-4B		*fh
	21		Klopper <i>et al.</i> 1998
	21+0-2B		*fh
<i>Phragmites australis</i>		36	Tischler 1942; Labadie 1976; Gervais 1981
		36, 44, 46, 48, 49, 50, 51, 52, 96	Gorenflot <i>et al.</i> 1972
		40	Kozuharov & Petrova 1991
		42-59	Gervais <i>et al.</i> 1993
		42,44,46, 49,50,51, 52,54	Gorenflot <i>et al.</i> 1975
		48,72,96	Gorenflot & Penahi 1979; Zong <i>et al.</i> 1991
		48, 96	Djebrouni 1992
		47- 49,72,96	González-Aguilera <i>et al.</i> 1990
		52	Fernandes & Queiros 1969

		48	Avdulov 1931; Tischler 1934, 1937; Hunter 1934; Rohweder 1937; Hagerup 1941; Saura 1948; Hubbard 1954; Löve 1954; Skalinska <i>et al.</i> 1968; Curran 1969; Fernandes & Queiros 1969; Vachova 1976; Löve & Löve 1981; Dimitrieva 1985; Parfenov & Dimitrieva 1987; Chen <i>et al.</i> 1993; Liang <i>et al.</i> 1994
		48,50,52, 56,72,96	Gorenflot <i>et al.</i> 1984; Gorenflot 1986
		84	Tarnavski 1948
		96	Avdulov 1931; Hubbard 1954; Chen <i>et al.</i> 1993; Liang <i>et al.</i> 1994
		116	Chen <i>et al.</i> 1993
<i>P. australis</i> subsp. <i>australis</i>		48	Sokolovskaya & Probatova 1976
<i>P. japonica</i>		48	Tateoka 1953, 1954; Gurzenkov 1973; Zong <i>et al.</i> 1991
<i>P. karka</i>	12		Bir & Sahni 1984
	24		Olorode 1975; Mehra 1982
		36	Ramanathan 1950
		36,38	Larsen 1963
		48	Tateoka 1955, 1956; Zong <i>et al.</i> 1991
<i>P. karka</i> var. <i>karka</i>	24		Kalia 1978
<i>P. mauritanus</i>		48	Dujardin 1979
<i>Prionanthium dentatum</i>	7		Davidse 1988; Visser & Spies 1994e

<i>P. ecklonii</i>	7		Davidse 1988; Visser & Spies 1994e
<i>P. pholioroides</i>	7		Davidse 1988
	7+0-2B		Davidse 1988; Visser & Spies 1994e
<i>Pseudopentameris macrantha</i>	6		*hdp
<i>Schismus arabicum</i>		12	Sokolovskaya & Probatova 1978
<i>S. arabicus</i>	6		Reeder 1977; Faruqi & Quraish 1979
		12	Gould 1958; Bowden & Senn 1962; Diaz Lifanté <i>et al.</i> 1992
<i>S. barbatus</i>	6		Raven <i>et al.</i> 1965; Humphries <i>et al.</i> 1978; Faruqi & Quirash 1979; Faruqi <i>et al.</i> 1987; Du Plessis & Spies 1988; Moinuddin <i>et al.</i> 1994; *hdp; *
	12		Du Plessis & Spies 1988
	18		Du Plessis & Spies 1988
		12	Gould 1958; Gould 1970
<i>S. barbatus</i> subsp. <i>calycinus</i>		12	Gould 1970
<i>S. inermis</i>	6		*hdp
	12		*hdp
<i>S. scaberrimus</i>	24		Spies & Du Plessis 1988
	36		Spies & Du Plessis 1988
<i>Styppeiochloa gynoglossa</i>	12		*
	24		*hdp
<i>Tribolium acutiflorum</i>	12		Spies <i>et al.</i> 1992; Visser & Spies 1994e; *
	12+0-1B		Visser & Spies 1994e
<i>T. brachystachyum</i>	12		Spies <i>et al.</i> 1992

	12+0-2B		Spies <i>et al.</i> 1992; Visser & Spies 1994d
<i>T. ciliare</i>	6		Spies <i>et al.</i> 1992; Visser & Spies 1994c
<i>T. echinatum</i>	6		Spies <i>et al.</i> 1992; Visser & Spies 1994c
	6+0-3B		Spies <i>et al.</i> 1992; Visser & Spies 1994c; Visser & Spies 1994d
<i>T. glomeratum</i>	12		Visser & Spies 1994e
	18		Visser & Spies 1994e
<i>T. hispidum</i>	6		Spies <i>et al.</i> 1992; Visser & Spies 1994c
	6+0-2B		Spies <i>et al.</i> 1992; Visser & Spies 1994c
	12		Spies <i>et al.</i> 1992; Visser & Spies 1994c; *
	12+0-7B		Visser & Spies 1994c
	18		Visser & Spies 1994c
<i>T. oblitterum</i>	12		Spies <i>et al.</i> 1992; Visser & Spies 1994e
	12+0-3B		Visser & Spies 1994e
	18		Spies <i>et al.</i> 1992
<i>T. pusillum</i> (= <i>Urochlaena pusilla</i>)	6		Spies & Du Plessis 1988; *hdp
	6+0-2B		Visser & Spies 1994e
<i>T. uniolae</i>	6		Spies <i>et al.</i> 1992
	6+0-1B		Visser & Spies 1994d
	12		Spies <i>et al.</i> 1992; Visser & Spies 1994d
	12+0-3B		Spies <i>et al.</i> 1992; Visser & Spies 1994d

	18		Spies <i>et al.</i> 1992; Visser & Spies 1994d
	18+0-4B		Spies <i>et al.</i> 1992; Visser & Spies 1994d
		28	De Wet 1960
<i>T. utriculosum</i>	6		Spies <i>et al.</i> 1992; Visser & Spies 1994c
	6+0-1B		Visser & Spies 1994c

- *Styppeiochloa gynoglossa* (Saayman 79), Fig. 3.5A-C ($n = 4x = 24$; octaploid); Spies 1485 and Spies 2642, Fig. 3.5D-G ($n = 2x = 12$; tetraploid).
- *Tribolium acutiflorum* (Nees) Renvoize (Spies 3866) Fig. 3.6A-C ($n = 2x = 12$; tetraploid);
T. hispidum (Thunb.) Renvoize (Spies 3509), Fig. 3.6D, E ($n = 2x = 12$; tetraploid).

A basic chromosome number of seven and multiples thereof, were also observed:

- *Pentameris thuarii* (Spies 3541 & Spies 6160), Fig. 3.7A, B ($n = x = 7$; diploid);
P. oreophila N.P.Barker (Spies 6166), Fig. 3.7C-E ($n = x = 7$; diploid);
P. macrocalycina (Spies 3644), Fig. 3.7F-H ($n = 3x = 21$; hexaploid).

A basic chromosome number of seven for the genus *Pentameris* is in contradiction with the chromosome numbers found by Barker (1993) of $2n = 12$ for *P. thuarii* and $2n = 36$ for *P. distichophylla* (Lehm.) Nees, respectively. In this study no univalents or B-chromosomes were observed that could have lead to erroneous chromosome counts. Therefore, seven seems to be the basic chromosome number for this genus. This is a new basic chromosome number for this genus.

The different chromosome numbers in the tribe can be attributed to evolutionary events, such as polyploidisation and aneuploidy (Table 3.2), as well as for example isolated geographical distributions, adaptations to extreme environmental conditions etc. The morphological similarities between many genera can also be attributed to similar basic chromosome numbers.

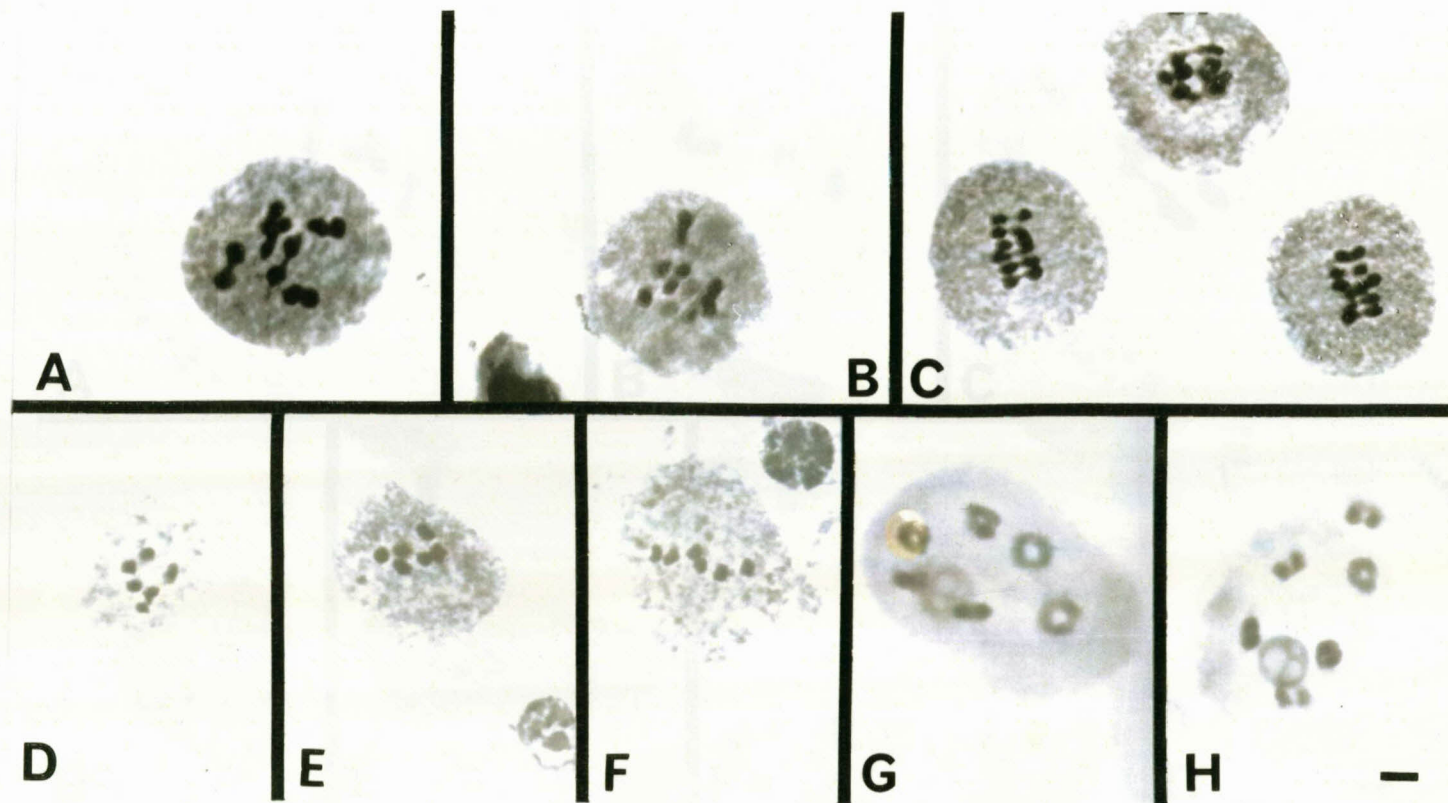


Figure 3.1 Meiotic chromosomes in diploid specimens with six as basic chromosome number. **A, B, C**, *Chaetobromus involucretes* subsp. *dregeanus*: **A, B**, *Spies 5691*, diakinesis, $n = 6$, 6_{IIK} ; **C**, *Spies 5691*, metaphase I, $n = 6$, 6_{IIK} . **D, E, F**, *Karroochloa purpurea*: **D**, *Spies 2477*, diakinesis, $n = 6$, $6_{II R}$; **E, F**, *Spies 2477*, metaphase I, $n = 6$. **G, H**, *Pseudopentameris macrantha*: **G**, *Spies 3431*, diakinesis, $n = 6$, $4_{II R}$, $2_{II K}$; **H**, *Spies 3431*, diakinesis, $n = 6$, $6_{II R}$. Scale bar: **A, E, H** = $4.5 \mu\text{m}$; **B, C** = $6.4 \mu\text{m}$; **D** = $5.6 \mu\text{m}$; **F** = $5.1 \mu\text{m}$; **G** = $4.8 \mu\text{m}$.

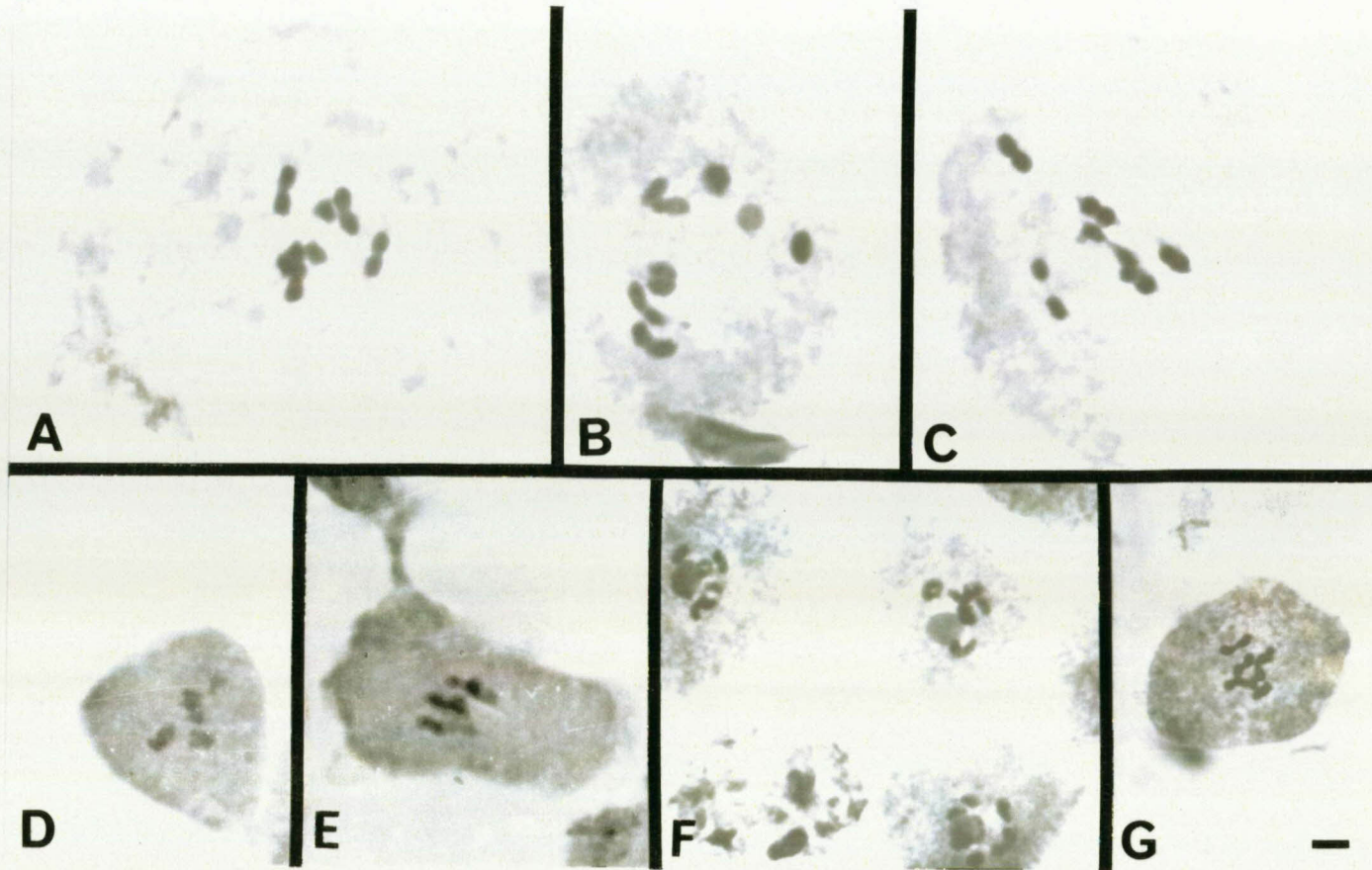


Figure 3.2 Meiotic chromosomes in diploid specimens with six as basic chromosome number. **A, B, C, *Tribolium pusillum***: **A, C, *Davidse 34022***, metaphase, $n = 6$, 6_{IIK} ; **B, *Davidse 34022***, diakinesis $n = 6$, 6_{IIK} . **D, E, F, G, *Schismus barbatus***: **D, *Spies 6596***, diakinesis, $n = 6$; **E, *Spies 6596***, metaphase I, $n = 6$. **F, *Davidse 34033***, diakinesis, $n = 6$; **G, *Davidse 34033***, metaphase I, $n = 6$. Scale bar: A, D = 5 μm ; B, E, F, G = 4.3 μm ; C = 4.6 μm .

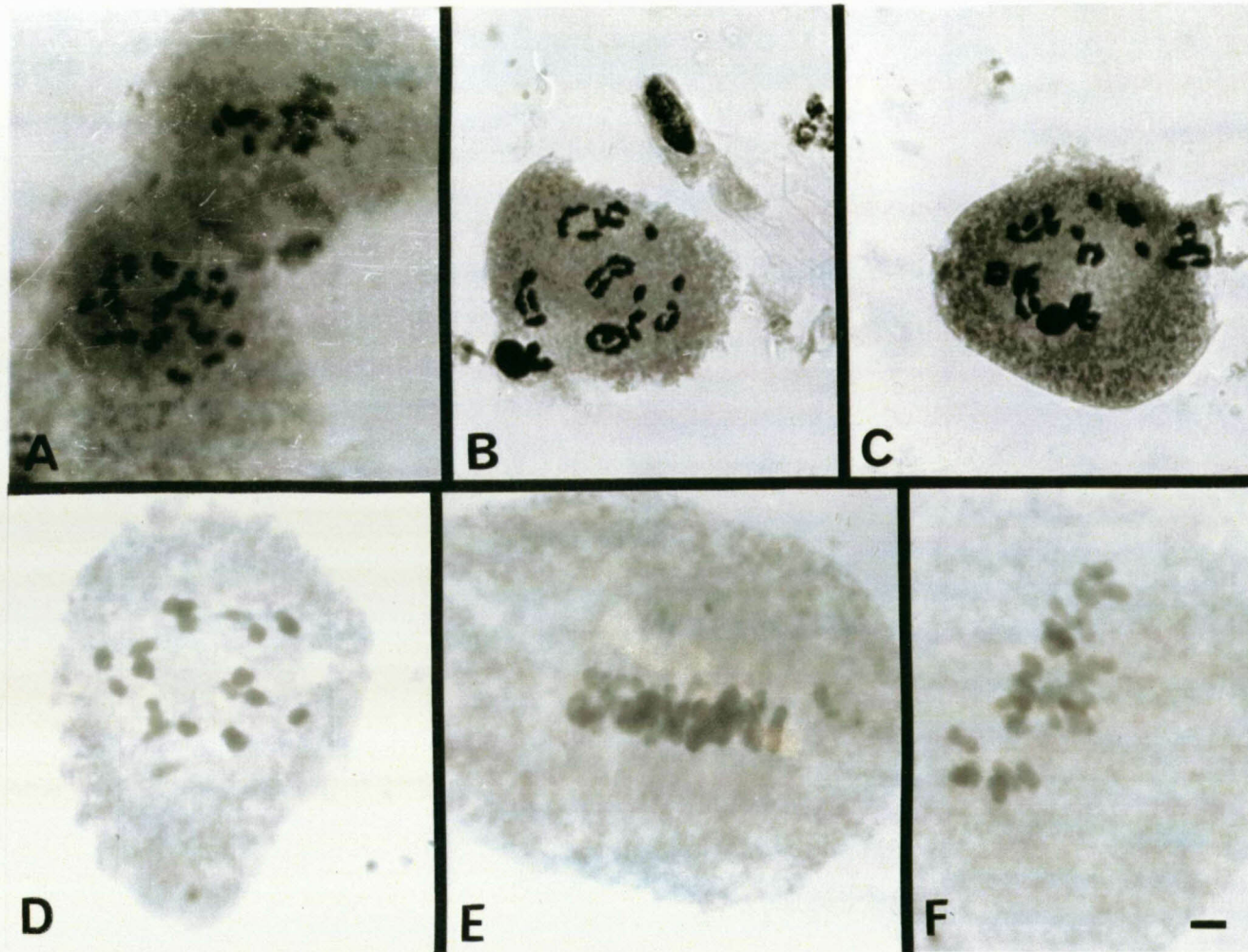


Figure 3.3 Meiotic chromosomes in polyploid specimens with six as basic chromosome number. **A**, *Centropodia glauca*, Spies 5706, diakinesis, $n = 18$. **B, C**, *Karooochloa purpurea*: **B**, Spies 2473, diakinesis, $n = 12+2-5B, 4_{II}, 4_{IV}$; **C**, Spies 2473, diakinesis, $n = 12+2-5B, 8_{II}, 2_{IV}$. **D**, *Merxmuellera cincta*, Spies 3504, diakinesis, $n = 18$; **E, F**, *M. decora*: **E**, Spies 3465, metaphase I, $n = 24$; **F**, Spies 3465, metaphase I, $n = 24$. Scale bar: A, C = 4.6 μm ; B = 4.0 μm ; D = 3.2 μm ; E, F = 3.5 μm .

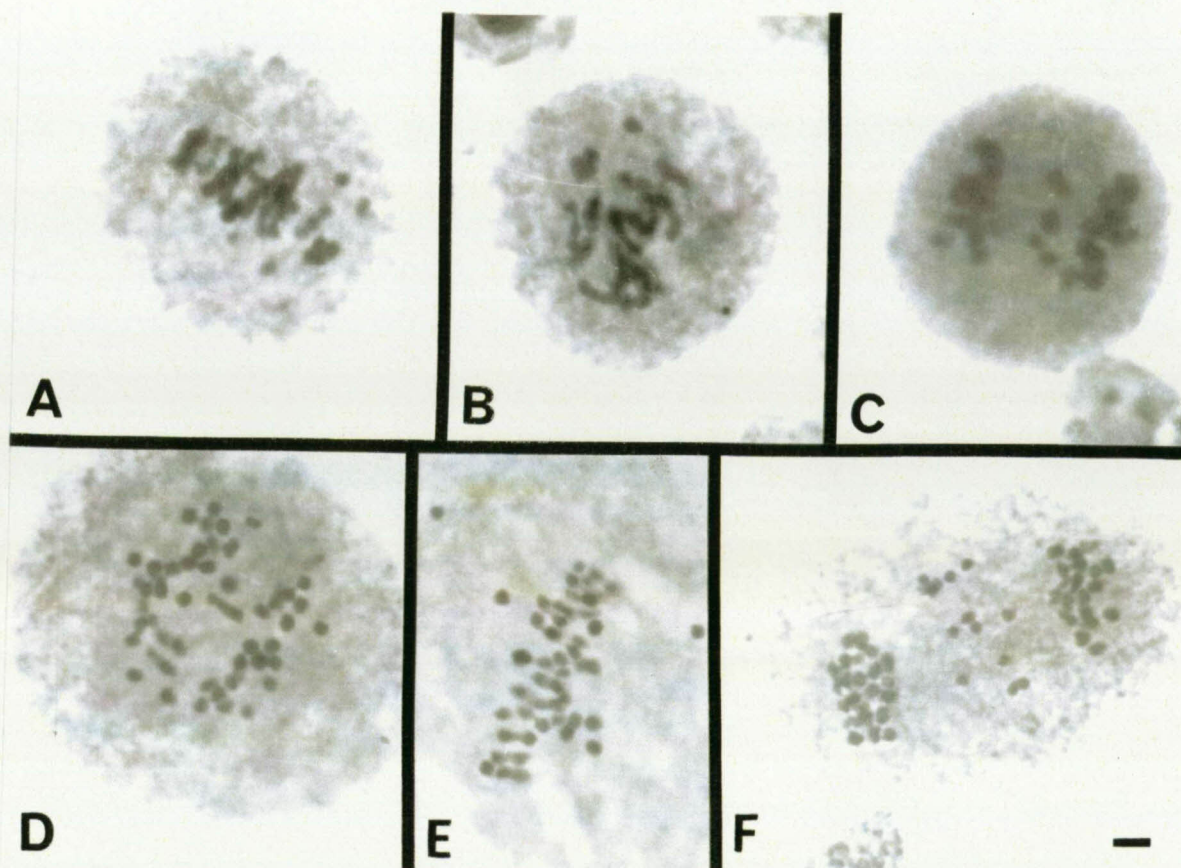


Figure 3.4 Meiotic chromosomes in *Merxmullera stricta*. **A, B**, *Spies 6288*, metaphase I, $n = 12$; **C**, *Spies 6288*, anaphase I, $n = 12$. **D, F**, *Spies 3140*, anaphase I, $n = 24$; **E**, *Spies 3140*, metaphase I, $n = 24$. Scale bar: **A** = 4.8 μm ; **B, C** = 4.1 μm ; **D, E**, = 6.5 μm ; **F** = 7.5 μm .

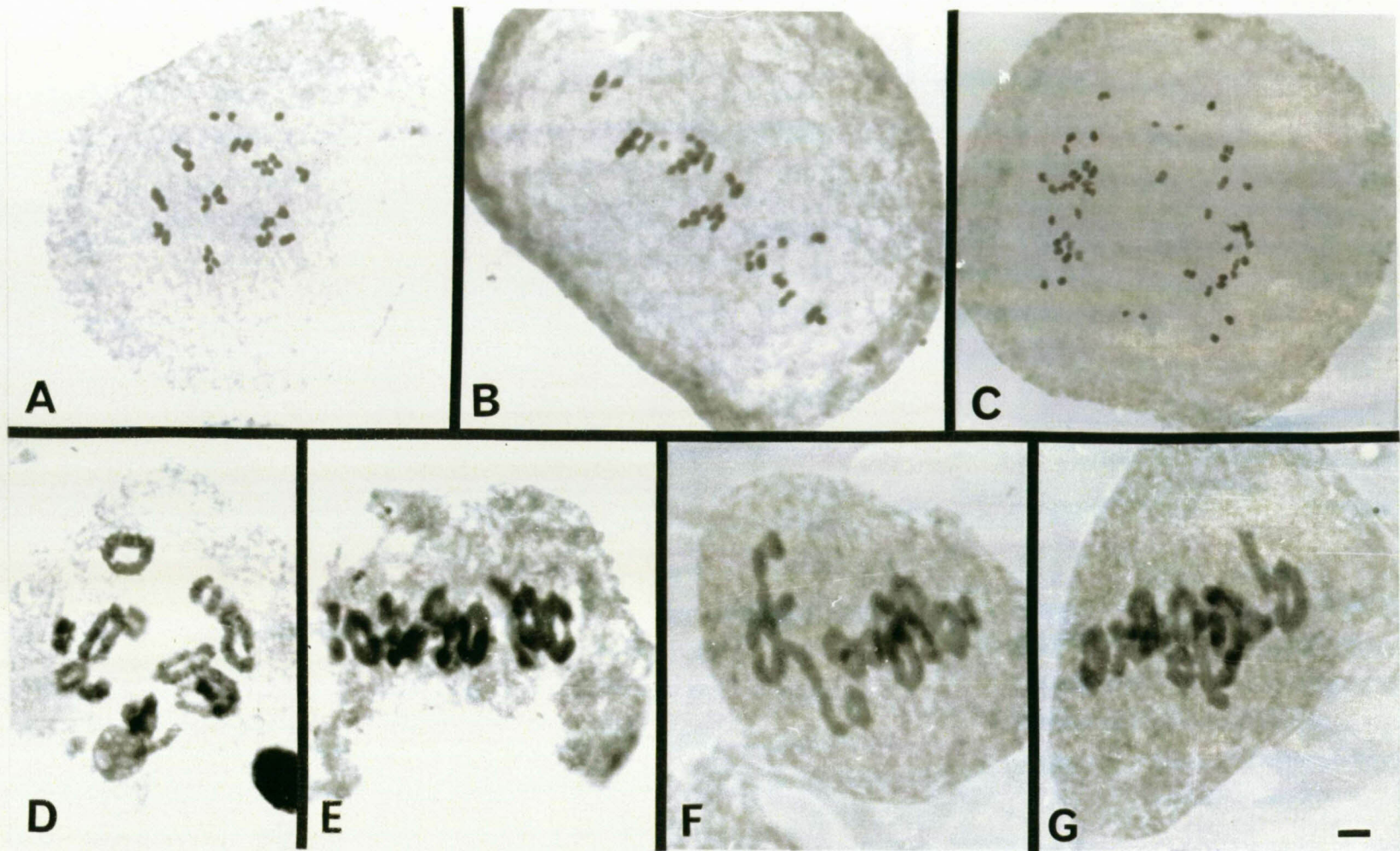


Figure 3.5 Meiotic chromosomes in polyploid specimens with six as basic chromosome number. **A-G**, *Styppeiochloa gynoglossa*: **A**, Saayman 79, early metaphase I, $n = 24$; **B**, Saayman 79, metaphase I, $n = 24$; **C**, Saayman 79, anaphase I (chromatid division), $n = 24$; **D**, Spies 1485, diakinesis, $n = 12$, $10_{II R}$, $2_{II K}$; **E**, Spies 1485, metaphase I, $n = 12$, $6_{II R}$, $6_{II K}$. **F**, **G**, Spies 2642, methaphase I, $n = 12$. Scale bar: A, B, C = $5.6 \mu\text{m}$; D = $3.7 \mu\text{m}$; E, F = $4.7 \mu\text{m}$; G = $4.2 \mu\text{m}$.

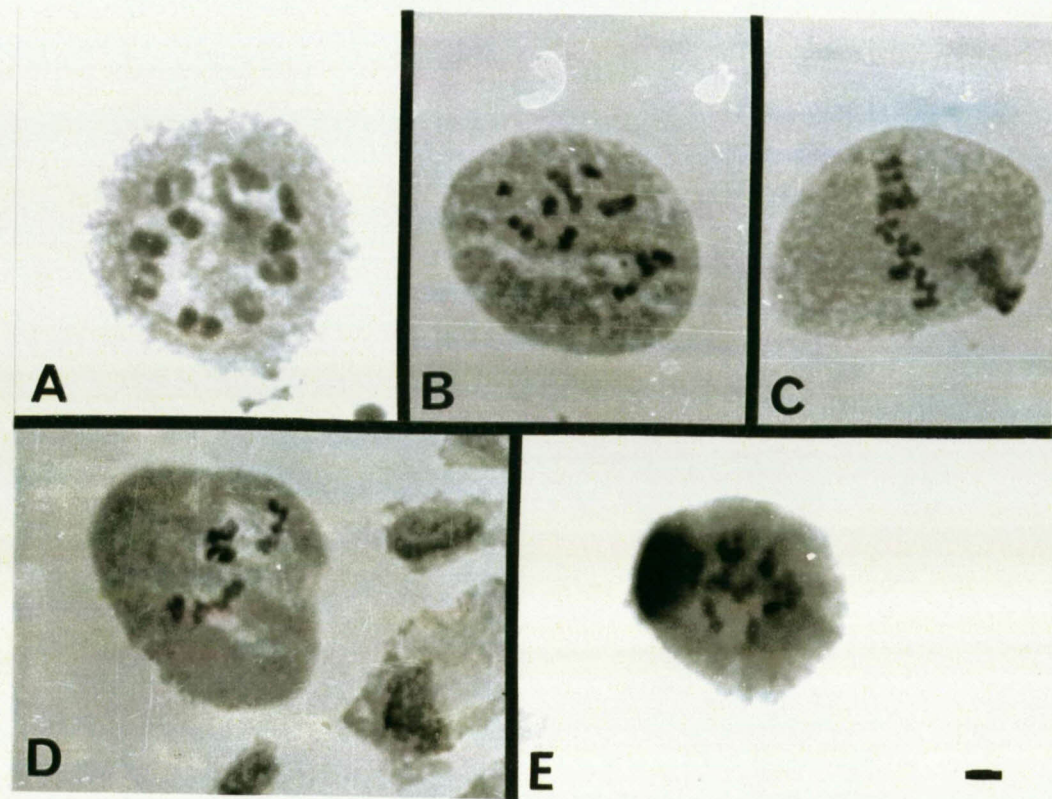


Figure 3.6 Meiotic chromosomes in polyloid specimens with six as basic chromosome number. **A, B, C**, *Tribolium acutiflorum*: **A, B**, *Spies 3866*, diakinesis, $n=12$, 12_{II} ; **C**, *Spies 3866*, metaphase I, $n=12$. **D, E**, *Tribolium hispidum*: **D**, *Spies 3509*, metaphase I, $n=12$; **E**, *Spies 3509*, diakinesis, $n=12$. Scale bar: **A, B, C** = 3.6 μm ; **D, E** = 5 μm .

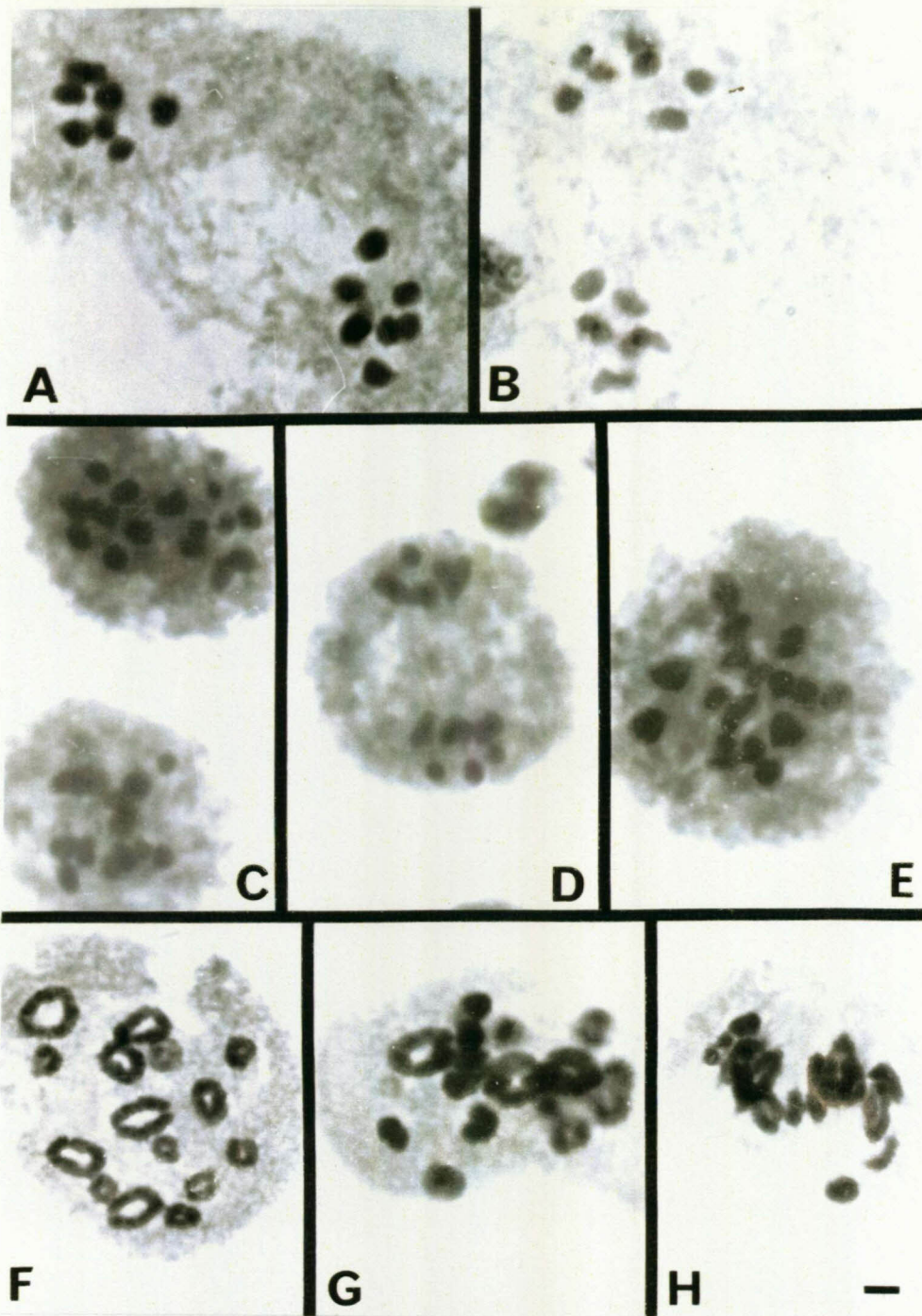


Figure 3.7 Meiotic chromosomes in *Pentameris* specimens. **A, B**, *P. thuarii*, *Spies 6160*, anaphase I, $n = 7$. **C, D, E**, *P. oreophila*: **C**, *Spies 6166*, diakinesis, $n = 7$; **D**, *Spies 6166*, anaphase I, $n = 7$; **E**, *Spies 6166*, metaphase I, $n = 7$. **F, G, H**, *P. macrocalycina*: **F**, *Spies 3644*, diakinesis, $n = 21$, 7_{IV} , $7_{II R}$; **G**, *Spies 3644*, diakinesis, $n = 21$, 6_{IV} , $8_{II R}$; **H**, *Spies 3644*, metaphase I, $n = 21$. Scale bar: **A, B** = $3.0 \mu\text{m}$; **C, D, E** = $3.6 \mu\text{m}$; **F, G, H** = $4.6 \mu\text{m}$.

In *Pentasthictis* the difference in basic chromosome numbers corresponds with geographical distribution. According to Du Plessis and Spies (1992), the basic number of seven predominates in southern African species, whereas $x = 13$ prevails in the remainder of Africa, except for *P. eriostoma*. A possible explanation for the occurrence of a basic chromosome number of 13 can be that this is the result of aneuploidy after polyploidisation of $x = 7$ ($n = 2x-1 = 13$) or of $x = 6$ ($n = 2x+1 = 13$). Thus, thirteen can be seen as a basic secondary chromosome number along with twelve (Stebbins 1956).

There are different views as to whether the primitive basic chromosome number for the Arundinoideae is six or seven. Davidse *et al.* (1986) suggested that $x = 7$ is a primitive number, as in the genus *Pentasthictis*, and that $x = 13$ was secondarily derived by an aneuploid reduction from $x = 14$. However, according to Hunziker and Stebbins (1987), the original primitive basic chromosome number is six, from which the secondary basic number $x = 12$ was derived and, thus, gave origin to higher polyploids. From $x = 6$ aneuploid increase probably resulted in $x = 7$. When the results in Table 3.2 are plotted (Fig. 3.8), it is clear that $x = 7$ forms a new polyploid complex and $x = 6$ an old mature complex. The majority of genera have a basic number of six.

From Table 3.2 the basic chromosome numbers for the genera studied can be deduced by investigating the various chromosome number reports.

Arundo – In this genus, three basic chromosome numbers are possible: four, six and ten. A basic chromosome number of four could be possible for all the observed chromosome numbers, except 70 and 110. This basic chromosome number, however, seems unlikely; because the occurrence of 15-ploid ($2n = 60$), 17-ploid ($2n = 68$) and 25-ploid ($2n = 100$) plants is highly unlikely. These uneven ploidy levels would undergo uneven segregation during meiosis and lead to more meiotic abnormalities. This is the result of the presence of an uneven number of genomes, which complicates chromosome pairing, chromosome segregation and could lead to sterility. The basic number of six seems to be the best option for a basic chromosome number, keeping the basic chromosome number of the rest of the tribe in mind. This number occurs in 40% of the observed cases.

Ten also seems to be a great possibility, representing 55% of the observed cases. The rest of the observations are deviations from chromosome numbers that can be derived from either six or ten. These deviations are probably the result of aneuploidy (gain or loss).

Centropodia – In this genus the basic chromosome number is six. The basic chromosome number of 12 is probably secondarily derived from six. A basic chromosome

Table 3.2 Total number of chromosome number reports for some of the genera in the tribe Arundineae. This data was used to obtain the basic chromosome number for each genus. Data taken from Table 3.1 was used to calculate the total number of chromosome number reports.

2n	24	60	64	66	68	70	72	100	110					
<i>Arundo</i>	1	2	2	1	1	2	4	2	5					
x = 4	60%													
x = 6	40%													
x = 10	55%													
2n	24	36	48											
<i>Centropodia</i>	3	1	2											
x = 12	100%													
2n	12	24	36	48	54	72								
<i>Chaetobromus</i>	5	4	21	2	2	2								
x = 4	94%													
x = 6	100%													
2n	70	72	76	90	108									
<i>Cortaderia</i>	1	4	2	3	2									
x = 4	66%													
x = 9	75%													
x = 10	33%													
x = 12	50%													
2n	42													
<i>Dregeochloa</i>	2													
x = 6	100%													
x = 7	100%													
2n	24	26												
<i>Elytrophorus</i>	3	1												
x = 12	75%													
2n	12	24												
<i>Karoochloa</i>	14	8												
x = 4	100%													
x = 6	100%													
2n	12	24	30	36	48	51	56							
<i>Merxmuellera</i>	2	19	11	2	5	1	1							
x = 4	70%													
x = 6	95%													

2n	12	14	36	42										
Pentameris	1	3	1	1										
x = 4	50%													
x = 6	25%													
x = 7	75%													
2n	14	26	28	40	42	52	56	70	78	84	98	104	180	
Pentastictis	86	10	37	1	6	7	4	1	1	2	1	1	2	
x = 7	86%													
x = 10	2.5%													
x = 13	12%													
2n	24	36	38	40	42	43	44	45	46	47	48	49	50	51
Phragmites	1	7	1	1	2	1	3	1	3	2	36	4	5	3
2n	52	53	54	55	56	57	58	59	72	84	96			
Phragmites (cont.)	6	1	3	1	3	1	1	1	5	1	9			
x = 6	63%													
x = 7	9%													
x = 13	6%													
2n	14													
Prionanthium	7													
x = 7	100%													
2n	24													
Pseudopentameris	1													
x = 4	100%													
x = 6	100%													
2n	12	24	36	48	72									
Schismus	26	5	1	1	1									
x = 4	100%													
x = 6	100%													
2n	24	48												
Styppeochloa	2	1												
x = 12	100%													
2n	12	24	36											
Tribolium	88	113	10											
x = 4	100%													
x = 6	100%													

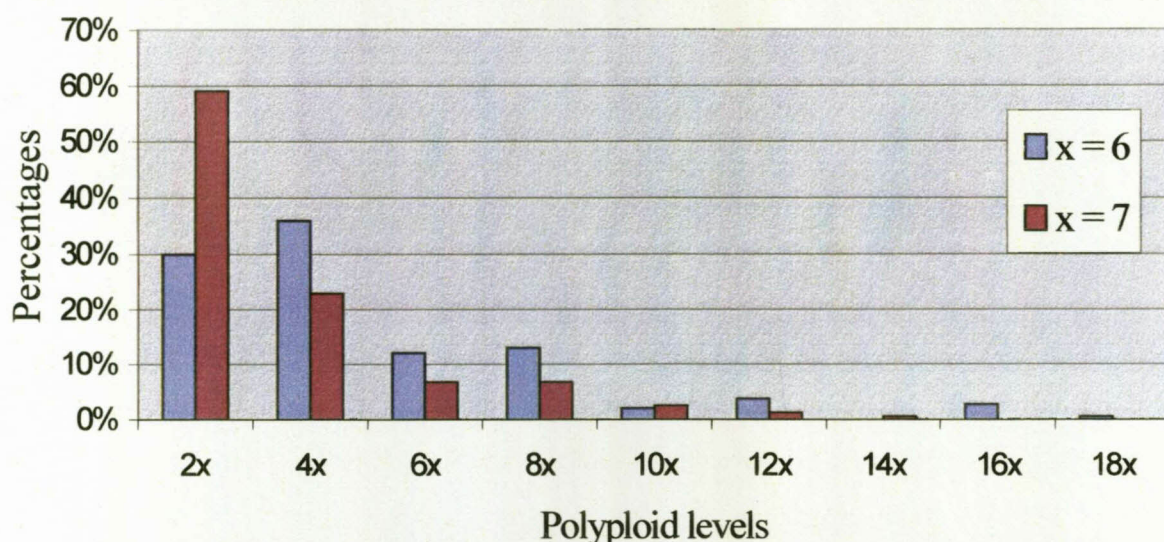


Figure 3.8 Percentage of polyploid levels present in specimens with 6 and 7 as basic chromosome numbers

number of twelve seems unlikely because of the observation of $2n = 36$. This would be a triploid organism if 12 were considered to be the basic chromosome number.

Chaetobromus – With this genus, two basic chromosome numbers are possible: four and six. Six is the only chromosome number that could account for all the observed chromosome number reports. A basic chromosome number of four forms uneven polyploid levels and does not account for all of the observed chromosome numbers. Twelve, as a basic chromosome number, is not considered because of the occurrence of haploid plants if this was the basic chromosome number.

Cortaderia – In this genus, the chromosome number of nine has the highest possibility (accounting for 75% of the observations) of being the basic chromosome number. The secondarily derived chromosome number of 12 accounts for 50% of the observed chromosome reports. Considering the placement of the genus in the Arundineae, a basic chromosome number of 12 seems likely to be highly possible. The other two chromosome numbers, four and ten, both have a lesser possibility and would indicate a great number of uneven ploidy levels.

Dregeochloa – Only two known reports of $2n = 42$ have been cited for this genus. This implies that six or seven could be the basic chromosome number. However, the chromosome number report on this genus does not mention any univalents or multivalents in these specimens (Spies & Du Plessis 1988) and, therefore, they are not heptaploid ($7x$ with six as a basic chromosome number). Seven would thus be the basic chromosome number, resulting in a hexaploid specimen.

Elytrophorus – Six is the basic chromosome number in this genus. The observation that 12 fits all the specimens can be attributed to the fact that 12 is a secondary basic chromosome number derived from six. A deviation of the expected chromosome number of $2n = 24$ or $2n = 30$ can be observed in the specimen with a somatic chromosome number of $2n = 26$. This probably resulted through loss or gain aneuploidy.

Karoochloa – In the genus *Karoochloa*, six is the only basic chromosome number which would best account for the observed chromosome numbers of 12 and 24. With four as a basic number, a great number of uneven polyploid levels would be created. The occurrence of so many uneven polyploid levels is highly unlikely. Twelve as a base number would imply a large percentage of haploid plants, which would not be viable.

Merxmullera – In *Merxmullera*, a basic chromosome number of six seems to be proven by the various chromosome number reports on this genus. Although four also seems to be a possibility, the large number of uneven polyploidy levels created by this basic chromosome number makes it less suitable.

Pentameris – Previous chromosome studies done on this genus, stated that the basic chromosome number is the same as that for the majority of the tribe, namely six. This study, however, unmistakably proves this to be erroneous. We place this genus in the minority group of genera in the Arundineae with seven as a basic chromosome number. Further studies are needed to investigate the extent of polyploidy and aneuploidy in this genus.

Pentaschistis – Seven is the basic chromosome number of this genus. This can be deduced from intensive chromosome number reports on this genus. Although 13 could only account for 12% of the observed cases, this chromosome number is believed to be a secondary basic chromosome number, derived through polyploidy and consequent aneuploidy. The basic chromosome number of ten occurs only in a minute fraction of the observed cases and is not considered relevant to the genus. The resultant somatic

chromosome numbers ($2n = 40$; $2n = 70$ and $2n = 180$) are thought to rather have been derived through aneuploidy.

Phragmites – In this pandemic grass, a basic chromosome number of six seems to be predominant in the whole genus. Despite this, a large number of aneuploid (either gain or loss) derived chromosome numbers, such as $2n = 43$; $2n = 47$; $2n = 51$; $2n = 53$ etc. also occur.

Prionanthium – This is also a genus, which has only been minimally investigated. This can be attributed to the extreme scarcity of this small grass genus. The studies done so far have undoubtedly shown seven to be the basic chromosome number of this genus.

Pseudopentameris – The chromosome count of $2n = 24$ for this genus, is, to the best of our knowledge, the first and only known chromosomal count for this genus. Four and six could easily be the basic chromosome number of this genus. Considering this genus' placement in the Arundineae and the chromosomal configurations observed, a basic chromosome number of six is recognized.

Schismus – In this genus two basic chromosome numbers can be recognised, namely four and six. Six definitely seems to be the basic chromosome number, with most of the observed specimens (76%) being diploid ($2n = 12$). The likelihood of four is limited and not considered to be the original basic chromosome number.

Styppeiochloa – This is another genus that has been neglected cytogenetically. The three specimens investigated in this study are the first known reports of this genus. Our study indicates that six is the original basic chromosome number. Twelve is probably a secondarily derived basic chromosome number.

Tribolium – The genus *Tribolium* has been thoroughly investigated over the last couple of years (Spies *et al.* 1992; Visser & Spies 1992a-e). These studies concluded that six is the basic chromosome number for this genus. Although four can also be considered, this possibility is eliminated because of the occurrence of uneven polyploid levels, as previously described.

In this study basic chromosome numbers of six (occurring in 12 genera and 48 species), seven (occurring in four genera and 48 species) and 13 (occurring in one genus and six species) have been confirmed for the tribe Arundineae. The genus *Cortaderia* needs

special consideration in light of the fact that it is not certain whether twelve (secondarily derived from six) or nine is the basic chromosome number for this genus.

3.3.2 Chromosomal abnormalities

Meiotic analysis can indicate whether a specimen (or sometimes even a species) is of hybrid origin. This evidence usually assists in the delimitation of biological species (Visser & Spies 1994e). An increased frequency of chromosomal abnormalities, particularly the presence of univalents during metaphase I, and chromosomal laggards during anaphase I, are expected in a hybrid. The occurrences of these abnormalities are usually correlated with the degree of divergence between the parental taxa (Visser & Spies 1994e). An increase in divergence would indicate a decrease in the degree of homology between the chromosomes present in the different genomes. Thus, an increase in the frequency of meiotic chromosome abnormalities in any hybrid between these taxa would occur as the degree of divergence between the two parental taxa increase. The frequency and types of abnormalities within each genus indicate the degree of homology between genomes and, consequently, aid in determining their phylogenies (Visser & Spies 1994e).

3.3.2.1 Polyploid levels

Polyploidy is frequent in the grasses and Stebbins (1985) postulated that more than 80% of the grasses in the world have undergone polyploidy at some time during their phylogenetic evolution. This figure is reflected in the South African grass flora as well. Cytogenetic studies on various species, show circa 81% of the grasses studied to be of polyploid origin (Moffett & Hurcombe 1949; De Wet 1954a, b; Pienaar 1955; De Wet & Anderson 1956; De Wet 1960; Vorster & Liebenberg 1977; Davidse *et al.* 1986; Liebenberg 1986; Spies & Du Plessis 1986a, b; Fossey & Liebenberg 1987; Spies & Du Plessis 1987a, b; Spies & Jonker 1987; Du Plessis & Spies 1988; Spies & Du Plessis 1988; Spies & Voges 1988; Spies *et al.* 1989, 1990, 1991; Du Plessis & Spies 1992; Spies *et al.* 1992; Liebenberg *et al.* 1993; Spies *et al.* 1994a, b; Strydom & Spies 1994; Visser & Spies 1994a-e; Spies & Van Wyk 1995; Spies *et al.* 1996a, b, 1997; Klopper *et al.* 1998; Visser *et al.* 1998a-c).

In this study three of the six tetraploid specimens, belonging to genera *Karoochloa* and *Styppeiochloa*, were analysed with the computer program "GENOOM". A part of this

program is based on various tetraploid models, formulated by Kimber and Alonso (1981). These four models are the 4:0, 3:1, 2:1:1 and 2:2 models and are based on the degree of homology between the genomes present. Chromosome configurations are used to determine the relative affinity between the genomes and this is expressed by an x-value. With this computer program, expected and observed chromosome configurations are compared and the sums of squares between these values for each specimen, are calculated (Kimber & Alonso 1981). The model with the lowest sum of squares is considered to be the model best suited for that particular specimen. In the case of a 3:1 model, an x-value of 0.5 indicates that the last genome corresponds to the first three and that the specimen has a genomic constitution of AAAA (autoploidy). With this same model, an x-value of 1 indicates no correspondence between the last genome and the first three and the specimen will have a genomic constitution of AAAB (alloploidy). The 3:1 model with an x-value of 1 would indicate a genomic composition of AAAB, and the 2:2 model with an x-value of 1 would indicate a genomic composition of AABB (Kimber & Alonso 1981). Values ranging from 0.5 to 1 indicate varying degrees of homoeology and would indicate segmental allopolyploidy. For example, a value of 0.6 for the 2:2 model would indicate a segmental allopolyploid, with a tendency towards autoploidy (Kimber & Alonso 1981). Due to an insufficient number of diakinesis cells, three of the six tetraploid species examined in this study (belonging to the genera *Merxmuellera* and *Tribolium*) could not be analysed.

The 2:2 model fitted all three of these specimens the best (Table 3.3). The *Karoochloa* specimen has an x-value which is very difficult to access as tending towards autoploidy or allopolyploidy. Therefore, this specimen is classified as a segmental allopolyploid. The high x-value seem to indicate segmental allopolyploidy with a tendency towards allopolyploidy in the case of *Styppeiochloa gynoglossa* (Spies 1485). The other *S. gynoglossa* specimen, Spies 2642, has a value of 1, which indicates that this is an allopolyploid specimen. In both *Styppeiochloa* specimens mostly bivalents were observed (Fig. 3.5D-G). In Spies 1485, multivalent formation was observed in some cells (Fig. 3.5D), but in Spies 2642 only rod and ring bivalents were observed (Fig. 3.5F, G): evident of allopolyploidy. The formation of mainly bivalents in specimens, such as Spies 2642, indicates an allopolyploid or segmental allopolyploid origin. This usually supports a hybrid origin for those particular taxa. Liebenberg (1986) suggested that the genomic composition of different segmental allopolyploids and possible autoploids within a particular polyploid level vary from area to area. This could indicate some karyotype evolution that occurred in the divergence of the different biotypes

and that the hybrid complex is made up out of various closely related homoeologous and homologous genomes. Homoeologous genomes account for segmental allopolyploids, whereas homologous genomes account for autopolyploids.

The polyploid complex represents the most common pattern of morphological variability and dispersal that is found among genera of grasses and other plants containing polyploidy. These complexes usually contain two or more diploid species or subspecies and/or their hybrids. Each taxon has a distinctive set of morphological characteristics and a well-defined ecological and geographical distribution (Stebbins 1956). However, these diploids represents, as a rule, only a small section of the complex and are outnumbered by the polyploids. These may include a few types which are barely distinguishable in outward appearance from their diploid ancestors, but a greater portion of the polyploids are easily recognisable by their outward appearance (Stebbins 1956). The polyploid members of these complexes are usually more widespread than their diploid ancestors, and are usually adapted to a great variety of habitats, instead of having neatly defined ranges of ecological tolerance (Stebbins 1956).

Table 3.3 Genomic relationships in the tetraploid specimens analysed according to the models of Kimber and Alonso (1981). Values indicated represents the sums of squares calculated for the four possible tetraploid models. The x-values are indicated in parentheses. The model best suited for each specimen is indicated in bold.

Specimen	Voucher number	Chiasma frequency	4:0 model	3:1 model	2:2 model	2:1:1 model
<i>Karroochloa purpurea</i>	<i>Spies 2473</i>	1.00	1.475	1.482 (0.5)	0.00005 (0.693)	0.0013 (0.836)
<i>Styppeiochloa gynoglossa</i>	<i>Spies 1485</i>	0.86	3.688	3.973 (0.9485)	0.416 (0.829)	0.458 (0.9389)
<i>Styppeiochloa gynoglossa</i>	<i>Spies 2642</i>	0.81	6.570	7.135 (0.92)	0.000003 (1)	1.835 (1)

Successful polyploid complexes evidently arise from recombination through hybridisation between genetic types adapted to entirely different environments or possessing different mechanisms of adaptation to the same environment. This genetic recombination builds up modes of adaptation to entirely new environments, to which none of the ancestral diploids were adapted (Stebbins 1956). The process of chromosome doubling may serve one of two purposes. Species, which are sterile hybrids because of differences in chromosome patterning of their parental species, may be turned into fertile hybrid polyploids. Fertile hybrids between races, or subspecies of the same species can, by doubling their chromosome number, become buffered against too rapid dispersion of their intermediate genotypes. This can be achieved by genetic segregation, since at the tetraploid level segregation is far more complex and each gene has a smaller effect on variation than it has on the diploid level (Stebbins 1956). On the other hand, the larger number of genes present in polyploids tend to reduce the efficiency of mutations, and consequently progressive evolution in the direction of completely new mechanisms of adaptation is slower in polyploids than in the diploids. For this reason polyploid complexes may pass through a series of stages:

1. Young polyploid complex: Most recent origin, diploid species are most common.
2. Mature polyploid complexes:
 - 2.1 Young mature polyploid complex: Diploids are far less common than polyploids and tend to be endemics.
 - 2.2 Old mature polyploid complex: Nearly all diploid species have become extinct and new cycles of polyploidy have arisen, based on the chromosome doubling and hybridisation of species which behave like diploids, but have basic chromosome numbers of ancient polyploid origin.
3. Old polyploid complexes: All the diploid ancestors have died out, but the basic chromosome number for the genus can still be determined.
4. Polyploid relicts: Monotypic genera of very high chromosome number, but which has no close existing relatives. It is impossible to determine the basic chromosome number of the genus (Stebbins 1956).

Intraspecific polyploidy is common in the following genera in the tribe Arundineae: *Arundo*, *Cortaderia*, *Chaetobromus*, *Merxmuellera*, *Pentaschistis*, *Phragmites*, *Schismus* and *Tribolium*. This leads to the existence of many polyploid complexes in the tribe as well. Studies done on the genus *Chaetobromus* by Spies *et al.* (1990) suggested a polyploid complex in this genus that ranged from diploid to duodecaploid. In this complex, polyploidy mainly occurs in the form of segmental allopolyploidy and occasionally as allopolyploidy (Spies *et al.* 1990). Polyploidy is frequently encountered in *Pentaschistis*. In the study by Du Plessis & Spies (1992), 13 of the 22 species investigated, were polyploid, or had different polyploid levels. Spies *et al.* (1994) suggested the existence of a polyploid complex in *Pentaschistis airoides* (Nees) Stapf. This polyploid complex includes specimens of hybrid origin on both diploid and polyploid levels. Klopper *et al.* (1998) reported on the existence of young polyploid complexes in 17 *Pentaschistis* species. Twelve species were found to be old polyploid complexes. These species were, however, not adequately studied and the age of the complexes should be verified. Furthermore, the genus *Pentaschistis* is suggested to be a young polyploid hybrid complex (Klopper *et al.* 1998). Du Plessis and Spies (1988) reported on a polyploid complex in *Schismus barbatus*, based on the meiotic behaviour of one diploid, three tetraploid and one hexaploid specimen studied in this species. In *Tribolium* polyploidy is common, but less frequent in the section *Tribolium*. This led Visser and Spies (1994c, d, e) to conclude that the genus *Tribolium* is a polyploid complex.

Polyploidy has also been observed in the following genera: *Centropodia*, *Dregeochloa*, *Elytrophorus*, *Karoochloa* and *Pentameris*. Because of the limited nature of the investigations on these genera, the occurrence of polyploidy could be higher. No polyploidy has as yet been observed in the genus *Prionanthium*. The reason for this could also be due to an insufficient number of specimens investigated. Furthermore, this genus comprises of annual species, with limited distributions, and annuals have a tendency towards lower ploidy levels.

Occasional or recurrent hybridisation and the complete breakdown of reproductive isolation between sympatric species result in the production of hybrid swarms. These swarms usually include the whole range of genetic variability of the parental species. This could be evident in species with a wide range of morphological, genetic and chromosomal variation (Visser *et al.* 1998a).

In this study polyploid levels were again confirmed in the genera *Centropodia*, *Karroochloa*, *Merxmuellera*, *Pentameris*, *Styppeiochloa* and *Tribolium*. These ranged from tetraploid (six species) to hexaploid (three species) and octaploid (two species).

When the genera with a basic chromosome number of six and those with a basic chromosome number are compared, six is the predominant base chromosome number. As can be seen from the graph in Fig. 3.8, six is the chromosome number found in most instances, as well as the chromosome number occurring in most of the genera (12) in the tribe Arundineae. Therefore, we agree with Hunziker and Stebbins (1987) that six is the basic chromosome number of the Arundineae and that seven was derived from this basic number through aneuploidy.

3.3.2.2 Cell fusion

No evidence for the occurrence of cell fusion (Spies & Van Wyk 1995) was found in this study, but the role that this phenomenon plays in polyploidy and evolution should not be underestimated.

Cell fusion is a process, which involves the cells adhering to each other and forming cytoplasmic bridges between them. These bridges are the means by which the nucleus of one cell moves to the other cell, by way of cytomixis. These bridges gradually widen and eventually the cells are completely fused (Spies & Van Wyk 1995). This will, consequently lead to polyploidy, since an additional chromosome complement is added to the gamete. This process can involve from two to many cells. Characteristics of these fused cells are size, presence of more than one nucleus during interphase and the formation of bivalents. This leads to gametes with higher chromosome numbers because of separate spindle formation (Spies & Van Wyk 1995).

A reason for the disregard of fusion cells may, in the past, have been due to the fear of contamination. Different polyploid levels in the same anther could have been attributed to contamination of some instrument during the squashing of the anther (Spies & Van Wyk 1995).

At present, cell fusion indicates dramatic changes in the polyploidy levels of the South African grasses, as a whole, for example in *Chaetobromus* (Spies & Van Wyk 1995), *Merxmuellera* (Spies & Van Wyk 1995), *Tribolium* (Visser & Spies 1994c, d, e) and *Pentaschistis* (Klopper *et al.* 1998). These genera belong to the tribe Arundineae.

The reason for the occurrence of this phenomenon can, as yet, not be attributed to any genetic or ecological determinant. According to Visser (1992), it may be assumed that fused cells do not usually adapt to such high chromosome numbers. Therefore, these cells are presumably sterile or semi-sterile. This can be the reason for the absence of specimens, with the high chromosome numbers associated with cell fusion, in nature (Visser 1992). Another reason could be that, for the progeny to be fertile, the seed donor must have a higher chromosome number than the pollen parent. In nature no such specimens exist that could contribute to this kind of fertilization (Visser 1992). Brown and Berttke (1969) suggested that cytomixis is under the influence of genes.

3.3.2.3. B-chromosomes

B-chromosomes, or accessory chromosomes, are present in many plant and animal cells and differ in many respects from normal chromosomes (A-chromosomes or euchromosomes) (Randolph 1928). B-chromosomes usually differ between different cells, tissues, individuals, populations and generations (Jones & Rees 1982). Therefore, it is usually not possible to use this phenomenon in determining phylogenetic relationships.

The most studies on B-chromosomes in plants have been made in the grasses (Bosemark 1957). According to Jones (1975), 14 families of the monocotyledons have B-carrying species. These species are concentrated largely in the Poaceae followed by Lilliaceae and Amarilladaceae (Jones 1975).

B-chromosomes are usually smaller than the normal A-chromosome complement and do not display Mendelian inheritance. They often exhibit nondisjunction during mitotic anaphase. Therefore, within an individual, their frequencies vary from one organ to another. A study done by Bosemark (1957) showed variation in B-chromosomes, not only between flowers, but also within flowers and individual anthers. The mechanism underlying this variation is not known. It may result from the loss of the B-chromosome at some stage during early development of the panicles combined with similar events in premeiotic mitosis in the anthers. In great numbers B-chromosomes could reduce fertility and diminish growth. These chromosomes are not known to carry any genes with major effects (Jones & Rees 1982).

B-chromosomes were observed in the following specimens in this study: *Karoochloa purpurea* (Spies 2473) (Fig. 3.9A-E) and *Merxmullera stricta* (Spies 6288) (Fig. 3.9F). These chromosomes behaved by clustering to one side of the metaphase plate

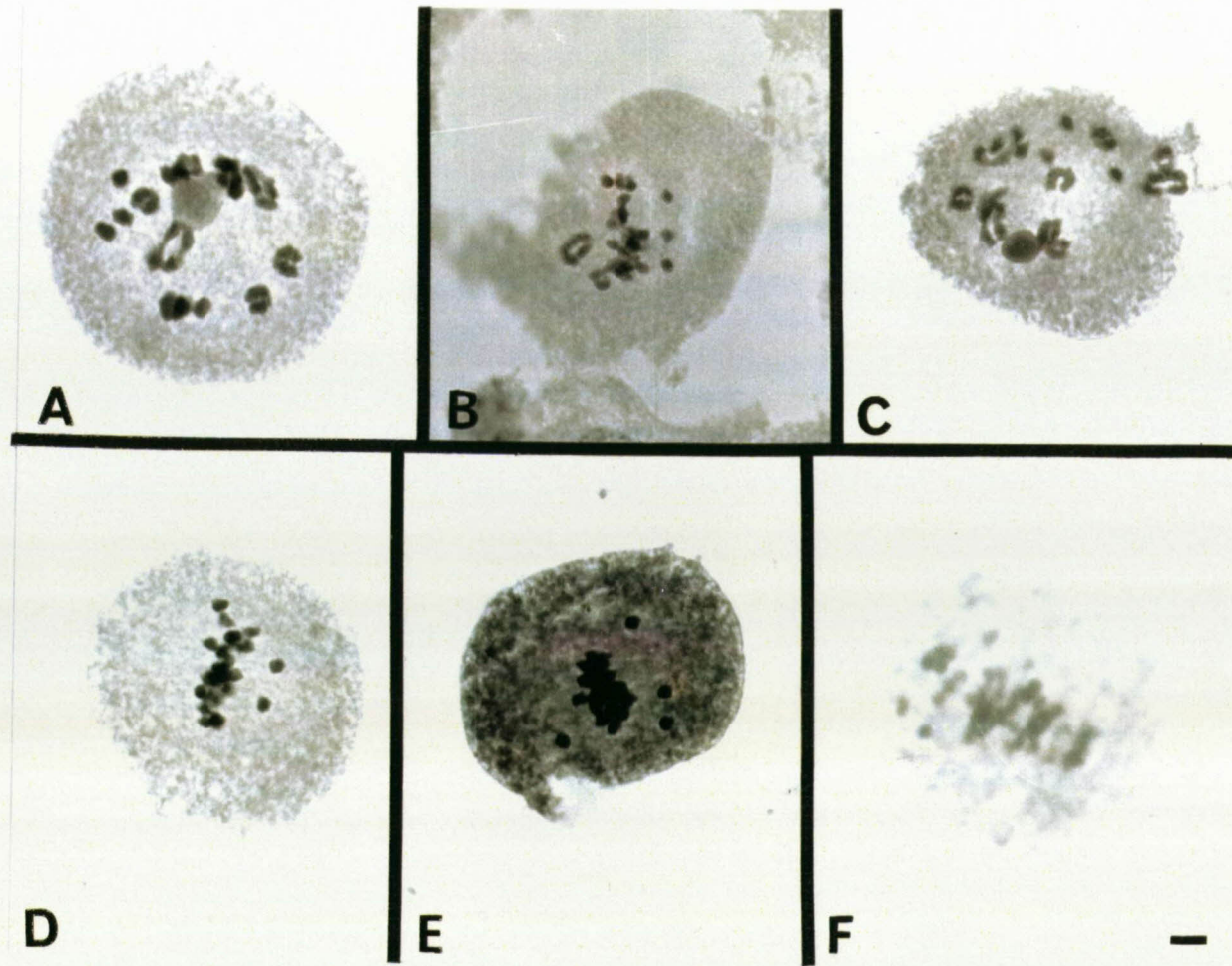


Figure 3.9 Meiotic chromosomes in studied specimens with B-chromosomes. **A-E**, *Karroochloa purpurea*, *Spies 2473*: **A - C**, diakinesis with 5 B's; **D**, metaphase I with 4 B's; **E**, metaphase I with 5 B's. **F**, *Merxmuellera stricta*, *Spies 6288*, metaphase I with 4 B's. Scale bar: A = 3.5 μm ; B, C, D, E = 4.2 μm ; F = 5 μm .

(Fig. 3.9D-F). B-chromosomes clustered to one side, as observed in this study, will result in an uneven distribution of chromosomes. B-chromosomes can also, however, form part of the metaphase plate. They do not usually divide normally and most often result in anaphase laggards and eventually micronuclei. B-chromosomes can pair strictly *inter se* and also have the capability to form multivalents when more than two are present, although they seldom pair with the same efficiency and regularity as do the A-chromosomes (Jones 1975).

B-chromosome have also been reported from the following genera:

- *Pentaschistis*. The number of B-chromosomes per cell varies within the same species and even within the same specimen. In the study by Du Plessis and Spies (1992), the paired B-chromosomes phenomenon was observed in four species, namely *P. capillaris* (Thunb.) McClean, *P. curvifolia* (Schrad.) Stapf, *P. eriostoma* and *P. pallida* (Thunb.) Linder. These chromosomes were morphologically similar to the euchromosomes and unpaired chromosomes that were smaller than the normal chromosomal complement. In the study by Spies *et al.* (1994), the frequency of B-chromosomes was low (15%) in the species *P. airoides* and *P. patula* (Nees) Stapf.
- *Prionanthium*. B-chromosomes have only been observed in two of the three species, namely *P. dentatum* (0-1) and *P. pholioroides* (0-3) (Visser & Spies 1994e). Davidse (1988) observed B-chromosome in *P. pholioroides* (0-1) only.
- *Tribolium*. In this genus B-chromosomes have a wide range of distribution, from 0-1 in *T. utriculosum* (Nees) Renvoize to 0-7 in *T. hispidum*. In *T. echinatum* (Thunb.) Renvoize the frequency of cells containing B-chromosomes ranged from 9% to 100% (Spies *et al.* 1992). B-chromosomes are thought to be associated with the phenomenon of precocious chromosome segregation (Visser & Spies 1994c). In *T. brachystachyum* (Nees) Renvoize B-chromosomes were present in all the specimens studied by Visser and Spies (1994d). In *T. pusillum* B-chromosomes have been observed, but only in low frequencies (0-2) (Visser & Spies 1994e).

Chiasma frequency and distribution are genetically controlled, but are also subject to environmental modification. It is not surprising that B-chromosomes should effect the pattern of A-chromosome recombination at meiosis (Jones 1975). Firstly, as chromosomes they change the genotype. Secondly, aside from genetic properties, they alter the immediate environment of the A-chromosomes themselves, that is the nucleus. Lastly, they are known to interfere with many other gene-controlled aspects of growth and development (Jones 1975). Various quantitative analyses have revealed that B-chromosomes have a wide-ranging effect on the phenotype, especially in plants. It could be that they have a wide spectrum of influence over various different gene-controlled processes or, alternatively, that they operate on some fundamental physiological process with pleiotropic effects (Jones 1975). Another aspect of B-chromosomes is the differential activity in relation to odd and even numbered combinations. Even numbered combinations seem to have a lesser effect on vigor and aspects of nuclear metabolism, including recombination, as can be seen from studies done on rye (Jones 1975).

3.3.2.4 Univalents

Univalents were only observed in *Stypeiochloa gynoglossa* (Saayman 79) (Fig. 3.10A) and *Merxmuellera stricta* (Spies 3140) (Fig. 3.10B-C). Univalents are lacking either a homologue, or result from asynapsis or desynapsis. Asynapsis is the absence of any meiotic pairing due to asynaptic genes or the influence of environmental factors (Rieger *et al.* 1976). During desynapsis bivalents fall apart during diplotene or diakinesis (Li *et al.* 1945).

These chromosomes usually lie randomly on the spindle during metaphase I and may follow one of two courses during anaphase (Darlington 1957). The chromosomes lying far from the spindle equator can be randomly distributed towards the spindle poles. On the other hand, those lying near the equator move onto the plate where they orientate axially and divide into the two chromatids which in turn pass onto the opposite poles (Darlington 1957). These chromatids will be smaller than a normal univalent. The univalent may not be included in one of the daughter nuclei and then becomes lost in the cytoplasm. Previously it was found that the presence of several univalents in a single meiotic cell may completely impair the separation of the chromosomes into two daughter groups and then form a restitution nucleus (Rieger *et al.* 1976).

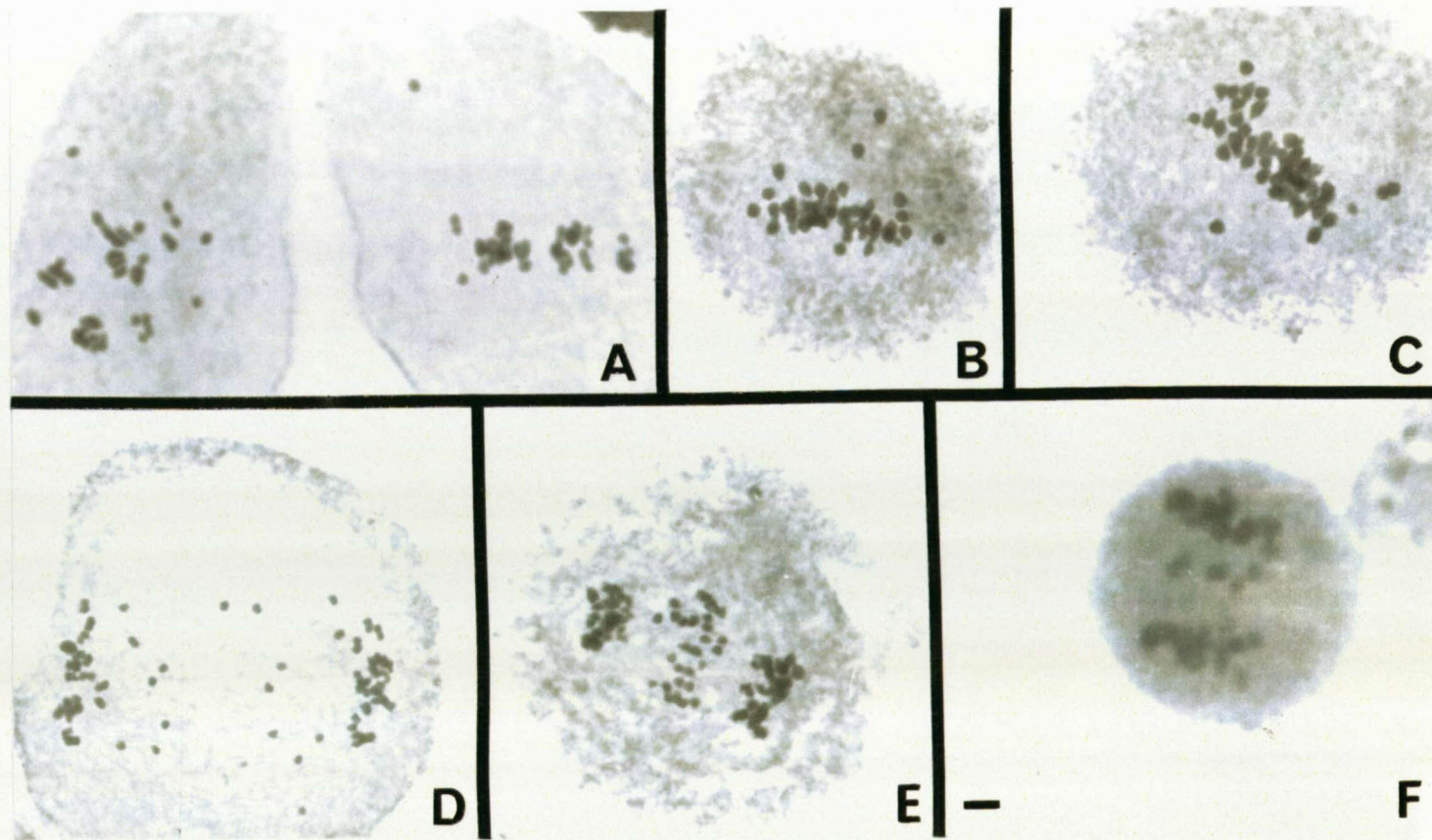


Figure 3.10 Meiotic chromosomes in some studied specimens with chromosomal abnormalities. **A**, *Styppeiochloa gynoglossa*, Saayman 79, metaphase with an univalent. **B**, **C**, *Merxmuellera stricta*: **B**, Spies 3140, metaphase I with 9 univalents; **C**, Spies 3140, metaphase I with 5 univalents. **D**, *Styppeiochloa gynoglossa*, Saayman 79, anaphase laggards (17). **E**, *Merxmuellera stricta*, Spies 3140, anaphase I with 27 laggards. **F**, *Merxmuellera stricta*, Spies 6288, anaphase I with 5 laggards. Scale bar: A, D = 6.2 μm ; B, E = 10 μm ; C = 7.5 μm ; F = 4.2 μm .

This phenomenon is not uncommon in the tribe Arundineae. Klopper *et al.* (1998) reported on the occurrence of univalents in nearly all the *Pentaschistis* specimens investigated in that particular study. In the study by Visser and Spies (1994c, d, e) univalents were observed in five species of *Tribolium*, namely *T. brachystachyum*, *T. echinatum*, *T. hispidum*, *T. obliterum* (Hemsl.) Renvoize and *T. uniolae* (L.f.) Renvoize. The greatest occurrence of univalents (11), was found in *Tribolium hispidum* by Visser and Spies (1994c).

In the study by Spies *et al.* (1992), it was proposed that the occurrence of univalents in a specific *Tribolium brachystachyum* specimen was probably the result of inadequate homology for chromosome pairing. In the genus *Tribolium* the presence of B-chromosomes is closely associated with univalents (Visser & Spies 1994c, d, e). Visser and Spies (1994e) did not observe this phenomenon in the genus *Prionanthium*.

3.3.2.5 Laggards

Chromosomes that lagged during anaphase I were observed in *Styppeiochloa gynoglossa* (Saayman 79) (Fig. 3.10D-E) and *Merxmullera stricta* (Spies 6288) (Fig. 3.10F). Laggards are chromosomes, which display no movement at all, or else retarded movement during anaphase. This is in comparison to the rest of the chromosomes, which undergo normal meiosis. A consequence of this is usually the failure of these chromosomes to be included in to one of the two daughter nuclei. This may lead to the formation of micronuclei during telophase I. Micronuclei are additional nuclei. These structures are formed by the lagging chromosomes during telophase. In the study on *Pentaschistis*, (Klopper *et al.* 1998) observed a small number of micronuclei throughout the whole genus. Furthermore, in *Pentaschistis* lagging chromosomes were observed in $\pm 25\%$ of the studied species (Klopper *et al.* 1998). In *Tribolium* laggards were observed in *T. acutiflorum*, *T. brachystachyum*, *T. echinatum*, *T. hispidum*, *T. obliterum*, *T. pusillum* and *T. uniolae*. Laggards were also observed in *Prionanthium pholioroides* (Visser & Spies 1994c, d, e). The univalents observed in *Tribolium* usually resulted in the lagging of chromosomes. These laggards were closely associated with the presence of B-chromosomes (Visser & Spies 1994c, d, e). Spies *et al.* (1992) found an abundance of laggards in *T. uniolae* and *T. brachystachyum*. This raises the question whether these species would be capable of sexual reproduction?

When we investigate the one *Merxmuellera* specimen more closely (Spies 3140) (Fig. 3.10F), the high occurrence of laggards, in this specimen, together with its chromosome number of $2n = 6x = 51$ would suggest this specimen to be of hybrid origin. Many *Merxmuellera stricta* specimens, investigated so far (Spies, personal communication), have been found to contain a high number of chromosomal laggards. *Merxmuellera stricta* is a variable perennial species. Chippindall (1955) states that "There is considerable variation in the plants referred to as *Danthonia stricta* (= *M. stricta*), and it is possible that they comprise more than one variety." Ellis (1980b) divided *M. stricta* into four "forms": the typical "form" (*M. stricta*), the Drakensberg "form" (*M. stricta*), the Cathedral Peak "form" (*M. guillarmodiae* Conert) and the alpine "form" (*M. guillarmodiae*). Each of these "forms" exhibits distinct epidermal structure and leaf anatomy. In the Drakensberg region, two *Pentaschistis* species displayed remarkable anatomical similarities with *M. stricta*. These are *Pentaschistis tysonii* Stapf and an unnamed *Pentaschistis* species. Anatomically they seem to show greater affinity with the *M. stricta* group than with *Pentaschistis* (Ellis 1980b). This raises the issue as to whether hybridisation occurred, or is still occurring, between these different *Merxmuellera stricta* forms, or between the *Pentaschistis* species and *M. stricta*, and whether this could clarify the possible hybrid nature of *M. stricta*. Presumably, diversity in hybrid taxa will be strongly affected by several factors, including the number of parental individuals involved in their origin, the degree of genetic divergence between the parental species, mating systems, species age, and phylogeny (Morrell & Rieseberg 1998).

Spies *et al.* (1990) reported on extensive morphological, cytogenetical and anatomical variation in *Chaetobromus*, indicative of hybridisation and polyploidy in this genus. According to Visser and Spies (1994c), the segmental allopolyploid nature of the polyploids in *Tribolium*, the intermediate morphological forms and the morphological overlapping, indicate that hybridisation played an important role in the evolution of the section *Tribolium* in the genus *Tribolium* (Visser & Spies 1994c). This is valid for the section *Uniolae* as well (Visser & Spies 1994d). The presence of a sexual reproduction system in *Tribolium* (Visser & Spies 1994b) contributes to the probability of hybridisation.

Laggards frequently occur in the tribe and can usually be attributed to the polyploidy status of the specimen. This is evident from the results found in this study: *Styppeiochloa gynoglossa* (Saayman 79) (octaploid) and *Merxmuellera stricta* (Spies 6288) (tetraploid).

3.3.2.6 Other chromosomal abnormalities

Other chromosomal abnormalities, such as anaphase bridges and fragments, were much less frequent and only rarely observed during this study. These are not thought to have had such a great influence on plant fertility or evolutionary adaptation.

Anaphase I bridges has been observed by Visser and Spies (1994c, d, e) in various *Tribolium* specimens. The occurrence of this abnormality is usually restricted to higher polyploid levels. In *T. brachystachyum*, (Spies *et al.* 1992) anaphase I bridges were observed in all the specimens studied (Fig. 3.11A, B). This is indicative of the lack of genome homology. A high percentage of univalents (much greater than the multivalents) along with the presence of anaphase bridges suggests a hybrid origin for this species (Spies *et al.* 1992). In the study by Visser and Spies (1994d) the highest frequency of anaphase bridges in *Tribolium* was also observed in *T. brachystachyum*. According to Linder and Davidse (1997), two putative parents could be *Tribolium hispidum* and *T. uniolae*. This is due to the fact that *T. brachystachyum* has the pubescence of *T. hispidum* and the leaf anatomy and inflorescence structure of *T. uniolae*. Another possibility is that the specimens are hybrids between "true" high-altitude *T. brachystachyum* species and the high-altitude forms of *T. uniolae* (Linder & Davidse 1997).

In *Pentasthis* anaphase I or II bridges were observed in \pm 11% of the studied specimens (Klopper *et al.* 1998).

The uneven segregation of chromosomes during anaphase I, was frequently observed in *Tribolium*. This abnormality is closely associated with the polyploid level of a specimen (Visser & Spies 1994c). Two factors, which could play an important role in the uneven segregation of chromosomes in polyploids, are the orientation and the configuration of the multivalents on the metaphase plate (Visser & Spies 1994d).

Deviation from normal meiosis occurs with the precocious segregation of bivalents during metaphase I. In this case a single bivalent segregates very early and it can even precede the segregation of multivalents (Spies *et al.* 1992). In *Tribolium*, this phenomenon was observed in *T. brachystachyum*, *T. echinatum*, *T. hispidum*, *T. oblitterum* and *T. uniolae* (Visser & Spies 1994c, d, e.). In a particular *T. brachystachyum* specimen this phenomenon was observed in 20% of metaphase I cells (Spies *et al.* 1992). This precocious segregation coincides with the presence of B-chromosomes, and was observed from diploid (*T. uniolae*) to higher polyploid levels (*T. brachystachyum*) (Visser & Spies 1994c, d, e). The phenomenon is attributed to either poor genetic control or poor chromosome

homology. This could also indicate the absence of Ph-like genes and their consequent inhibition on homoeologous chromosome pairing (Sears & Okamoto 1958).

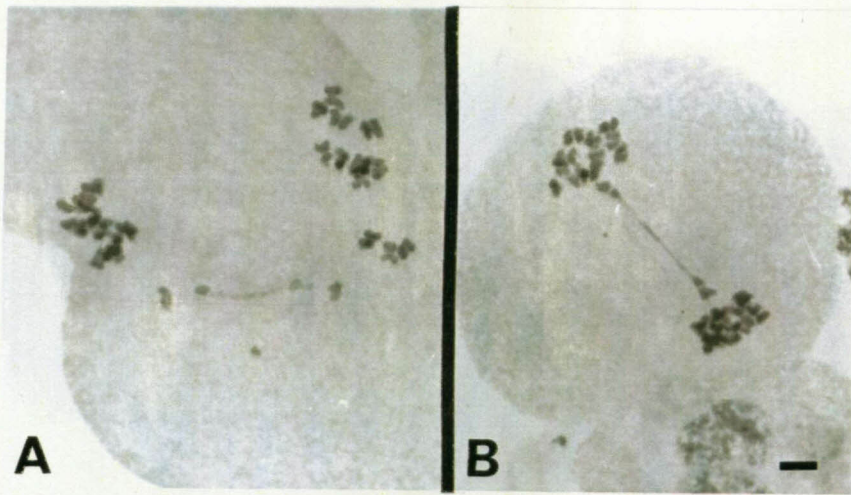


Figure 3.11 Meiotic chromosomes in *Tribolium brachystachyum*. **A, B**, Spies 3875, anaphase I bridges. Scale bar: A, B = 5.6 μm .

3.4 Phylogenetic relationships

In general, chromosomal data is difficult to interpret and to use in phylogenetic relationship determination. This is due to the effects of the varying chromosomal characteristics.

1. Basic chromosome number cannot be used, because two unrelated genera or even species may share the same chromosome number. This parameter can only be used in the cladistic analysis of closely related taxa or in cases where genome homology has been proven (Burger 1995). For example, a basic chromosome number of six, is a plesiomorphic character which occurs widely in the Arundineae, and is thus not very informative in deciding generic relationships (Spies *et al.* 1992).
2. Polyploid levels based solely on basic chromosome numbers cannot be used. In closely related taxa (eg. in a genus) polyploid levels may contribute to a cladistical study. In the study of a polyploid complex many specimens,

representative of the whole geographical distribution of the group, should be studied (Burger 1995).

3. B-chromosomes vary in number between different cells, tissues, individuals, populations and generations and the use of these chromosome numbers, as parameters for phylogeny, are usually not feasible (Burger 1995).
4. B-chromosomes, laggards, anaphase bridges and micronuclei, have a direct influence on the evolutionary process and are probably also the result of environmental influences. Consequently, these characters are also not suitable for phylogenetic purposes (Burger 1995).

A reasonable appraisal of the taxonomic value of chromosomal parameters depends on knowledge of the nature and amount of chromosomal variation between and within populations, and between closely related but reproductively more or less isolated gene pools or major populations (Greilhuber 1984).

Phylogenetic relationships found in the tribe so far are supported by this study. Since only a few genera share seven as a basic chromosome number it is necessary to look at this relationship between *Prionanthium*, *Pentaschistis*, *Dregeochloa* and *Pentameris*. *Pentaschistis* and *Prionanthium* share the feature that both possess multicellular glands. According to Davidse (1988), this seems to be a very important character in relating these genera, because multicellular glands are very rare in the family Poaceae. Furthermore, according to Davidse (1988) the genera *Pentaschistis* and *Prionanthium* also share large chromosomes, among the largest in the subfamily. *Prionanthium* and *Dregeochloa* are, lastly, very rare grass species with very limited distributions. In the molecular phylogenetic study on the Arundinoideae, based on rDNA sequences by Hsiao *et al.* (1998a), they found that *Pentaschistis aspera* (Thunb.) Stapf, *Prionanthium ecklonii* and *Pentameris macrocalycina* form a monophyletic clade with a bootstrap support index of 100%. In this article these species are referred to as "a closely related trio".

Klopper *et al.* (1998) suggested that *Pentaschistis eriostoma* and *P. borussica*, two of the species in the tribe with 13 as a basic chromosome number, must be further removed from the other *Pentaschistis* species. Since *P. eriostoma* shows little morphological, anatomical and cytogenetic similarity to the other species in the genus, it was suggested that this species is not closely related to the other species in the genus *Pentaschistis*. *Pentaschistis borussica* is also probably the result of polyploidisation and subsequent

aneuploidy of a *Pentaschistis* species, or hybridisation between two different *Pentaschistis* species and subsequent aneuploidy (Klopper *et al.* 1998).

Visser and Spies (1994d) reported on the presence of a *T. uniolae* hybrid swarm in which polyploidy was frequently encountered (namely tetraploidy in 86% of the specimens studied). This swarm would consist of *T. uniolae*, *T. amplexum* Renvoize and *T. alternans* (Nees) Renvoize, which are nearly impossible to separate on morphological grounds (Visser & Spies 1994d). A hybrid swarm is considered to be a complex mixture of parental forms, hybrids, backcross types and segregation products (Grant 1981). These three species have now been united under the name *T. uniolae* in the latest classification (Linder & Davidse 1997). The species in the section *Acutiflorae* was also proposed to form a hybrid swarm, with only *T. acutiflorum* justifiable as a separate taxon on morphological basis (Visser & Spies 1994e). This is also supported by evidence of allopolyploid origin within the species. The progenitors of these hybrid species may be some representatives of the sections *Tribolium* or *Uniolae* (Visser & Spies 1994e).

The apparent capability to hybridise and to exploit the advantages of hybrid species complexes or hybrid swarms, with ranges of genomes and chromosome numbers, is ancient in the grasses (De Wet 1987). Natural hybridisation is common in Poaceae and within a hybrid population the variability will naturally increase. This level of genetic variability allows the grasses to take advantage of new habitats (Ehrendorfer 1980).

The Arundineae have retained an apparently primitive chromosomal condition of a basic chromosome number of $x = 6$. They have remained primitive in some other respects as well, such as the lack of reduction in the spikelets and the maintenance of diversity in epidermal and anatomical structures (Stebbins 1956). Isolated genera of this group, such as *Phragmites* (the common reed), though very old, are still successful throughout the world, whereas others, such as *Cortaderia* (Pampas grass) and the South African endemic species in the tribe Arundineae, have developed chiefly in the Southern Hemisphere.

3.5 Conclusions

The evolutionary "tree" of most grass genera is not a simple branching affair, but a highly complicated system. Most of the common species of grasses are not descended from a single ancestral type, but consist of gene combinations in variable proportions, derived

from two, three, four or more separate and often widely differing ancestors (Stebbins 1956).

The position of the group of grasses with a basic chromosome number of seven seems to be isolated in the tribe Arundineae. The occurrence of this chromosome number apparently unites *Dregeochloa*, *Pentameris*, *Pentaschistis* and *Prionanthium* as a monophyletic grouping in the Arundineae. Morphological similarities and dissimilarities, although useful, do not clarify the apparent relationship between these four genera.

Although B-chromosomes are not well understood, their effect in the grass subfamily seems to be substantial. Many of the chromosomal abnormalities observed result from, or occur with, the presence of B-chromosomes.

The role that hybridisation and polyploidy play seems to be the driving force of evolution in this subfamily. Not a single genus in the Arundineae is known to be without reports of polyploidisation. The small and monotypic genera are exceptions, but this can be attributed to the lack of cytogenetic work done on these genera and to their scarcity. Their annual nature could also be a contributing factor.

The different factors influencing grass phylogeny from a cytotaxonomic point of view are overwhelming. Cytotaxonomy should be used as a guideline and ultimately be used in conjunction with morphology, anatomy and molecular studies. More insight into hybridisation and perhaps even the origin of B-chromosomes should be sought in hybridisation techniques such as GISH.

CHAPTER 4

DNA AMPLIFICATION FINGERPRINTING

4.1 Introduction

Molecular genetic approaches have enriched the resolution of plant genome analysis. The ability to sequence and clone specific genomic regions, has added sequence-based information to the understanding of plant genomes derived from cytogenetics and large-scale DNA analysis (Gresshoff 1994). Although the information base of nucleotide sequences is growing exponentially, methods are needed to investigate plant genomes at a complexity level above the primary sequence, but below the karyotype and cytogenetic arrangements (Gresshoff 1995).

Single, short primer based DNA amplification techniques have been developed over the last few years and have been applied to plant genomes. These techniques have collectively been labeled as MAAP (Multiple Arbitrary Amplicon Profiling) (Caetano-Anollés *et al.* 1992a, 1993, 1994). In essence MAAP involves the use of a short, arbitrarily chosen oligonucleotide primer, which annealed to DNA, will direct DNA amplification of multiple genomic regions (amplicons, Mullis 1991). In contrast to standard PCR, MAAP uses a single primer, which is of arbitrary sequence. MAAP generates multiple products and a primer used for one species can be used repeatedly for others as well, even if the evolutionary distance between the DNA templates are large (Gresshoff 1995). Variation in primer sites on the target DNA, length variations between the primer sites and possible changes in the secondary structure of the target DNA between or flanking the primer recognition sites, creates molecular polymorphisms. These molecular polymorphisms then define molecular regions of the genome (Gresshoff 1994).

DNA amplification fingerprinting (DAF) is a MAAP technique which was developed by Caetano-Anollés *et al.* (1991a, b). This MAAP technique is the enzymatic amplification of arbitrary stretches of DNA, which is directed by very short oligonucleotide primers (5-15 nucleotides in length) of arbitrary sequence to generate complex, but characteristic fingerprints (Caetano-Anollés *et al.* 1992b).

DAF can be distinguished from the other genome scanning techniques by the high primer to template ratios, excellent reproducibility and high multiplex ratios. Furthermore,

DAF uses over ten times more primer than RAPD, and uses high primer/template mass ratios in the range of 5/50000 (Caetano-Anollés 1997a). In general DAF profiles contain about 20-40 scoreable fragments. The number varies with primer and template DNA. Co-migration is likely and requires cloning to generate verified single molecular markers (Gresshoff 1995).

DAF detects changes in DNA sequences at arbitrary sites in the genome, which are defined by the primer. These changes are manifested in the number and lengths of the amplified products, and are not linked to particular loci. A general observation is that the DNA fragments fall into two categories: Fragments that are phylogenetically conserved (these could be classified as plesiomorphic characters for phylogenetic analysis) and fragments that are individual specific (these could be classified as autapomorphic characters for phylogenetic analysis) (Caetano-Anollés *et al.* 1991a).

DAF patterns are characterised by numerous fragments of differing intensities and mobility (Bassam *et al.* 1992a). The ability to generate many amplification products suggests that DAF is a very efficient technique in scanning the genome of an organism for variable sites (Gresshoff 1995). The large number of products allow a high density genotyping and genotype differentiation (Gresshoff 1992). The fragments of varying intensity results from products amplified to varying extents. For example, less efficiently amplified secondary or tertiary products could result from weaker mismatch annealing of the primer to one or both target sites. More intense products could arise when amplified regions are present in higher copy number in the template. Finally, in the initial rounds of amplification, template effects like steric hindrance of annealing and/or primer extension could effect product yield (Bassam *et al.* 1992b).

The amplification products can be separated and recorded by a variety of methods of detection. In all the cases, linear signal arrays generates a profile, which is representative for the target DNA (Gresshoff 1995). The use of polyacrylamide gel electrophoresis and DNA silver staining can adequately resolve the spectrum of DAF amplified products into detailed and reproducible patterns that reveal more than just a few predominant products (Bassam *et al.* 1992a).

Fingerprints can be tailored to vary in the number of both polymorphic and monomorphic fragments (Caetano-Anollés *et al.* 1991a; Bassam *et al.* 1992a). Tailoring can target the number and range of amplification products, template complexity, the nature of amplified sites and the level of polymorphic DNA detected (Caetano-Anollés 1996). A few of these tailoring techniques are the following:

- **Arbitrary mini-hairpin primers**

Mini-hairpin primers contain stable and compact hairpin structures at the 5' end of the primer that interferes with the formation of mini-hairpin loops in the resulting amplification products (Caetano-Anollés & Gresshoff 1994b, 1996). These primers increase detection of polymorphic DNA and direct the controlled amplification of small template molecules, thereby generating complex and reliable "sequence signatures" from even small template molecules such as plasmids, cloned DNA and PCR amplified fragments (Caetano-Anollés & Gresshoff 1996). The enhanced resolving power of mini-hairpin primers may result from an increase in the size of the genome being probed. This is because the annealing of these primers appears to be influenced by the secondary structure of the DNA and interactions between amplicon termini (Caetano-Anollés & Gresshoff 1994b).

- **Multiple primers**

Multiple primers can also generate DNA fingerprints. This technique is termed multiplex DAF. This technique also permits the combinatorial use of a limited set of primers (Caetano-Anollés 1997a). As expected, certain fragments disappear and new ones will be generated. Each primer amplifies discrete and limited portions of a genome, producing a characteristic set of amplification products. When more than one primer is used, new products will arise from the overlap of the extension products initiated by each primer, while others disappear. The extent to which this will occur depends on template characteristics (Caetano-Anollés *et al.* 1991b).

- **Endonuclease digestion of template DNA**

Digestion of template DNA with restriction enzymes prior to amplification (or tecMAAP), enhances the detection of polymorphic DNA (Caetano-Anollés *et al.* 1993). This strategy can be used to efficiently identify sequence tagged markers linked to genes of interest, for high-resolution linkage mapping of specific genomic regions, and potentially for chromosome walking (Caetano-Anollés *et al.* 1993). This technique has been used for the amplification of near

isogenic soybean lines and closely related plant accessions (Caetano-Anollés *et al.* 1993).

The reason for an increase in the generation of polymorphic DNA is still speculative, but may stem from an increase in the size of the genome being probed during annealing or restriction. Digestion of the template DNA could result in the differential destruction of amplicons and the selective amplification of those products that lack these internal restriction sites (Caetano-Anollés 1994).

The appearance of fragments, not amplified under normal undigested conditions, could arise from the exposure of amplicons normally hidden by secondary DNA structure. These changes will become competitively important at low annealing and extension temperatures, resulting in not all the genomic regions being equally amplifiable (Caetano-Anollés *et al.* 1993). Alternatively, the digestion of the template DNA can cause the extending primer to jump to another template during amplification to produce a recombinant product (Paäbo *et al.* 1990).

- **Endonuclease digestion of amplification products**

Digestion of the amplification products can also increase the information content of fingerprints. In contrast to template digestion, the total mass of amplified DNA is maintained. Every disappearing fragment should produce at least two smaller DNA fragments. With many organisms, smaller fingerprint fragments appear and larger amplification fragments disappear, following digestion of amplified products (Caetano-Anollés *et al.* 1993).

- **Arbitrary Signatures from Amplification Profiles**

ASAPS are fingerprints generated from fingerprints, by re-amplification of DAF profiles with mini-hairpin or standard arbitrary primers (Caetano-Anollés & Gresshoff 1996). ASAPS are also fingerprints obtained by re-amplification of any amplification product, ranging from those produced in PCR to those generated with arbitrary primers (Caetano-Anollés 1997b).

ASAP analysis is a dual-step amplification approach that provides additional scanning of primary sequence within pre-selected amplicons. The

process requires the use of more than one primer in each amplification step, allowing the combinatorial use of primers in fingerprinting. Providing that the sequence of the primers differ significantly from each other, distinct fingerprints can be generated in each particular combination (Caetano-Anollés 1997b).

The aim of this study is to use DNA amplification fingerprinting, without any tailoring, to locate differences within some species in the tribe Arundineae that could be of phylogenetic importance and assist in the classification of the subfamily. The use of DAF in the elucidation of relationships above species level, thus between different genera, will also be investigated. Furthermore, DNA amplification fingerprinting will be used in the calculation of genetic distances between the different genera to determine the genetic diversity within and between genera.

4.2 Results

The DAF analyses were optimised with the Taguchi method. Due to the fact that the same fragment pattern is obtained for all the reaction with a specific DNA specimen, the fragments were only scored for yield, i.e. the amount of amplification per reaction. Reaction optima for the following four components were read from the graphs (Fig. 4.1). These reaction optima were 3 μ l template DNA (0.5 ng/ μ l), 3 μ l MgCl₂ (2 mM/ μ l), 3 μ l primer (50 pmol/ μ l) and 3.5 μ l dNTP (50 μ M/ μ l). The amount of Taq polymerase and buffer were constant at 0.16 μ l and 2.5 μ l respectively.

The results are presented as a photographic representation of the gel profile for each primer (Figs. 4.2-4.10). The legend for each of the schematic representations are as follows, 1 = *Tribolium echinatum* (Spies 6084), 2 = *T. hispidum* (Spies 5967), 3 = *T. hispidum* (Spies 6106), 4 = *T. hispidum* (Spies 6240), 5 = *T. uniolae* (Spies 6025), 6 = *T. uniolae* (Spies 6096), 7 = *T. uniolae* (Spies 6203), 8 = *T. obtusifolium* (Spies 6085), 9 = *T. uniolae* (Spies 6201), 10 = *T. acutiflorum* (Spies 6291), 11 = *T. uniolae* (Spies 6181), 12 = *T. brachystachyum* (Spies 6249), 13 = *Chaetobromus involucretes* subsp. *dregeanus* (Spies 5976), 14 = *C. involucretes* subsp. *dregeanus* (Spies 6237), 15 = *Prionanthium dentatum* (Spies 6047), 16 = *P. dentatum* (Spies 6286), 17 = *P. ecklonii* (Spies 6061), 18 = *P. ecklonii* (Spies 6254), 19 = *P. pholioroides* (Spies 6101), 20 = *P. pholioroides* (Spies 6213), 21 = *P. pholioroides* (Spies 6252), 22 = *Tribolium pusillum* (Spies 6256), 23 =

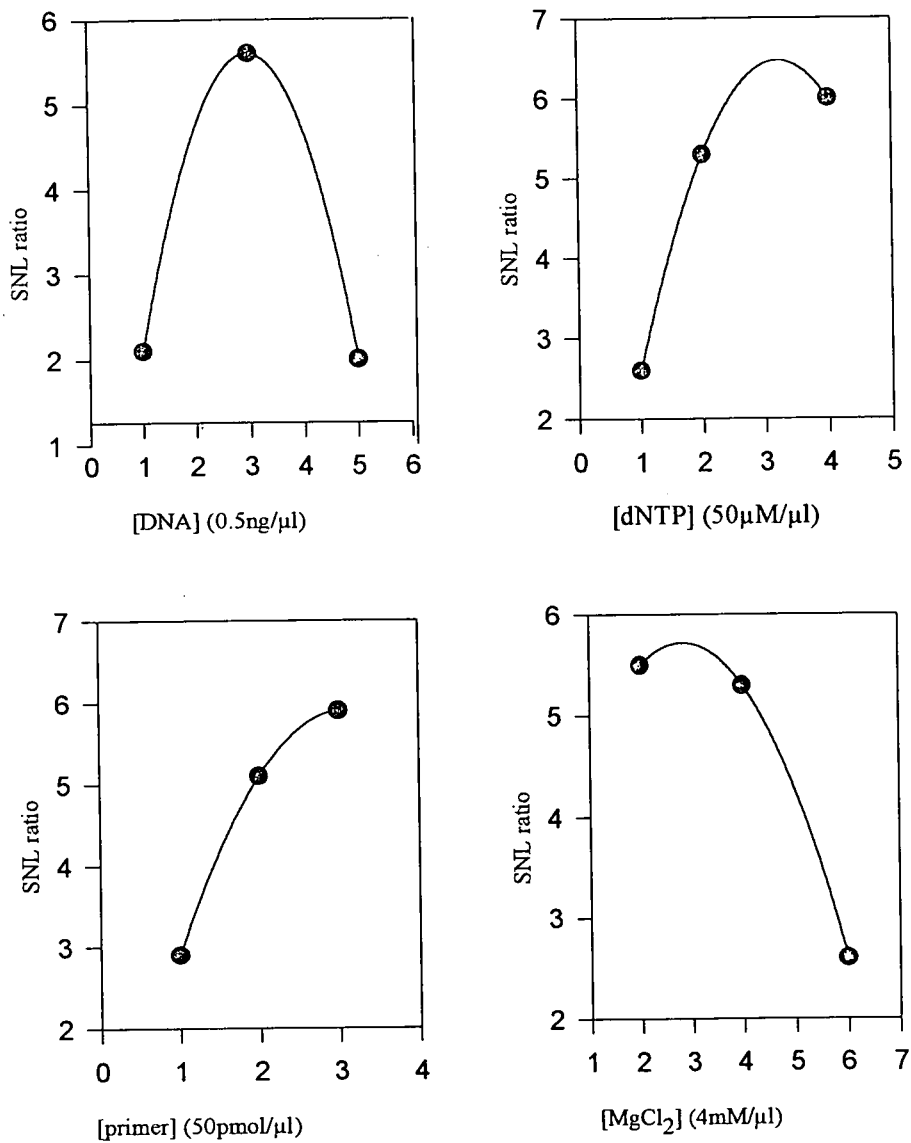


Figure 4.1 Taguchi optimisation reactions for each of the four variables tested in the DNA amplification fingerprinting reaction, namely primer, MgCl₂, dNTP and DNA quantities. Optimal reaction volumes are 3 μl primer, 3 μl MgCl₂, 3.5 μl dNTP's and 3 μl DNA.

Pentameris longiglumes (Spies 6072), 24 = *P. longiglumes* (Spies 6154), 25 = *P. longiglumes* (Spies 6225), 26 = *P. thuarii* (Spies 6160), 27 = *P. oreophila* (Spies 6166), 28 = *P. macrocalycina* (Spies 6235), 29 = *P. macrocalycina* (Spies 6316), 30 = *Merxmuellera disticha* (Spies 6140), 31 = *M. dura* (Spies 6285), 32 = *M. stricta* (Spies 6227), 33 = *M. stricta* (Spies 6145), 34 = *M. stricta* (Spies 6288), 35 = *M. arundinacea* (Spies 6257), 36 = *Karoochloa purpurea* (Spies 6241), 37 = *K. purpurea* (Spies 6244), 38 = *Phragmites australis* (Spies 6575), 39 = *Arundo donax* (Spies 6574), 40 = *Cortaderia selloana* (Spies 6573).

The average number of fragments ranged from 26 (DAF 11) to 46 (DAF 3 and DAF 5) per specimen. The fragment lengths ranged from 100 to 1200 bp and varied from a light to a dark intensity. In the study only nine of the eleven primers used for amplification of the DNA could be used for analyses. DAF 7 and 8 did not amplify.

The fragments observed for each primer were scored (1 = fragment present, 0 = fragment absent in a specific position) and a data matrix was compiled (Appendices B-J). The similarity indices (F) and genetic distances (D) between the different specimens were calculated (Appendices K-S), using the combined datamatrix.

4.2.1 Genetic variation

The F and D values obtained for each primer (Appendices K-S) was used to calculate the genetic distances between, and within, the different genera studied.

In the genera *Arundo*, *Cortaderia* and *Phragmites* only one specimen per genus was investigated. Thus, no infrageneric distances could be calculated in these genera. In the genera *Chaetobromus* and *Karoochloa*, two specimens per genus were investigated. This, however, only constituted one species in each of these genera and no infraspecific distances could be calculated. In the remaining genera (*Merxmuellera*, *Pentameris*, *Prionanthium* and *Tribolium*), the infra- and interspecific genetic distances between the different species in each of these genera were calculated (Tables 4.1 – 4).

The D values (Appendices K-S) were also used to calculate the calculate infra- and intergeneric distances for the genera studied (Table 4.5).

The high infrageneric distances could be attributed to the fact that these genera are representative of a wide distribution range and not geographically isolated groups in which these distances would be much lower. It can also be attributed to the fact that a whole genus is used for the calculation and not a particular species.

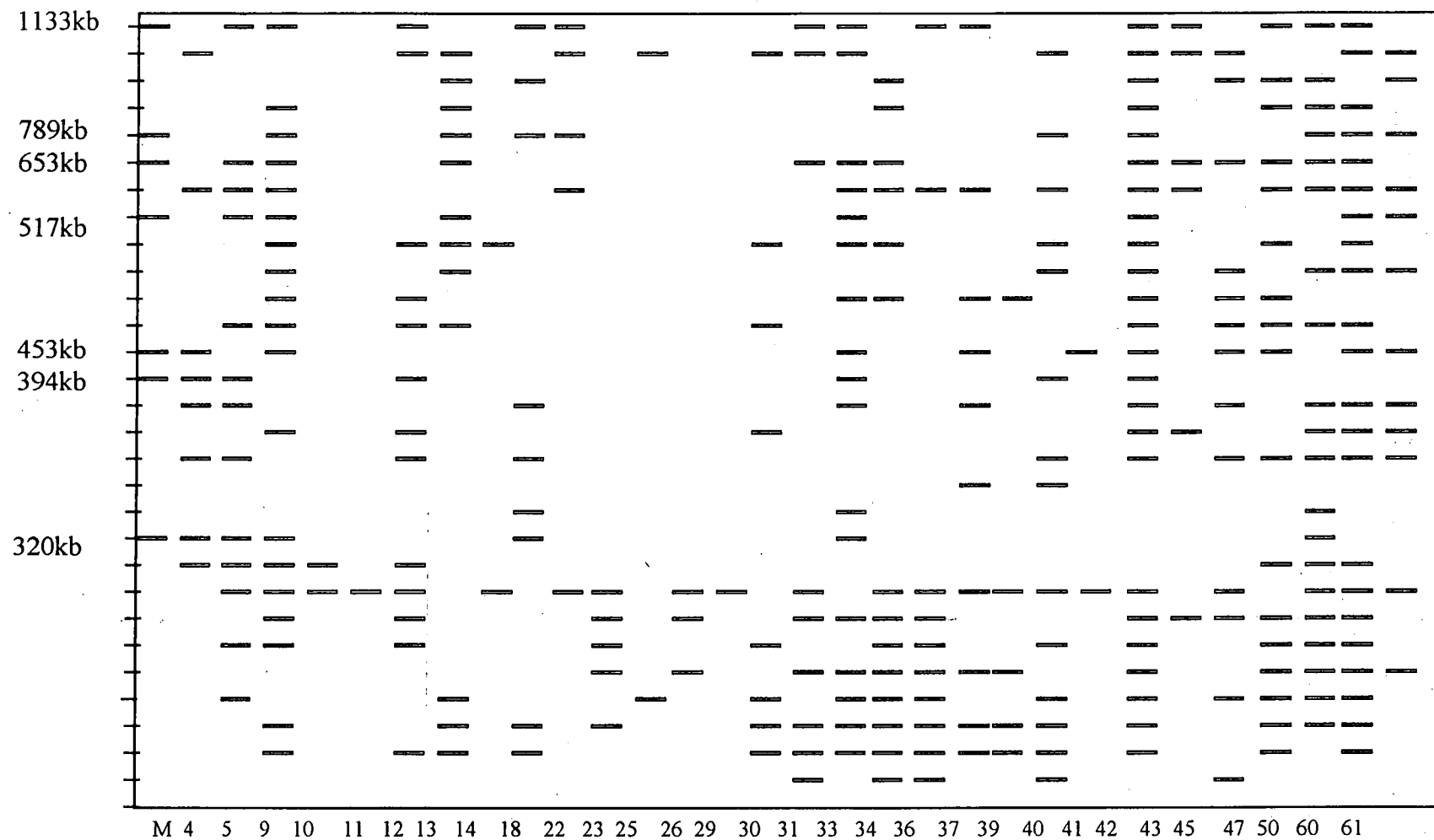


Figure 4.2 Schematic representation of amplification products produced by DAF primer1. Marker IV is indicated by M.

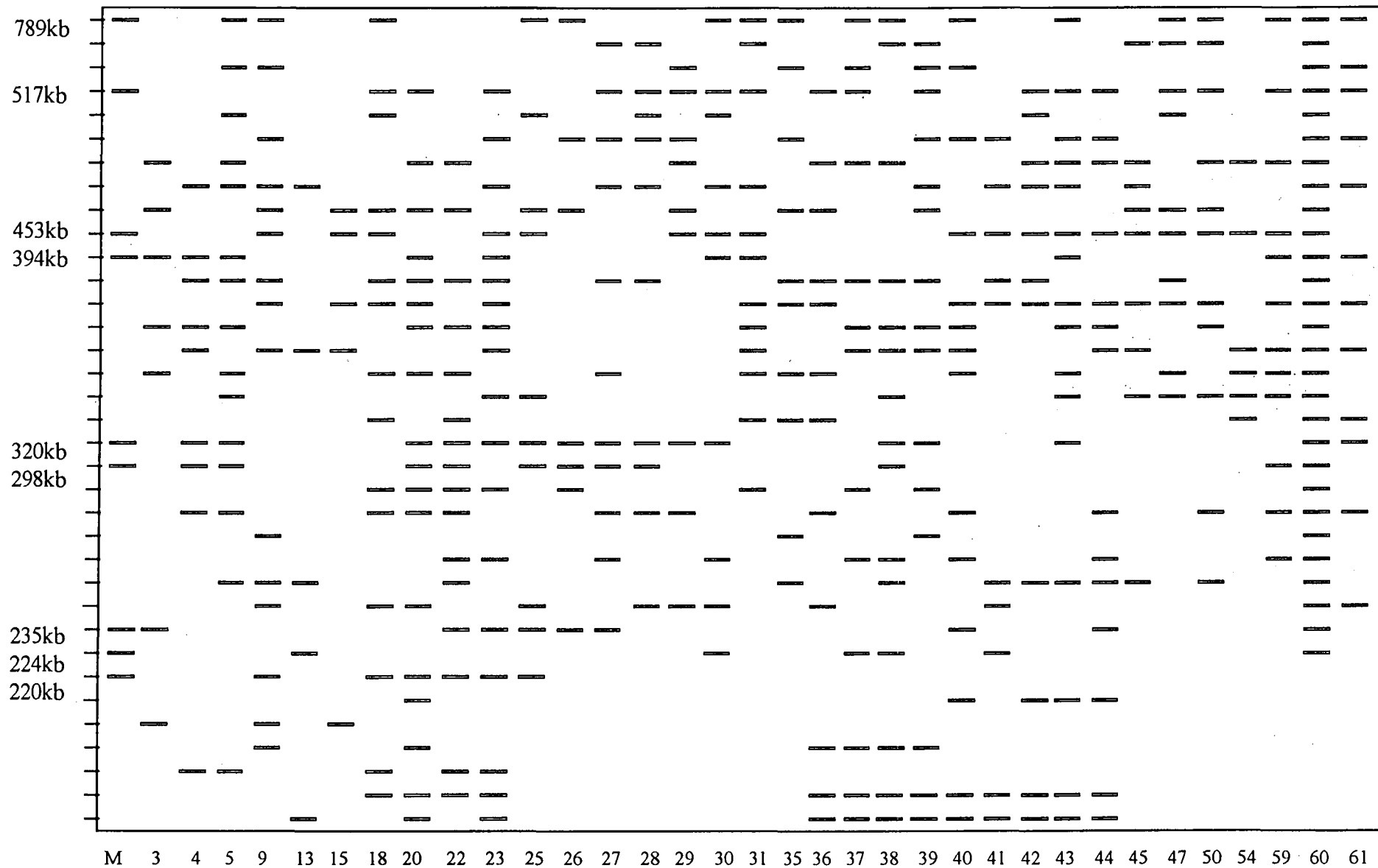


Figure 4.3 Schematic representation of amplification products produced by DAF primer 2. Marker IV is indicated by M

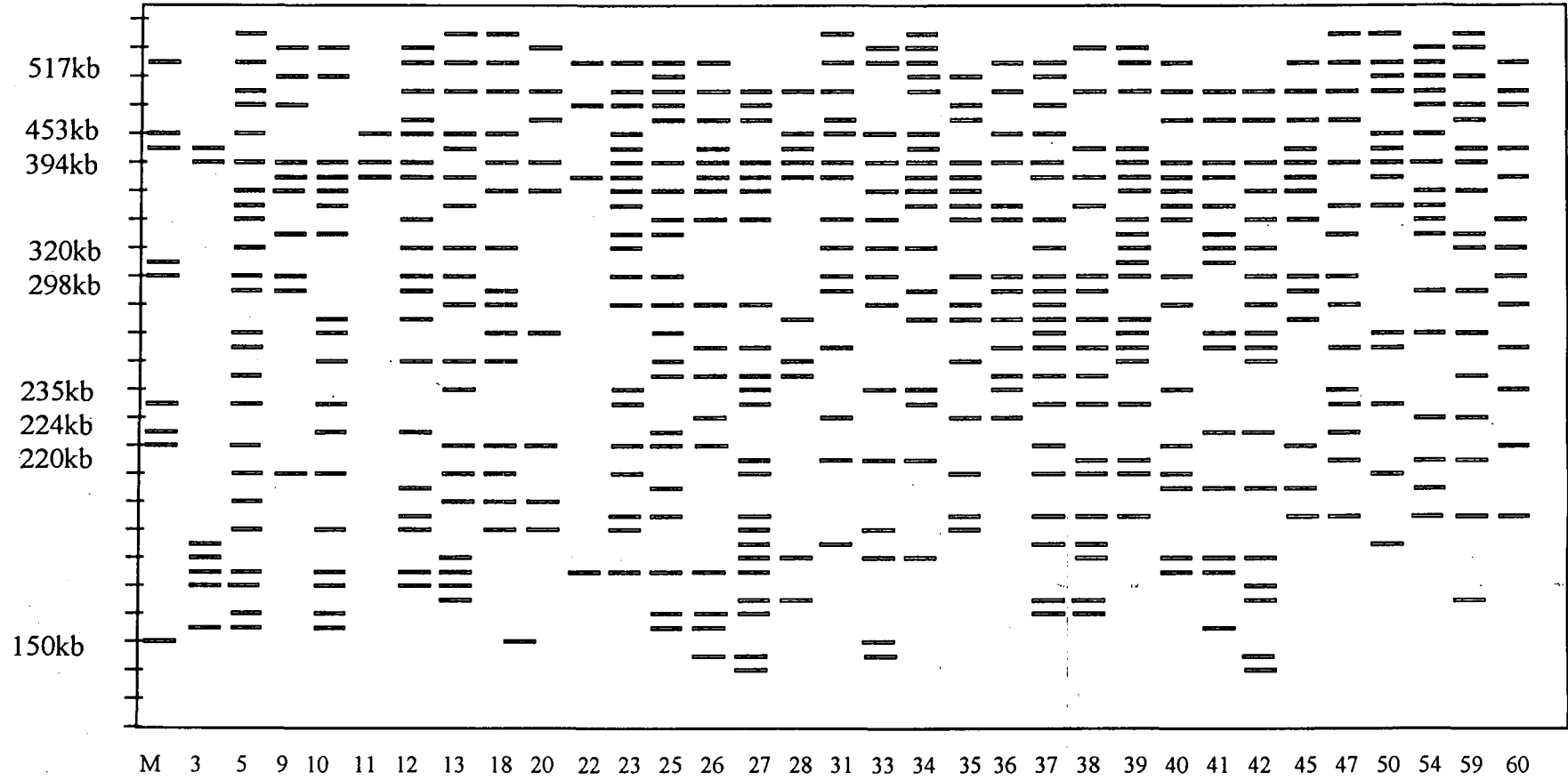


Figure 4.4 Schematic representation of amplification products produced by DAF primer 3. Marker IV is indicated by M.

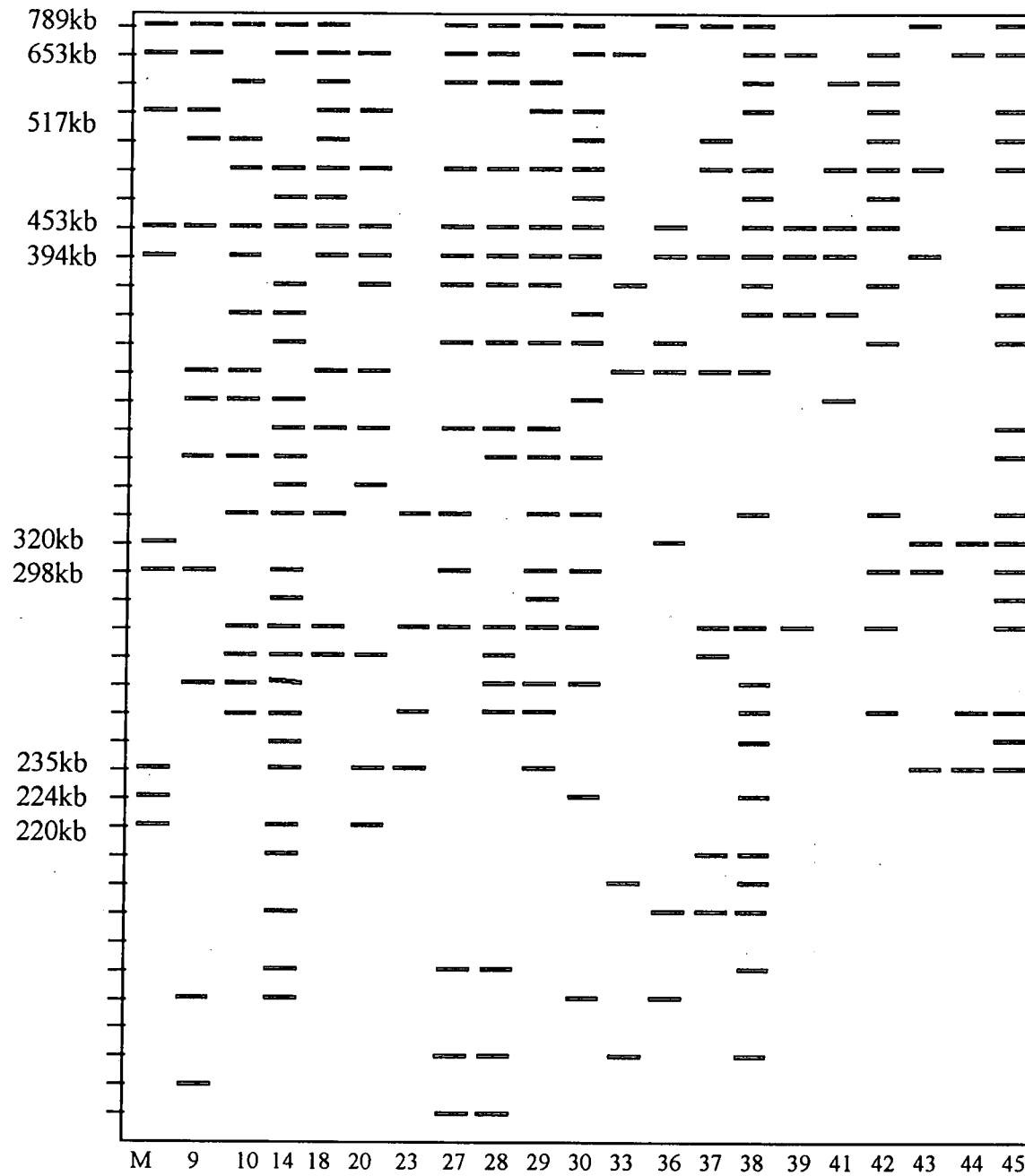
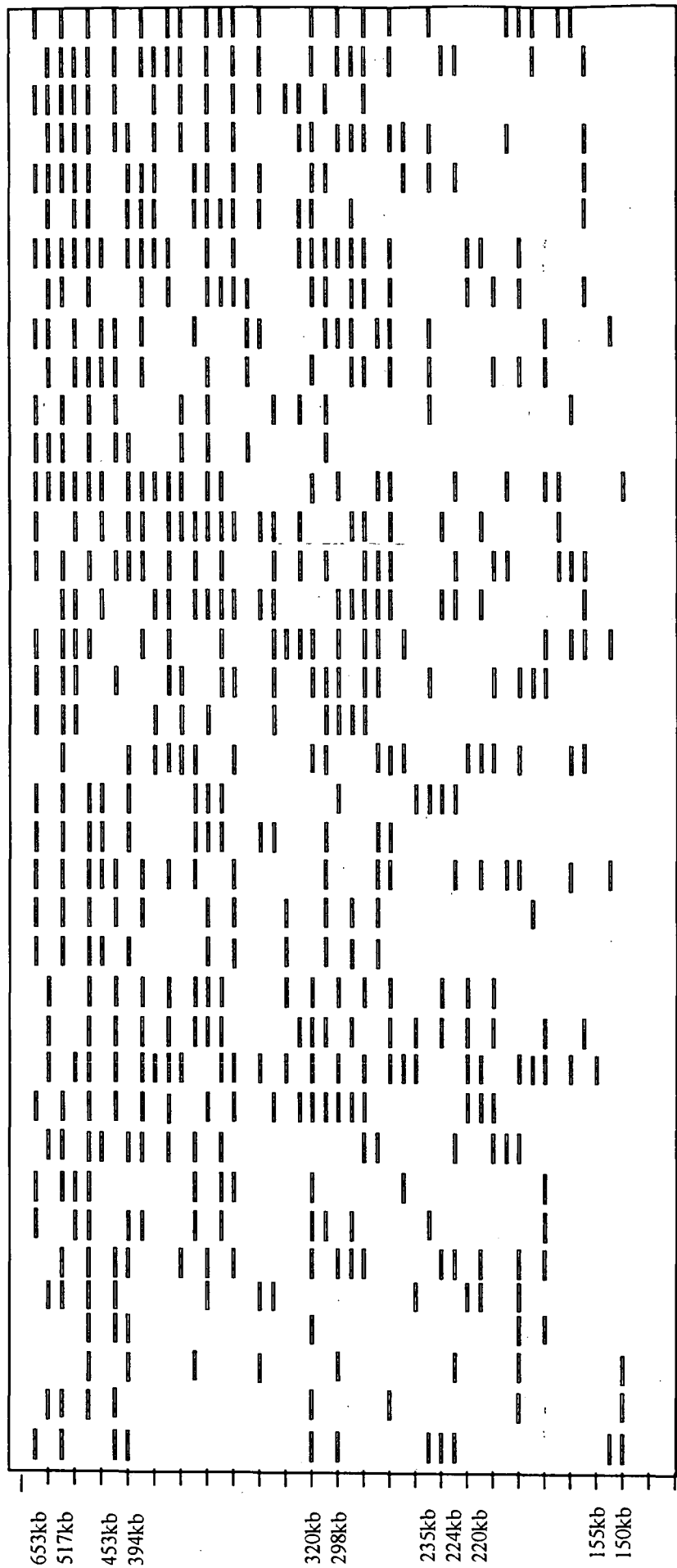


Figure 4.5 Schematic representation of amplification products produced by DAF primer 4. Marker IV is indicated by M.



M 3 4 5 9 10 11 12 15 18 20 22 23 25 26 27 28 29 30 31 33 34 35 36 37 38 39 40 41 42 43 44 47 50 54 59 60 61

Figure 4.6 Schematic representation of amplification products produced by DAF primer 5. Marker IV is indicated by M.

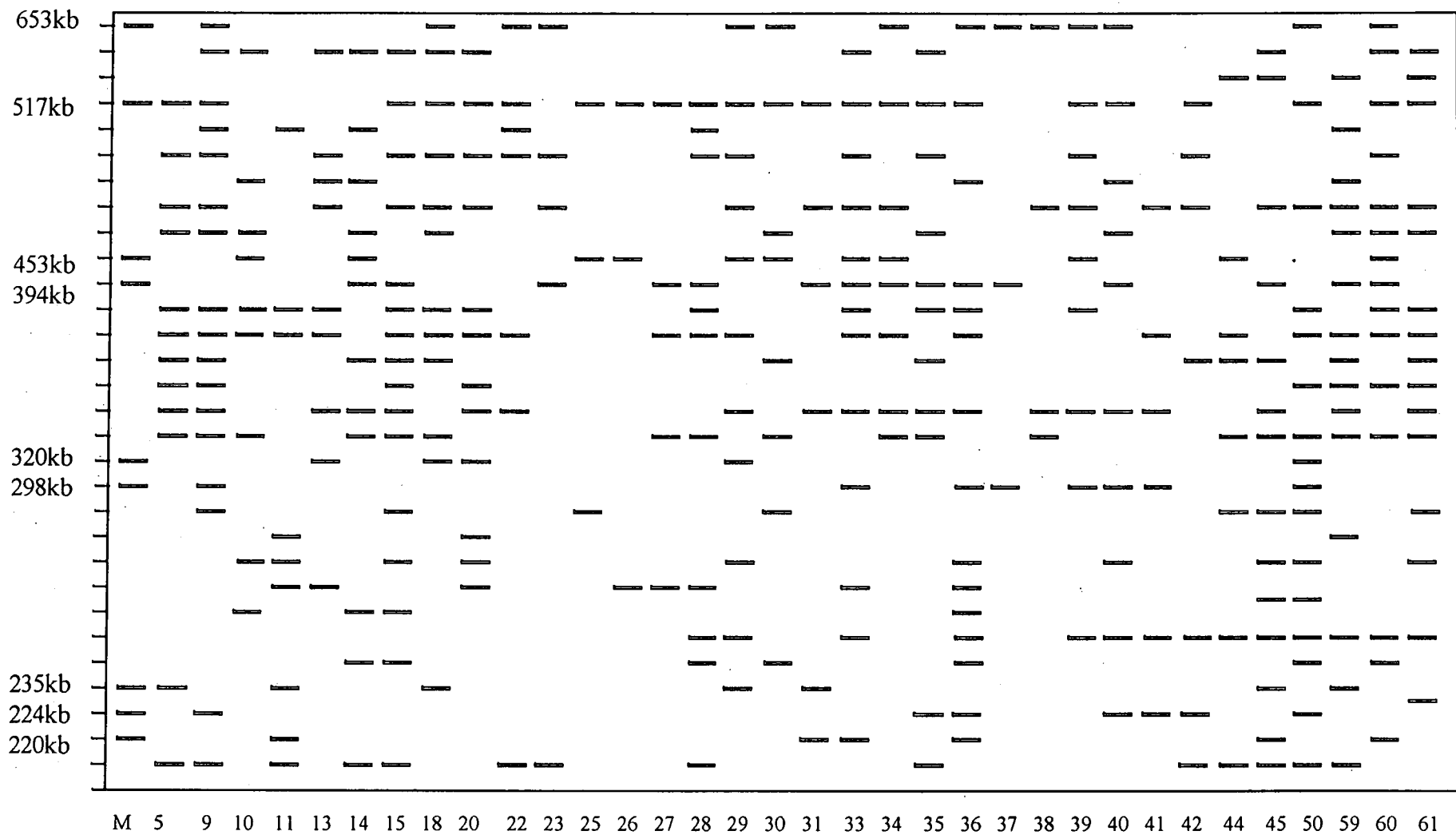


Figure 4.7 Schematic representation of amplification products produced by DAF primer 6. Marker IV is indicated by M.

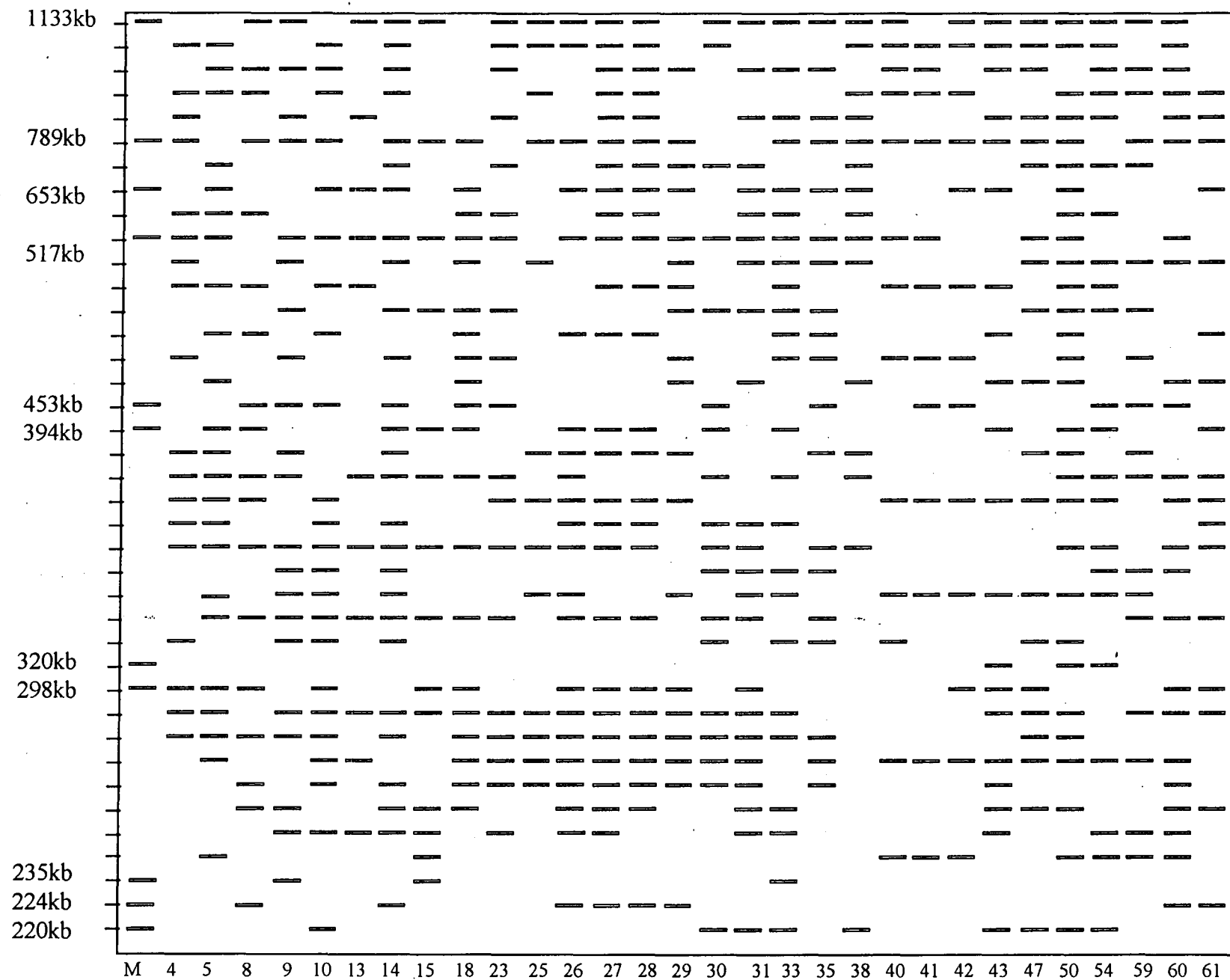


Figure 4.8 Schematic representation of amplification products produced by DAF primer 9. Marker IV is indicated by M.

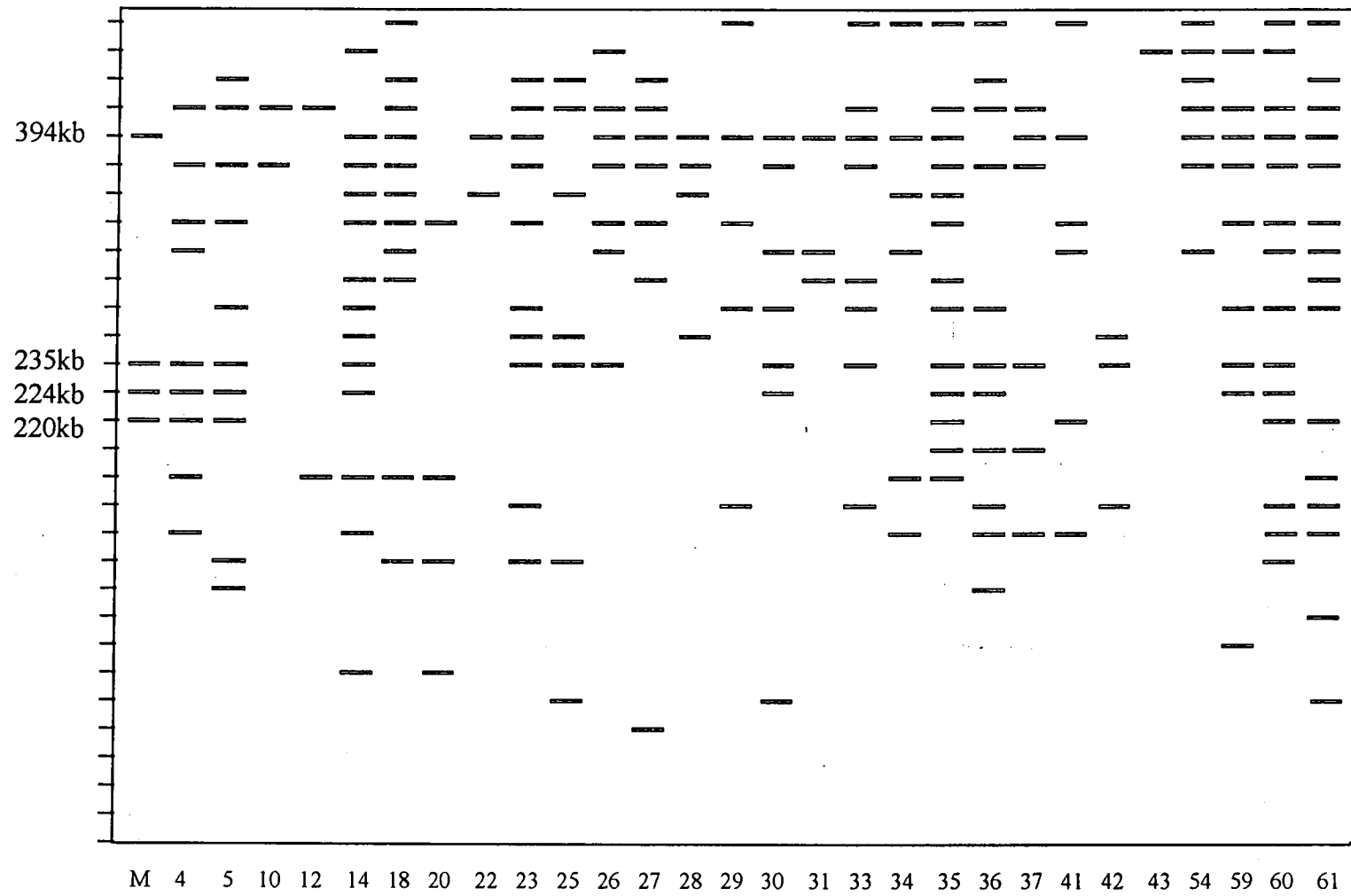
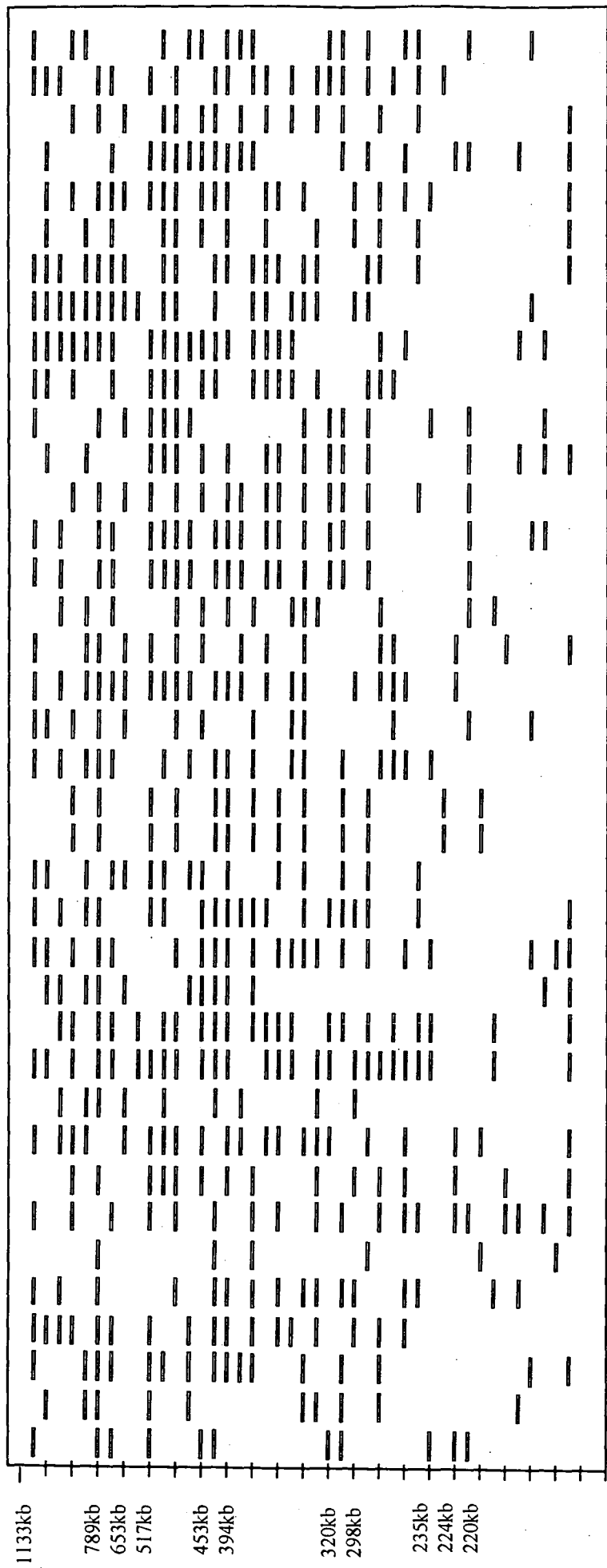


Figure 4.9 Schematic representation of amplification products produced by DAF primer 11. Marker IV is indicated by M.



M 3 4 5 9 11 12 13 14 15 18 20 22 23 25 26 27 28 29 30 31 33 34 35 36 37 38 39 40 41 42 43 44 45 50 59 60 61

Figure 4.10A Schematic representation of amplification products produced by DAF primer 12. Marker IV is indicated by M.

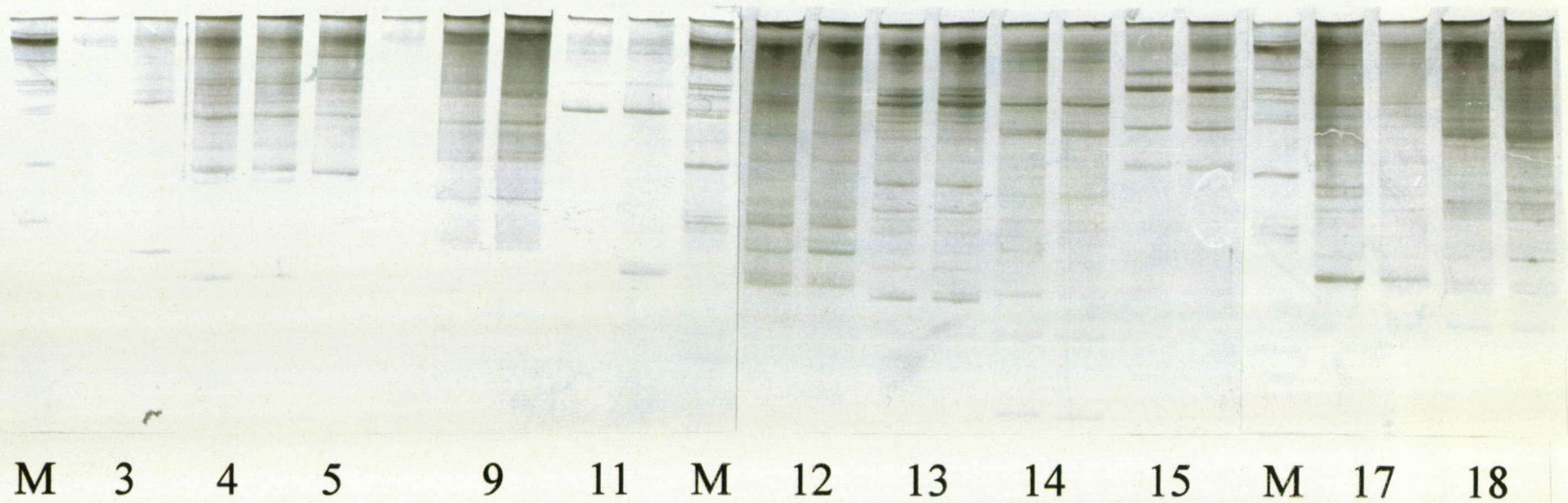


Figure 4.10B Photographic representation of the amplification products of some of the studied specimens. Markers are indicated by M. Amplification products were produced by DAF primer 12.

Table 4.1 Infra- (in parentheses) and interspecific genetic distances (D values) in the genus *Tribolium*. *Tech* = *T. echinatum*; *This* = *T. hispidum*; *Tunio* = *T. uniolae*; *Tobt* = *T. obtusifolium*; *Tacut* = *T. acutiflorum* and *Tbrac* = *T. brachystachyum*.

	<i>Tpus</i>	<i>Tech</i>	<i>This</i>	<i>Tunio</i>	<i>Tobt</i>	<i>Tacut</i>	<i>Tbrac</i>
<i>Tpus</i>	(-)						
<i>Tech</i>	0.96	(-)					
<i>This</i>	1.00	1.07	(0.87)				
<i>Tunio</i>	1.06	1.41	1.10	(0.95)			
<i>Tobt</i>	0.62	1.16	0.86	1.05	(-)		
<i>Tacut</i>	0.81	1.13	0.91	1.08	1.03	(-)	
<i>Tbrac</i>	0.86	1.53	0.88	0.87	1.28	1.07	(-)

Table 4.2 Infra- (in parentheses) and interspecific genetic distances (D values) in the genus *Prionanthium*.

	<i>P. dentatum</i>	<i>P. ecklonii</i>	<i>P. pholioroides</i>
<i>P. dentatum</i>	(0.51)		
<i>P. ecklonii</i>	0.84	(0.76)	
<i>P. pholioroides</i>	0.90	0.84	(1.02)

Table 4.3 Infra- (in parentheses) and interspecific genetic distances (D values) in the genus *Pentameris*.

	<i>P. longiglumes</i>	<i>P. thuarii</i>	<i>P. oreophila</i>	<i>P. macrocalycina</i>
<i>P. longiglumes</i>	(0.72)			
<i>P. thuarii</i>	0.79	(-)		
<i>P. oreophila</i>	0.81	0.63	(-)	
<i>P. macrocalycina</i>	0.82	0.85	0.88	(0.81)

Table 4.4 Infra- (in parentheses) and interspecific genetic distances (D values) in the genus *Merxmuellera*.

	<i>M. disticha</i>	<i>M. dura</i>	<i>M. stricta</i>	<i>M. arundinacea</i>
<i>M. disticha</i>	(-)			
<i>M. dura</i>	0.84	(-)		
<i>M. stricta</i>	0.89	0.73	(0.67)	
<i>M. arundinacea</i>	0.86	0.67	0.70	(-)

Table 4.5 Genetic distances within (in parentheses) and among the different genera studied. 1 = *Tribolium*, 2 = *Chaetobromus*, 3 = *Prionanthium*, 4 = *Pentameris*, 5 = *Merxmuellera*, 6 = *Karoochloa*, 7 = *Phragmites*, 8 = *Arundo* and 9 = *Cortaderia*.

	1	2	3	4	5	6	7	8	9
1	(1.02)								
2	1.00	(1.02)							
3	1.00	0.97	(0.85)						
4	1.05	0.93	0.94	(0.80)					
5	0.98	0.87	0.94	0.92	(0.76)				
6	0.95	0.93	0.84	0.76	0.75	(0.58)			
7	0.92	0.93	0.84	0.90	0.75	0.56	(-)		
8	0.87	0.77	0.81	0.70	0.88	0.59	0.58	(-)	
9	0.93	1.13	0.95	0.87	0.93	0.75	0.79	0.52	(-)

4.2.2 Phylogenetic analyses

4.2.2.1 PAUP

A Strict, Semistrict and Adams consensus cladogram was computed during the phylogenetic analysis with PAUP. These consensus trees were identical. The total number

of equally parsimonious cladograms computed was two trees (Fig. 4.11A, B) with a CI of 0.15 and RI of 0.34 and tree length of 2251.

Bootstrap branch support values are indicated in parentheses on the particular branches. Only the *Pentameris thuarii*-*P. oreophila* clade (with 72% support) and the *Prionanthium ecklonii* clade (with 85% support) was sufficiently supported by the bootstrap technique (with values above 65%). Most of the basal clades were not supported at all.

In all of the above searches the computer software already removed the uninformative characters. These were characters 109, 111, 144, 147, 194, 287, 288 and 291. Heuristic searches after additional character removal (approximately 40% of the original characters were removed), yielded four most equally parsimonious trees with a tree length of 1215, CI of 0.16 and RI of 0.43. The Strict and Semistrict consensus trees computed were identical, but showed a lack of resolution in most of the clades. The Adams consensus tree best resolved the relationships between the genera and species, with the formation of a single polytomy in the upper part of the cladogram (Fig. 4.12).

Bootstrap replicate values are indicated in parentheses on the branches. The highest support values were again for the *Prionanthium ecklonii* clade with a support value of 94%. Most of the basal clades were once again not supported in the bootstrap replicates.

A final heuristic search was conducted on the datamatrix to which successive weighting was applied. Successive weighting according to rescaled consistency (RC) values was applied to the datamatrix three times, before the tree stabilised (the cladograms on successive repetitions are identical). A single most parsimonious tree (Fig. 4.13) with length of 9977, CI of 0.18 and RI of 0.45 was computed. Bootstrap support was again the greatest for the *Prionanthium ecklonii* clade with a value of 98%. More branch support is evident in this search, compared to the two previous searches.

No decay values could be computed due to insufficient memory. In all instances *Cortaderia selloana* (Spies 6573) was used as outgroup. The reason is that generally *Cortaderia* is considered to be further separated from the tribe Arundineae. This genus was initially placed in Arundineae with *Arundo* and *Phragmites*, whereas the rest of the genera studied here were placed in Danthonieae (Conert 1987). Later *Cortaderia* was moved from Arundineae to Danthonieae, removing it from the reedlike grasses *Arundo* and *Phragmites* (Watson 1990). Furthermore, there is confusion in this genus as to whether nine or six (as in the rest of the tribe) is the basic chromosome number of this genus.

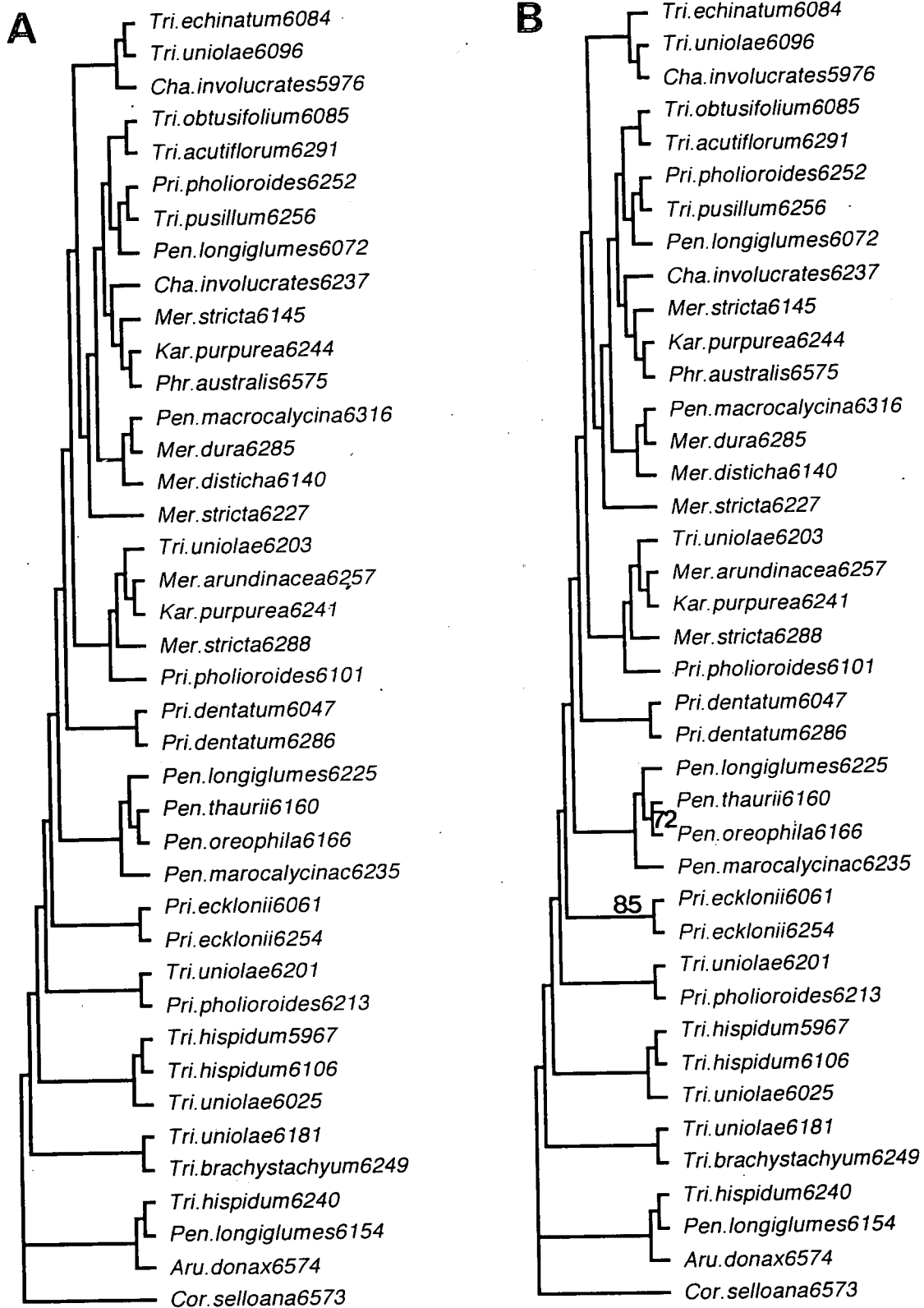


Figure 4.11 The two most equally parsimonious trees (A & B) obtained with the first heuristic search. The trees have a CI of 0.15, RI of 0.34 and tree length of 2251. The Strict, Semistrict and Adams consensus computed from these two trees are identical in forming a trichotomy at the position where the two cladograms differ in the relationship between *Chaetobromus involucretes* subsp. *dregeanus* Spies 5976, *Tribolium uniolae* Spies 6096 and *T. echinatum* Spies 6084 at the top of the respective cladograms. Bootstrap support values are indicated on the relevant branches.

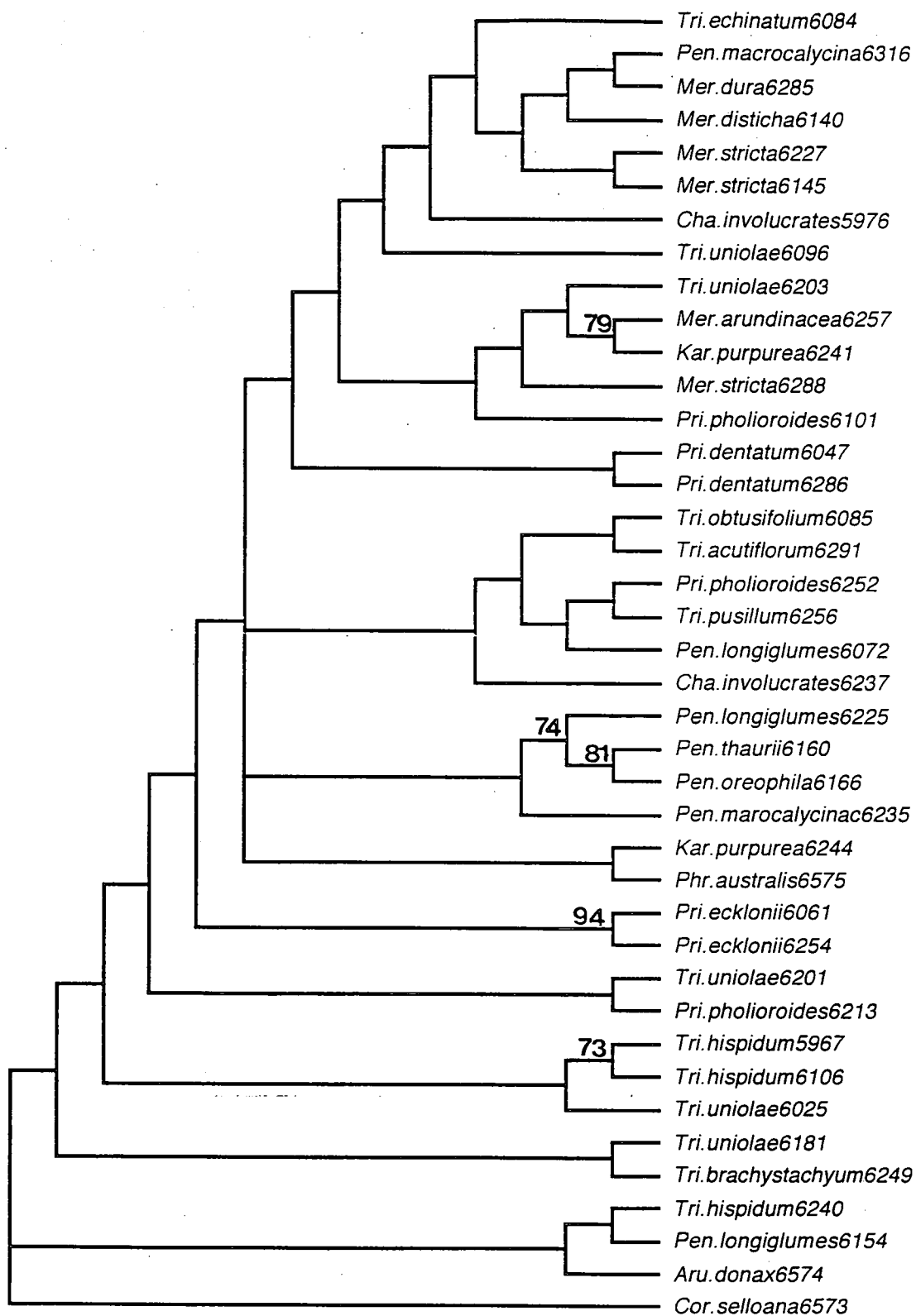


Figure 4.12 The Adams consensus cladogram obtained after a heuristic search was conducted on the matrix from which some characters were removed. The four equally most parsimonious trees from which this tree was computed, has a length of 1215, CI of 0.16 and RI of 0.43. Bootstrap values are indicated on the relevant branches.

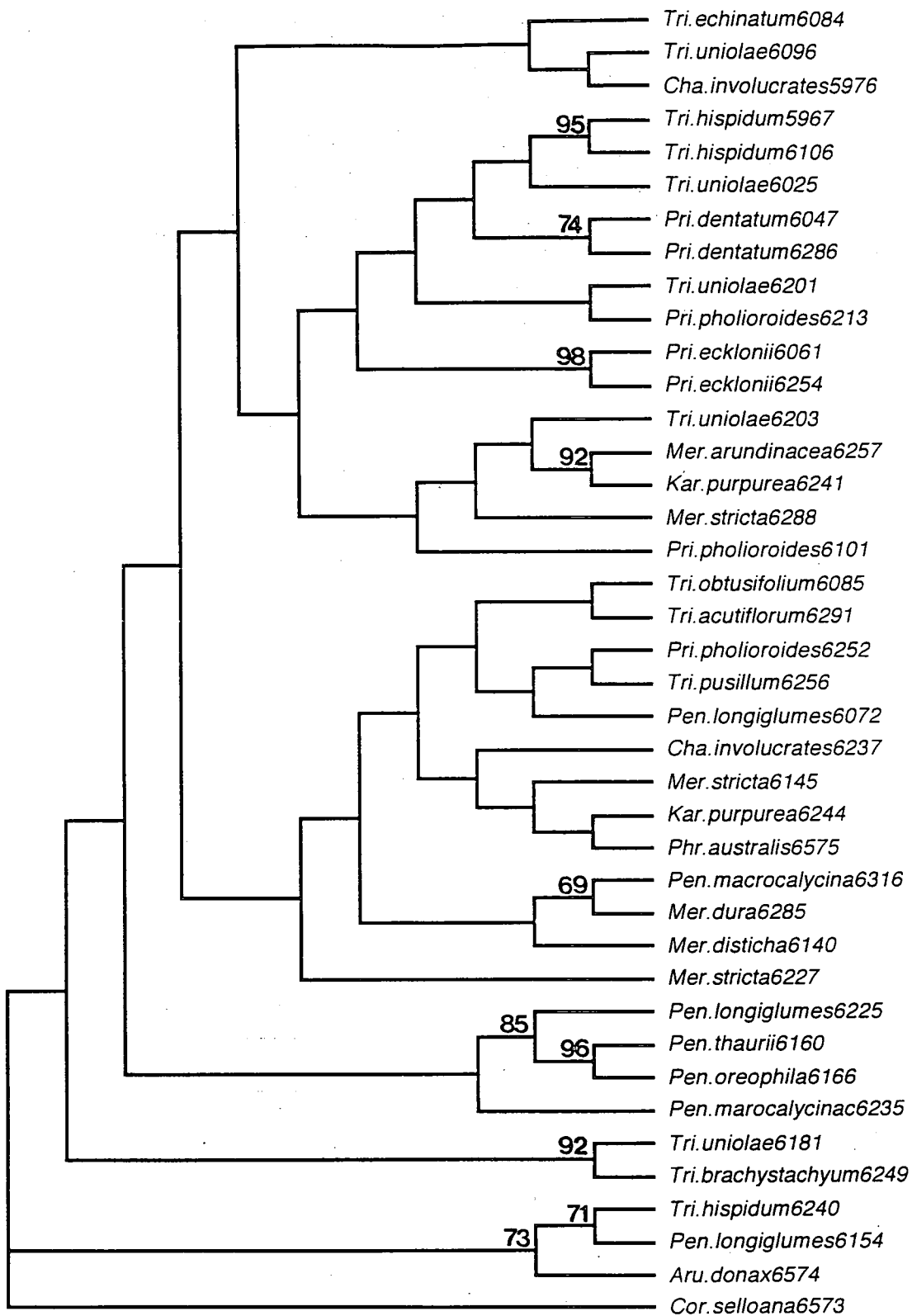


Figure 4.13 The single most parsimonious tree with length of length of 9977, CI of 0.18 and RI of 0.45, computed after successive weighting was applied to the data set. Bootstrap support values are indicated on the relevant branches.

4.2.2.2 HENNIG86

The only difference between the PAUP and HENNIG86 searches conducted in this study, was that in PAUP the data was unordered and in HENNIG86 the data was ordered (according to default settings).

With the HENNIG86 computer program, using the mhennig and branch swapping search option, a single most parsimonious tree (Fig. 4.14) was obtained with a length of 2249, CI of 0.14 and RI of 0.33.

After characters were removed from the datamatrix according to the CI:RI ratio's, the multiple hennig and branch swapping search option yielded two equally parsimonious trees with a length of 1166, CI of 0.16 and RI of 0.43, from which the nelsen consensus tree (Fig. 4.15) was computed.

A final mhennig and branch swapping search was conducted after successive weighting. This search yielded over 100 equally parsimonious trees with a length of 225, CI of 0.38 and RI of 0.68, from which the nelsen consensus tree (Fig. 4.16) was computed. Successive weighting was applied five times at which time the trees stabilised. Again *Cortaderia selloana* (Spies 6573) was used as outgroup.

When the datamatrix was analysed with the jackknife monophyly index (JMI), (LANYON option) in the Random Cladistics software package, one single most parsimonious cladogram was obtained. In this tree 13 of the 37 monophyletic groups analysed had JMI values of above 0.73.

Bootstrap monophyly index (BS) values calculated by this software (HEYJOE option) also computed one single most parsimonious tree. In this tree only two of the 37 monophyletic groups analysed had bootstrap support values of > 0.65 .

4.3 Discussion

The DAF analysis produced clear and distinct reproducible fragments with good resolution. Using polyacrylamide gels and silver staining increased the resolution of the minor fragments, when compared to agarose gel electrophoresis along with ethidium bromide detection. Considerable variation was detected with the primers within the genera and species studied. Some amplified fragments were common to all individuals evaluated, whereas others were present in some individuals but absent from others and were, thus phylogenetically informative.

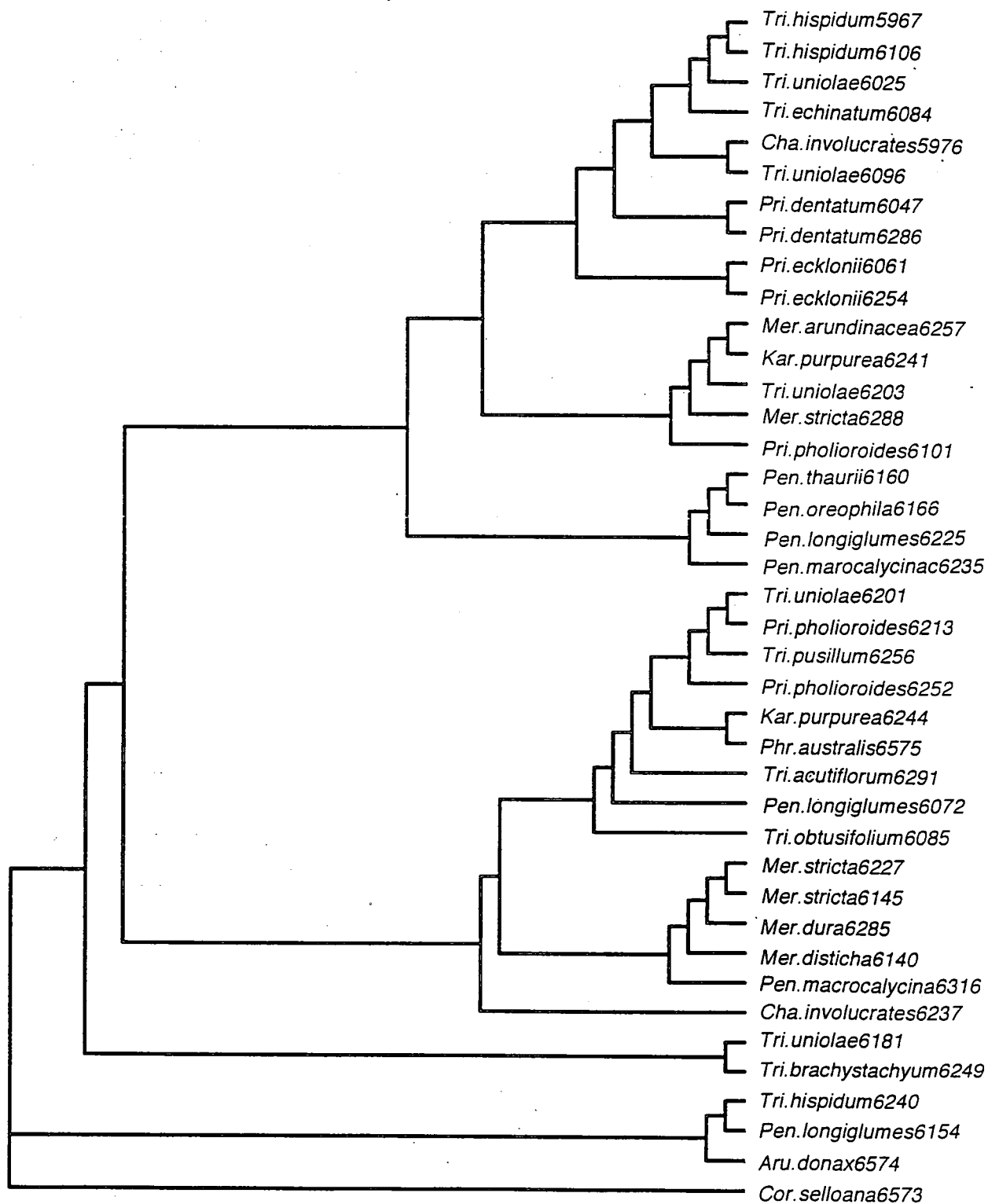


Figure 4.14 The single most parsimonious cladogram obtained with a mh, bb search in HENNIG86. The tree has a length of 2249, CI of 0.14 and RI of 0.33.

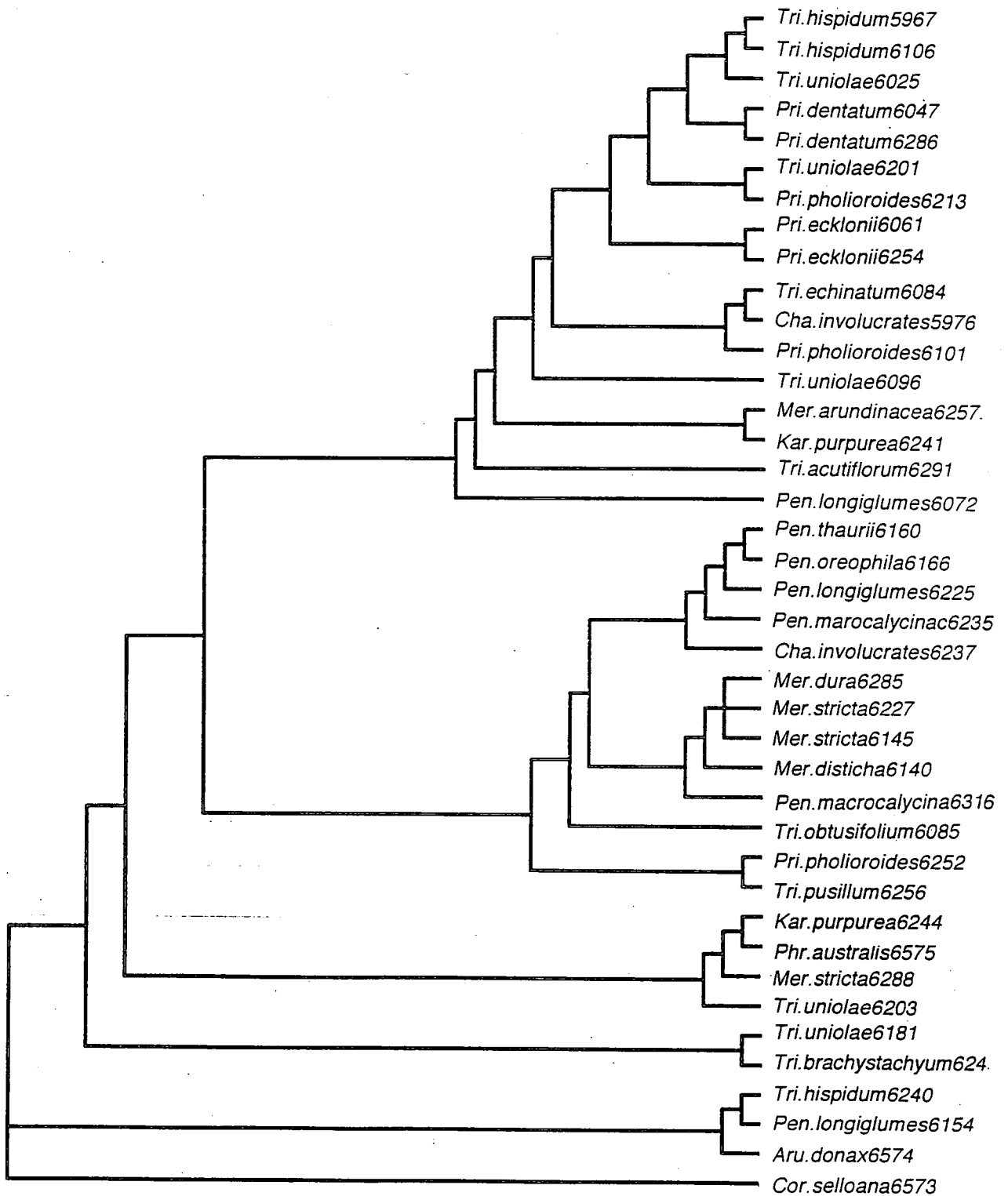


Figure 4.15 The nelsen consensus tree obtained after character removal from the original data set. The nelsen consensus tree was computed from nine equally parsimonious trees with a length of 1166, CI of 0.16 and RI of 0.43.

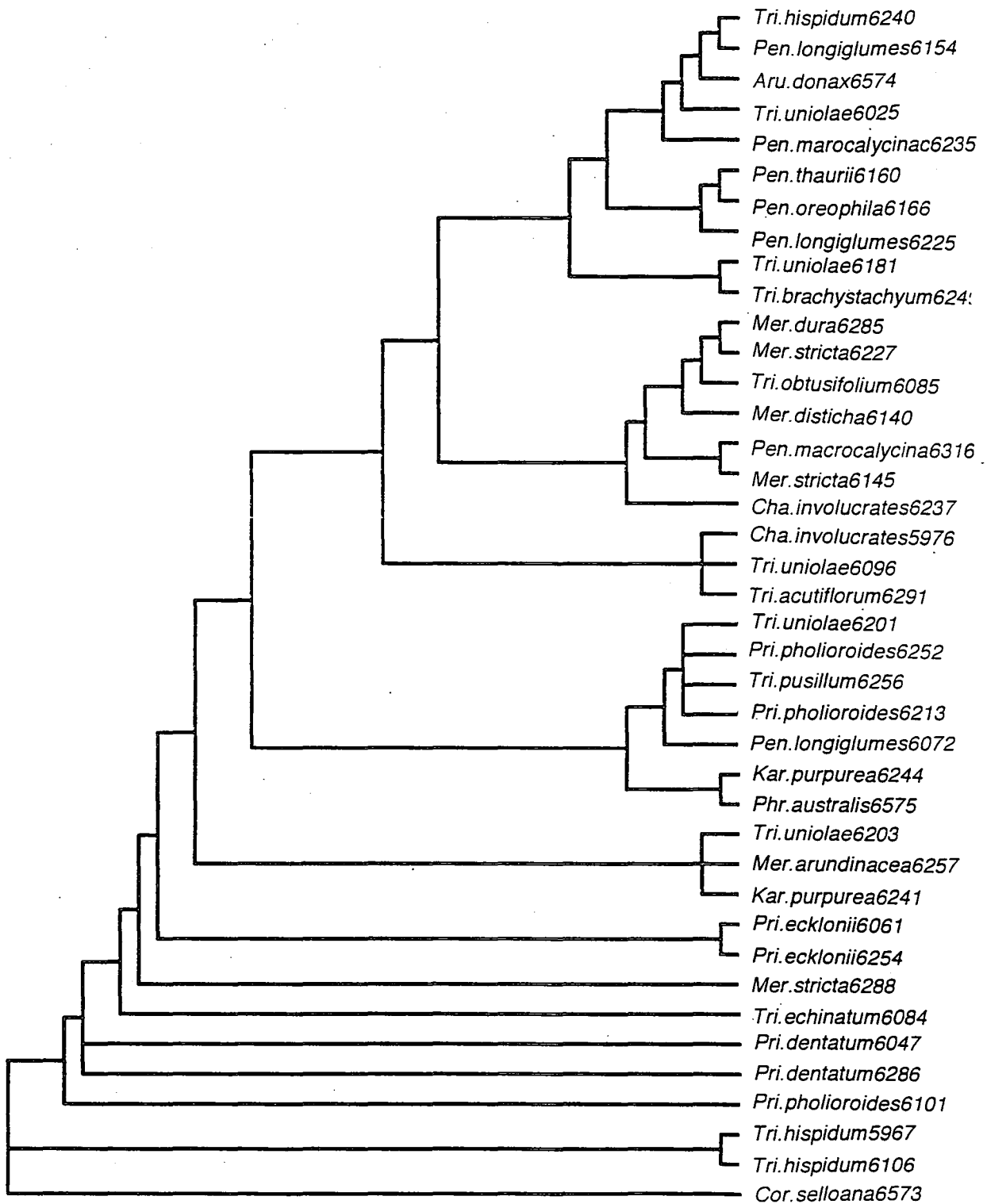


Figure 4.16 The nelsen consensus tree of more than 100 equally parsimonious trees computed after successive weighting of the data set. The trees have a length of 225, CI of 0.38 and RI of 0.68.

Due to the sensitivity of this fingerprinting technique, control reactions (without template DNA) are important components of the reaction (Sambrook *et al.* 1989). Even minor contamination from pipettes, reagents or tubes can lead to false results. This would be evident if a common fragment were to be found in the control reactions. There can be instances in which primers generate fragments in the absence of any template DNA. This phenomenon is attributed to primer multimer formation (Williams *et al.* 1990). Fragments present in both the control lanes and in the template containing reactions should, therefore, not be used for analysis as was done in this study.

4.3.1 Genetic variation

In the genus *Tribolium* great infra- and intergeneric distances were observed. Most of the D values are above one. In many species the variation is more in a specific species than between the different species. In this genus the distribution areas of the different species differ, but their habitats coincide. Most species are found in disturbed habitats such as on agricultural land and along roadsides (Gibbs Russell *et al.* 1990). According to Spies *et al.* (1992), most *Tribolium* species exhibit great genetical and morphological variation. This could explain the variation, which is evident from the calculated genetic distances (Table 4.1). This morphological and genetical variation is particularly evident in the species *Tribolium uniolae*. The infraspecific distances calculated for this species, (Table 4.1) confirm this. These variations could possibly be attributed to hybridization (Visser & Spies 1994d). The relationship between *T. brachystachyum* and *T. uniolae* is indicated by the genetic variation. These genera form part of the same morphological grouping, namely section *Uniolae* (Visser & Spies 1994d; Linder & Davidse 1997). Furthermore, the close relationships within the three groupings recognised in *Tribolium*, namely *Tribolium* (*T. echinatum*, *T. hispidum* and *T. pusillum*), *Acutiflorae* (*T. acutiflorum* and *T. obtusifolium*) and *Uniolae* (*T. brachystachyum* and *T. uniolae*) (Linder & Davidse 1997), can be inferred from Table 4.1.

In the genus *Prionanthium* the genetic distances are what is to be expected from a genus that has such a limited distribution range. *Prionanthium pholioroides* is distinct in the genus *Prionanthium* on the basis of a number of leaf anatomical features. Furthermore, *P. pholioroides* possesses sessile glands, whereas the other two species share stalked, multicellular glands (Ellis 1989). This is evident from the genetic distances calculated for this species: *Prionanthium pholioroides* is further separated from the rest of the species in

the genus (Table 4.2). Furthermore, there seems to be a greater amount of variation in this species as opposed to the rest of the genus.

Close affinities within the genus *Pentameris* are indicated by the genetic variation within the genus. A biosystematic study done by Barker (1993) found that within *Pentameris* two smaller clades are recognised: *Pentameris thuarii* would group in the one clade with other species not studied here and *P. longiglumes*, *P. macrocalycina* and *P. oreophila* would form the other clade along with *P. hirtiglumes* N.P.Barker. According to genetic distances, *P. thuarii*, *P. oreophila* and *P. longiglumes* share a close relationship (Table 4.3). This is in contrast with morphology. According to leaf anatomy, *P. thuarii* differs greatly from the other species in the genus (Ellis 1985b). The leaf anatomy of *P. thuarii* differs so much from that of the other species that a generic difference seems to be indicated (Ellis 1985c). Furthermore in the study by Barker (1993), *P. oreophila* and *P. macrocalycina* formed a monophyletic grouping in the genus by sharing conical tertiary ribs and permanently rolled leaf blades. *Pentameris oreophila* is distinguished by the autapomorphy of long prickles. In this study the genetic variation between these two species are the greatest, with *P. oreophila* showing closer affinities with *P. thuarii* and *P. longiglumes* (Table 4.3).

In the genus *Merxmuellera*, *M. dura* (Stapf) Conert and *M. stricta* are closely related (Table 4.4). These two genera resemble one another greatly on morphological level and are often confused for one another, especially in the north-western Cape region (Ellis 1980b). This corresponds with the localities in which the particular specimens representative of these species were collected. On anatomical level, these two species can, however, be easily distinguished (Ellis 1982b). Furthermore, affinities also exist between *M. dura*, *M. stricta* and *M. arundinacea* (Berg.) Conert (Table 4.4). *Merxmuellera dura* and *M. arundinacea* occupy very similar niches, with *M. dura* distributed in a more northerly direction. Their distributions, however, do not appear to overlap (Ellis 1982b). The anatomy of *M. arundinacea* is typical of the genus and it resembles the *M. disticha* (Nees) Conert group of species in leaf anatomy and epidermal histology (Ellis 1981b). These relatives in the *M. disticha* group are *M. disticha*, *M. davyi* C.E.Hubb. and *M. macowanii* (Stapf) Conert. This affinity can be seen from the genetic variation between *M. disticha* and *M. arundinacea* (Table 4.4). In *M. stricta* there are four anatomical "forms": the typical "form" (*M. stricta*), the Cathedral peak "form" (*M. guillarmodiae* Conert), the Drakensberg "form" (*M. stricta*) and the alpine "form" (*M. guillarmodiae*). Each of these "forms" exhibits characteristic leaf anatomy and epidermal structures (Ellis 1980b). In *M.*

disticha three anatomical "forms" exist: the typical *M. disticha* "form", the Drakensberg "form" and the alpine bog "form". It appears as if each of these three "forms" have different habitat requirements (Ellis 1980a). These two species share the same sort of infraspecific variation and occur in the same habitats in the Drakensberg mountain range (Ellis 1980b). According to Ellis (1980b), the anatomical differences between the different "forms" are of considerable magnitude. In many cases these differences are greater than those between other *Merxmuellera* species and even between some of the genera of Danthonieae. The amount of variation in these two species is not portrayed in the values of Table 4.4. This is due to the fact that in many cases the total variation present in the species was not sampled.

The genetic distances calculated within and between genera (Table 4.5), mostly do not correspond to recognised relationships in the tribe Arundineae. In many instances the variation within a genus is greater than the variation between genera. The reason for this could be attributed to phenomena such as co-migration, in which single fragments often comprise of several co-migrating fragments. It could also be attributed to the occurrence of apparently identical molecular weight fragments, which are not homologous fragments between different individuals.

This is evident from example the genera *Phragmites* and *Karroochloa* (Table 4.5). Within the genus *Karroochloa* more genetic variation exists than between *Karroochloa* and *Phragmites*. This may also be attributed to sample size and because only one of the four species in this genus was investigated.

Furthermore, from the DNA fingerprinting no apparent affinity between the genera *Prionanthium* and *Pentameris* (the only genera with seven as a basic chromosome number) can be found. Due to the fact that all the specimens were not investigated at a cytogenetic level, and the fact that we are dealing with different genera, polyploid levels could not be brought into consideration with genetic distances.

4.3.2 Phylogenetic analyses

Several measures are used to calculate the efficiency of the cladogram constructed using different cladistic software packages. Three main parameters used to determine these indices (Farris 1989 a, b) are the following:

s = length (number of steps) required by the character on the cladogram being evaluated,

m = minimum amount of change that the character may show on any conceivable cladogram,

g = maximum possible amount of change that a character could possibly require on any conceivable cladogram. In other words the length of the character on a completely unresolved bush.

The consistency index (ci) for a single character equals m/s (Kluge & Farris 1969). If ci is 1, the cladogram explains the data as well as possible. In 1989 (a, b), Farris proposed two new indices, the retention index (ri) and the rescaled retention index (rc). For a single character the retention index is defined as $(g-s) / (g-m)$. When a character poorly fits the cladogram the retention index will be zero. For uninformative (e.g. autapomorphic) characters, $m = g$, so that ri is undefined. Farris (1989a, b) recommends using ri as a factor for scaling ci between 0 and 1, defining the rescaled consistency index as the product of ri and ci ($rc = ri \times ci$).

The overall consistency index, CI (Farris 1989b) for a suite of characters is calculated as M/S where M and S are the sums over all the characters in the suite of individual-characters m and s values, respectively. The ensemble retention index, RI is similarly defined to the ensemble consistency index; $(G-S) / (G-M) = (\sum g - \sum s) / (\sum g - \sum m)$. The product of RI and CI is referred to as the ensemble rescaled consistency index, RC ($RC = CI \times RI$). The homoplasy index, HI is equal to $1 - CI$.

According to Farris (1989b), the retention index (RI) also expresses the homoplasy on the tree as a fraction of the maximum homoplasy that can be required for the data.

Some authors suggest that homoplasy as measured by the consistency index is an inappropriate measure of the reliability of datasets (for example, see Donoghue & Sanderson 1992). It, however, remains an indication of how well the data fits the tree and is an appropriate measure of the homoplasy itself (Goloboff 1991).

4.3.2.1 PAUP

Because large data sets can contain "messy" data, which would slow down calculation times, it is often necessary to look at a smaller set of the representative taxa, but possibly miss important character combinations. This is an opposition to looking at as

many species as possible, yet not being able to search the data adequately. These results almost certainly are not maximally parsimonious, whereas the results from the smaller data set may represent an overall simplified picture (Linder & Verboom 1996). The approach dealing with a smaller data set of representative taxa was implemented in this study.

The following interpretations can be made from the results obtained:

- From the phylogenetic analysis can be seen that an increase in CI and RI values was obtained after successive weighting (Fig. 4.13) of the datamatrix. Successive weighting (Farris 1969; Carpenter 1988) is a technique in which characters are successively down-weighted as a function of their unit consistencies on initial trees. Thus, higher weights are allocated to characters that are less homoplasious and smaller weights to characters that are more homoplasious. The advantage is thus that homoplasy is reduced in the matrix, but this would on the other hand be a disadvantage if the amount of homoplasy in the original datamatrix were informative.
- The tree obtained after the removal of characters from the matrix (Fig. 4.12), showed an increase in CI and RI values from the values obtained from the original data set (uninformative characters removed). The characters removed from the data set were those exhibiting a low CI:RI ratio (in other words characters exhibiting high levels of homoplasy). CI values are indicative of the amount of homoplasy (Higher homoplasy – lower CI value) and RI values are evident of the amount of synapomorphies predicted by the datamatrix, which are retained on the tree (Lipscomb 1998). Because synapomorphies (shared derived characters) are the bases of cladistic analyses, higher RI values are indicative of a tree in which many of the predicted synapomorphies are retained and, thus, represents the tree which probably predicts the phylogenetic history of a organism or group of organisms the best. So, even though a character has a low CI value due to homoplasy, a higher RI value would be indicative of the character still being informative and even indicating branch support (Lipscomb 1998). The ratio is not an absolute value, but was taken were the individual CI values were less than 0.2 and the accompanying CI:RI ratio was also less than two. These characters were temporarily removed.
- The tree obtained after character exclusion (Fig. 4.12), although showing an increase in CI and RI value, shows a loss in resolution in the upper part of the

cladogram. Character exclusion on the basis of low CI:RI values, therefore, does not seem to be an accurate way of dealing with homoplasy in this instance. Homoplasy, therefore, does not seem to show great branch support or evolutionary significance in this assemblage. This is also why successive weighting, and the consequent reduction in homoplasy, showed an increase in resolution in the topology of the cladogram (Fig. 4.13). Furthermore, it could also be that the characters excluded were highly homoplasious in a certain part of the cladogram, but consistent and phylogenetically informative in another part (Kellogg & Watson 1993).

- The CI and RI values are not very high in any of the studied trees. However, data which provides a good basis to choose among trees are not necessarily those with low homoplasy (or high CI or RI values), but instead are those for which some trees can require considerably greater amounts of homoplasy (higher values of CI and RI) than others (Goloboff 1991). Furthermore, the decisiveness of a data set is not a function of the homoplasy and, therefore, it is possible that adding characters to the data set could lower the homoplasy, but the decisiveness of the data set as well. Furthermore, cladists are not necessarily in search of data with a minimum homoplasy, but data for which the homoplasy can be minimised (Goloboff 1991).
- As can be seen from Fig. 4.11, most of the genera and species studied do not form definite monophyletic groupings. In the instances where more than one specimen for a species was investigated, usually two specimens are grouped together with a third specimen found elsewhere on the cladogram (for example *Tribolium hispidum*), while other species are not grouped very closely at all (see for example, *Merxmuellera stricta*, *Tribolium uniolae* and *Prionanthium pholioroides*). Often species of a genus are shown to be sisterspecies. Only the genus *Pentameris* forms a largely uniform grouping, with single specimens of the species *P. longiglumes*, however, scattered over the cladogram.
- The genus *Arundo* shows affinities with the outgroup *Cortaderia*. *Phragmites*, however, shows great affinities with the genus *Karroochloa*. Such a relationship is highly unlikely. Almost the only characteristic that these species share is the fact that both are perennial, rhizomatous species (Gibbs Russell *et*

- al.* 1990). According to Clayton and Renvoize (1986), *Arundo* is probably related to *Phragmites* but not as closely as the similarity in habit would suggest.
- The two specimens representing the genus *Chaetobromus* do not show any close relationship. This is consistent with known variability in the genus. Populations of *Chaetobromus* plants in the field are exceedingly variable, even within limited populations. Each plant usually differs in vegetative morphology. This appears even in homogeneous habitats where all the individuals are subject to the same environmental constraints and seem to be of equal age (Ellis 1988b). In habitats that are less uniform, such as rocky outcrops where water availability, soil depth and insolation can vary over very short distances, this variation can become even more, especially when the areas are also subject to grazing. This type of variation seems to suggest that large genetic distances exist between individuals, even in small populations (Ellis 1988b). This has been confirmed by cytogenetic studies (Spies & Du Plessis 1988).
 - As with the genetic distance analysis, no close affinity between the genera with seven as a basic chromosome number, *Prionanthium* and *Pentameris*, can be deduced.
 - One question that arises is whether *Prionanthium* should be grouped with *Tribolium* and *Stypeiochloa* to form, what Clayton and Renvoize (1986) refer to as, primitive genera. These three genera are the South African component of “ a heterogeneous assortment of genera brought together by their short glumes and multinerved lemmas with entire tips.” This is the basic form of unspecialised spikelet common to a number of unrelated tribes.
 - Gibbs Russell *et al.* (1990) did not place the monotypic genus *Urochlaena* in the genus *Tribolium*, because it was considered to be a type of “tumbleweed”. *Tribolium* and *Urochlaena* are, however, reproductively, morphologically, chemotaxonomically and cytogenetically related and are distributed in the same geographical area (Visser & Spies 1994e). Furthermore, *Tribolium* and *Urochlaena* are grouped together by Barker (1994) on basis of external fruit morphology. They share ovate, strongly dorsiventrally compressed fruit with a flaking pericarp. The embryo is a quarter of the length of the fruit. On the basis of these fruit characters they form a natural grouping. In particular the nature of the flaking and separable pericarp indicates a close relationship between the two

genera (Barker 1994). When Linder and Davidse (1997) revised the genus *Tribolium*, they, however, included *Urochlaena* in the genus *Tribolium* under the name *Tribolium pusillum*.

- In this study *Tribolium pusillum* grouped with *Prionanthium pholioroides*. Molecular studies, based on *rbcL* sequence data, indicate that *Tribolium pusillum* and *T. uniolae* are sister species (Barker *et al.* 1995). This is true for some *T. uniolae* specimens in this study.
- According to Clayton and Renvoize (1986), *Prionanthium dentatum* shows a distant relationship with *Tribolium*. Barker *et al.* (in press), by using a grass specific insert in the *rpoC2* gene, found *Prionanthium* to be included in the *Pentaschistis-Pentameris* clade, and not grouped with *Tribolium*. Furthermore, Linder and Davidse (1997) found *Prionanthium pholioroides* to be a sister species to *Tribolium ciliare* (Stapf) Renvoize, based on morphological analysis. This data suggests that *Prionanthium* is only distantly related to *Tribolium*, but that there has been extensive convergence between these two genera. In this study *Prionanthium* and *Tribolium* are grouped as sisterspecies to one another.
- The other possibility that arises is that *Prionanthium*, *Pentameris* and *Pentaschistis* form a grouping. Barker (1994), on the basis of external fruit morphology, found that *Pentameris* would belong in his group 4 with *Pentaschistis* and *Prionanthium* on the basis of overall similarity. He, however, treated *Pentameris* as an exceptional taxon on the basis of the phenetic groupings he employed in that particular study. In 1995, Barker *et al.* found that *Prionanthium* was included in the *Pentaschistis* clade, along with *Pentameris* and did not group with *Tribolium*, in the molecular *rbcL* sequence data study. This was confirmed by molecular studies based on *rpoC2* data (Barker *et al.* in press).
- *Pentameris*, *Pentaschistis* and *Prionanthium* are the only genera with seven as a basic chromosome number, along with the genus *Dregeochloa*. Furthermore, *Prionanthium* and *Pentaschistis* share many fruit characters, supporting the hypothesis that these two genera are closely related. Another character that is unique to these two genera is the possession of glands (Linder *et al.* 1990). This contrasts close relationships between *Tribolium* and *Prionanthium* (Clayton & Renvoize 1986).

- In this study *Pentasthictis* was not included. The *Pentameris* clade shows sister relationships with the various *Prionanthium* species-clades. No further apparent close relationships seem evident from the fingerprinting data.
- No clear-cut answer can be given to which of the two above-mentioned relationships is the correct one. Elements of *Pentameris*, *Prionanthium* and *Tribolium* show many unexpected affinities within the *Tribolium-Prionanthium* and/or the *Prionanthium-Pentameris* group.

4.3.2.2 HENNIG86

The mhennig (multiple hennig) search option, which was one of the options used, is a refinement of the hennig option. The hennig option builds a tree using the Wagner algorithm. This proceeds by successively adding the taxa in the matrix in a way to minimise the length of the tree (Farris 1988). However, as the Wagner algorithm proceeds there can be more than one way to add the next taxon to the growing tree. Using the hennig command will only follow one of these paths. The mhennig option will follow multiple paths in building the tree if there are multiple paths available (Farris 1988). For complicated datasets with homoplasy, hennig and mhennig are not likely to find the most parsimonious tree/trees. Branch breaking (bb) is then used to compute a better tree. The bb command applies branch swapping to trees constructed by another command, mhennig in this case. The branch swapping algorithm swaps the branches of the cladogram to search for equally parsimonious or more parsimonious trees.

The trees obtained with the entire data set (Fig. 4.14) and from the data set from which characters were excluded (Fig. 4.15), correspond greatly to those obtained from the same data sets with PAUP (Figs. 4.11 and 4.12). They correspond in tree length as well as CI and RI values. However, the topologies of the trees differ slightly from those obtained with PAUP.

After successive weighting the CI and RI values are dramatically increased from 0.14 to 0.38 and 0.33 to 0.68 respectively. The tree length is reduced from 2275 to 225. In contrast with the tree obtained with PAUP, this tree (Fig. 4.16) showed a decrease in resolution, with the creation of three trichotomies and a polytomy. The reason why the successive weighting trees in PAUP and HENNIG86 differ, could be that although successive weighting is done according to RC values in both instances, higher weights are not necessarily given to less homoplastic characters (Goloboff 1991).

Thus, although successive weighting in this instance, increased the consistency and retention index (and decreased the homoplasy), it decreased the resolution of the tree. It would seem to indicate that homoplasious characters support these clades that lost resolution.

Bootstrap and jackknife are both "resampling" techniques because they operate by estimating the form of the sampling distribution by repeatedly resampling data from the original data set. The bootstrap and the jackknife differ in the manner in which similarity between trees has been calculated. In the bootstrap the data points are sampled randomly, with replacement, from the original data set, until a new data set containing the original number of observations is obtained. Some data points will, therefore, not be included in the replicate while others may be included more than once. For each replication the statistic of interest is computed (Swofford & Olsen 1990).

The jackknife, however, resamples the original data set by dropping one data point at a time and recomputing the estimate (pseudo-estimate) from the remaining data. The change that results due to the dropping of one observation is as large as the change in the estimate that would result when a new sample of observations from the larger population is taken. Thus, the variance of the estimate can be calculated by extrapolating from the pseudo-estimates to the population at large (Swofford & Olsen 1990).

Because clade stability indices are monophyletic-dependant, such indices cannot be applied to unrooted networks and Siddall also argues that it is not suitable to jackknife outgroup taxa in the generation of pseudoreplicates (Siddall 1995). Therefore, the outgroup was identified before analyses were conducted and removed from the data set.

The jackknife monophyly procedure identified more monophyletic groupings with support values of above 0.65 from the available data set, than the bootstrap. This implies that when characters are removed one after the other, the monophyletic groupings are supported better than when a pseudoreplicate of the underlying data is constructed using the data in a random manner. The clades supported correspond to the results obtained with PAUP. Less bootstrap support was, however, found with the Random Cladistic software. This could be due to the fact that only 100 replicates were used in this software as opposed to the 200 replications used in PAUP. Both jackknife and bootstrap support values should not be used to reject or accept groupings. There might be argued that the values indicate the amount of statistical support for a clade, but inversely they could not indicate the amount of support that there is against a clade. The values only indicate which clades

would be more or less stable than other clades within and among most parsimonious trees (Siddall 1995).

According to Siddall (1995), the JMI has certain more advantageous qualities over the bootstrap and other methods of investigating the effects of homoplasy. The bootstrap technique is falsely biased by the number of synapomorphies supporting a group. The JMI is not biased in this manner by the influence of uninformative characters whether they are universally conserved or autapomorphic.

4.4 Conclusions

The use of arbitrarily primed DNA for phylogenetic purposes at species level has been reported widely (for example, Halward *et al.* 1992; Böhm *et al.* 1993; Baum *et al.* 1994). The genetic polymorphisms created by these techniques provide a powerful tool for organismal identification. DNA amplification fingerprinting (DAF) with arbitrary primers has emerged as a strategy to generate relative complex DNA profiles with high information content (Caetano-Anollés *et al.* 1991a).

In this study the technique was used in the elucidation of relationships at generic level. Although relationships between species were resolved in most cases, the relationships between genera remain uncertain. The ability of this technique to identify relationships at a level above that of species was not successful in this study.

In multilocus DNA fingerprints, such as those produced in DAF analysis, population genetics rest on the basic assumptions that fingerprint fragments (characters) are independent allelic products from different loci, allelic frequencies at each locus are uniformly distributed, and that co-migrating fragments are orthologous (i.e. representing the same locus) (Schierwater 1995). This may, however, not always be true.

In this study co-migration of paralogous fragments possibly did occur and certain non-homologous fragments possibly share the same molecular weight.

Even though DAF-analysis did not assist in the elucidation of the generic relationships in this study, the technique seems to be useful at lower taxonomic levels, such as within and between species.

CHAPTER 5

SEQUENCING

5.1 Introduction

Segments of the genome can be sequenced to obtain phylogenetically important characters. These characters can be obtained from sequences of most genes. However, the evolution of the sequence, not that of the organism is reflected in phylogenetic relationships based on sequence data (Doyle 1992).

For larger scale phylogenetic studies, DNA sequencing of slowly evolving protein coding genes or ribosomal RNAs is the more conventional approach in plants, as it is in other groups (Chase *et al.* 1993; Doyle 1993). The ubiquity of rRNA throughout nature and the development of DNA sequencing for the rapid determination of the primary nucleotide sequence of rRNA molecules, makes rRNA an excellent tool for inferring evolutionary relationships (Hamby & Zimmer 1992). In 1992, Hamby and Zimmer reviewed a broad rRNA survey with more than 60 sequences in the flowering plants.

Variation, or the lack of it, between different members of a rDNA family is of theoretical and applied interest. Variation can be seen in the internal transcribed spacers (*ITS1* and *ITS2*) and the intergenic spacer (*IGS*). *ITS* regions are more variable than the functional *18S*, *5.8S* and *28S* genes, but they are more conserved than the *IGS* region (Jorgensen & Cluster 1988). Variation between the *18S*, *5.8S* and *28S* genes are homogenised by processes like unequal crossing over and gene conversion, which are known collectively as molecular drive (Dover 1982).

In many angiosperm families, the internal transcribed spacer regions (*ITS*) of the *18-26S* nuclear ribosomal DNA (nrDNA) have proved to be a useful source of sequence-level characters for phylogenetic studies, at species and generic levels (Buckler & Holtsford 1996a). The two spacers that occur within this region (*ITS1* and *ITS2*) can be readily amplified with the PCR technique and sequenced, using universal primers (White *et al.* 1990). In most plant groups, both *ITS1* and *ITS2* appear to be variable enough to differentiate between closely related species. Variation between *ITS* sequences is attributed mainly to point mutations and less to insertions/deletions (indels) of nucleotide sequences (Grebstein *et al.* 1998). Alignment attempts across angiosperm families earlier indicated that plant *ITS1* and *ITS2* have diverged further at the level of their nucleotides than the

nrDNA subunits (Yokota *et al.* 1989), with the exception of the expansion segments subregions (Hassouna *et al.* 1984) or the large subunit (26S) divergent domains. Restriction site analysis of the nuclear DNA of closely related plant species have shown consistently that a high proportion of variable sites match to the *ITS* region, as well as intergenic spacer (*IGS*) and external transcribed spacer (*ETS*) regions (Appels & Dvorak 1982).

Properties of the *ITS* region that make it favourable for studies are the following:

1. The *ITS* region is one of the most highly repeated sequences in the plant nuclear genome.
2. It undergoes rapid concerted evolution (Arnheim *et al.* 1980) by means of gene conversion and unequal crossing over.
3. The small size of the *ITS* region (<700 bp in angiosperms).
4. The presence of highly conserved sequences flanking each of the two spacers makes the region easily amplifiable (Baldwin *et al.* 1995).

Within the family Poaceae, *ITS* sequences have been successfully used to resolve phylogenetic relationships at the subfamilial (Hsiao *et al.* 1994, 1995a) and tribal (Hsiao *et al.* 1995b) levels. These include species of the genus *Zea* (Buckler & Holtsford *et al.* 1996a, b), and the tribe Triticeae (Hsiao *et al.* 1995b). Phylogenetic relationships between 23 species of Aveneae, such as *Avena* and its close existing relatives *Arrhenatherum* and *Helictotrichon* (Greibenstein *et al.* 1998) and further members of the subfamily Pooideae (Hsiao *et al.* 1994, 1995a) have also been investigated in this way. A comprehensive molecular study on the phylogeny of the subfamily Arundinoideae, based on rDNA sequences, has also recently been conducted by Hsiao *et al.* (1998a).

The aim of this study is to use the information provided by the *ITS* region in determining the phylogenetic relationships within the tribe Arundineae.

5.2 Results

5.2.1 *ITS* region: length, variation and GC content

During this study we were not able to amplify the entire 5.8S gene or the *ITS2* region. Therefore only the *ITS1* region will be used for analyses. The grass genus *Ehrharta* Thunb. was used as outgroup. Two specimens were used, namely *Ehrharta capensis* Thunb. and *E. villosa* Schult. f. var. *villosa*.

The alignment of the *ITS1* sequences of the 34 specimens with CLUSTALW produced a consensus length of 241 (Appendix T). The alignment program MALIGN could not align the sequences, due to the computational time involved with the running of the program and the large size of the data set. The lengths of the specimens ranged from 189 (*Ehrharta villosa*, Spies 6299) to 228 (*Schismus barbatus*, Spies 6353). Alignment for all the taxa required one or more gaps at 28 of the 241 possible nucleotide positions or 12% of the sites. The percentage guanine (G) and cytosine (C) in the 34 specimens ranged from 62% [*Karroochloa purpurea* (Spies 4748); *K. tenella* (Spies 6290); *Schismus barbatus* (Spies 6353); *K. tenella* (Spies 4530); *S. scaberrimus* Nees (Spies 4660)] to 74% (*Centropodia glauca*). The average GC content was 67 %.

Of the 241 positions compared, 38 (16%) were identical in the 34 specimens (including the outgroup). Only 28 (12%) insertion/deletion events were postulated at the other 203 variable positions. Therefore, multiple alignments were readily accomplished. In nine of the 203 variable positions, the ingroup taxa were identical, but differed from the outgroup, *Ehrharta*. These positions are phylogenetically uninformative in the Arundinoideae. Another 51 sites were phylogenetically uninformative. They represent autapomorphies within the different genera. As more genera are added these autapomorphies may, however, become synapomorphies (Hamby & Zimmer 1988). There remain 135 (56%) truly phylogenetic informative variable sites. These are sites at which at least two species possess a common synapomorphic character (in other words the same nucleotide at the same position) (Hamby & Zimmer 1988). Of these 135 sites, 64% are due to base substitutions. This constitutes 22 unambiguous transitions and 13 unambiguous transversions. Fifty-two of the 135 variable sites were multiple hits, in other words at least three of the four nucleotides were observed at that position among all the specimens (Hamby & Zimmer 1988). Insertions or deletions represent only about a third (36%) of the variable sites.

Some gaps at the end of the sequence can be attributed to the shorter sequences of two specimens, *Tribolium utriculosum* (Spies 5892) and *T. pusillum* (Spies 6296), which were not fully sequenced.

There are two main phylogenetic informative gaps: A 12-13 bp deletion in the genera *Pentaschistis aristifolia* Schweick., *P. aspera*, *P. rupestris* (Nees) Stapf and *Prionanthium ecklonii* and a 12 bp deletion in *Merxmuellera macowanii*. Various smaller indels are found at positions 59, 68, 105, 112, 177 and 239.

Pairwise distances between taxa were calculated, by using the mean distance option in PAUP (Appendix U). These values ranged between genera from 0.061 (*Merxmuellera dura* and *Schismus scaberrimus*) to 0.581 (*Schismus barbatus* and *Karroochloa purpurea*). The values within genera ranged from 0.076 (*Pentaschistis*) to 0.419 (*Karroochloa*). In the species in which the values could be calculated, the variation ranged from 0.099 (*Merxmuellera dura*) to 0.117 (*Schismus barbatus*) and 0.119 (*Tribolium pusillum*). This variation is for the ingroup taxa only. When the outgroup, *Ehrharta*, is included the variation between genera range from 0.385 (*Karroochloa purpurea* and *Ehrharta capensis*) to 0.632 (*Merxmuellera macowanii* and *Ehrharta villosa*). Within the genus *Ehrharta*, the variation was 0.069.

5.2.2 Phylogenetic analyses

Phylogenetic analysis using heuristic search options yielded two equally parsimonious trees with a length of 518, CI of 0.51 and RI of 0.66. They only differ in the placement of the two genera *Merxmuellera stricta* and *Cortaderia selloana*. The Strict and Semistrict consensus cladograms computed were identical and differed from the Adams consensus cladogram (Figs. 5.1 and 5.2). The Adams consensus tree was the only tree to resolve the relationship between *Merxmuellera stricta* and *Cortaderia selloana*, whereas the Strict and Semistrict consensus trees yielded a polytomy at this position in the cladogram. The CI values indicate that just over 50% of the character state changes observed in the data set, are actual synapomorphies. Bootstrap support values of more than 65% support most of the clades, although some clades show a definite lack of support.

After successive weighting was applied to the data set three times, one single most parsimonious cladogram (Fig. 5.3) was computed with a length of 16996, CI of 0.67 and RI of 0.81. The bootstrap branch support values for this cladogram support most of the groupings with values ranging from 70% to 100%. More clades are supported than with the first heuristic search.

A search using mhennig and branch swapping in HENNIG86 computed two equally parsimonious trees with a length of 582, CI of 0.55 and RI of 0.66. From these two trees the nelsen consensus tree (Fig. 5.4) was computed.

Successive weighting was not implemented with HENNIG86, due to the fact that the two searches used in this study (PAUP and HENNIG86), correspond to such a great extent. Character removal was also not implemented again due to the fact that in the pre-

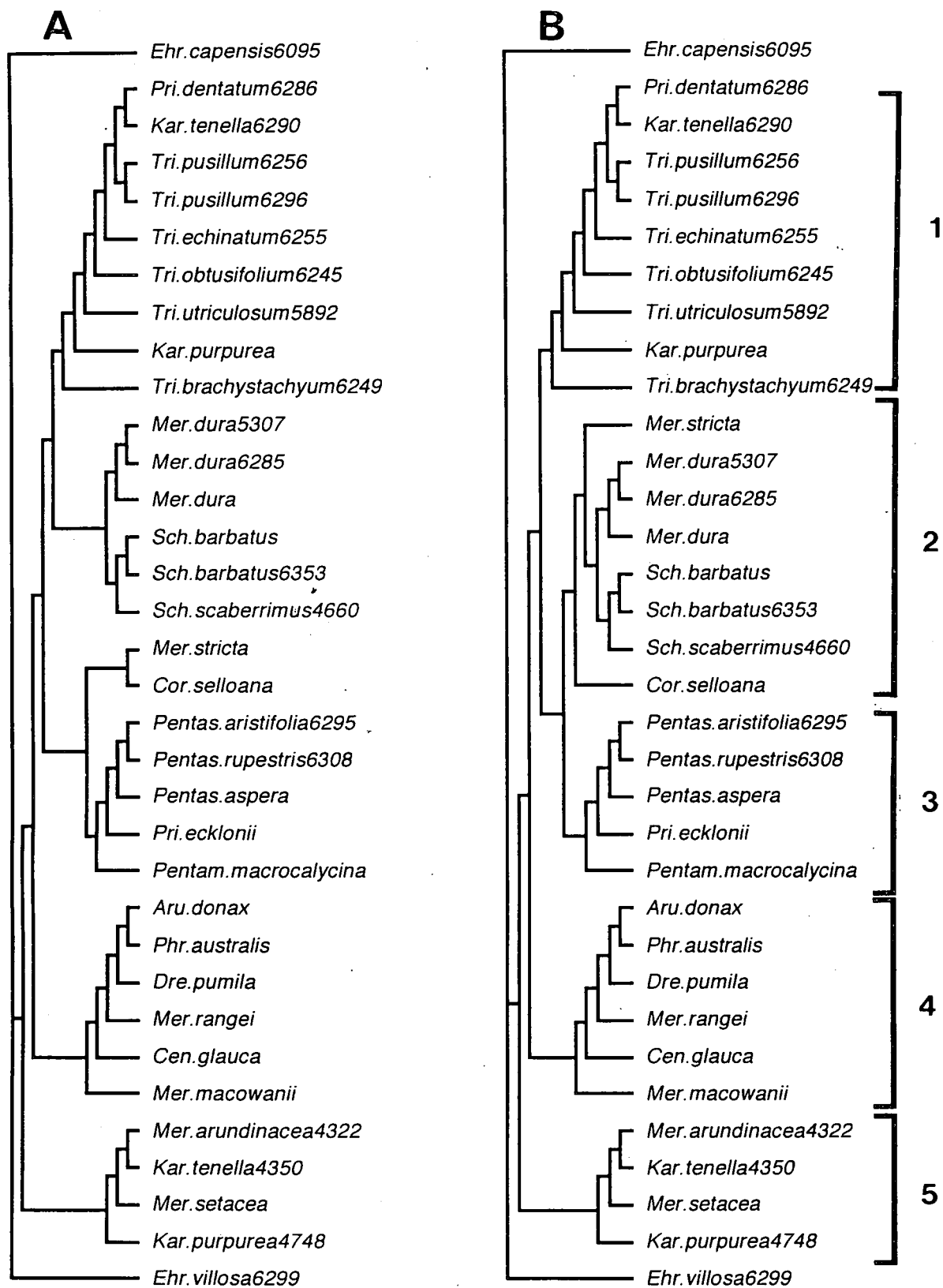


Figure 5.1 The Strict consensus cladogram constructed from the two equally parsimonious cladograms (Fig. 5.1A, B) with a length of 518, CI of 0.51 and RI of 0.66. The Semistrict consensus is identical to this cladogram. Bootstrap support values are indicated on the relevant branches in the Adams consensus tree. Clade numbers are referred to in the text.

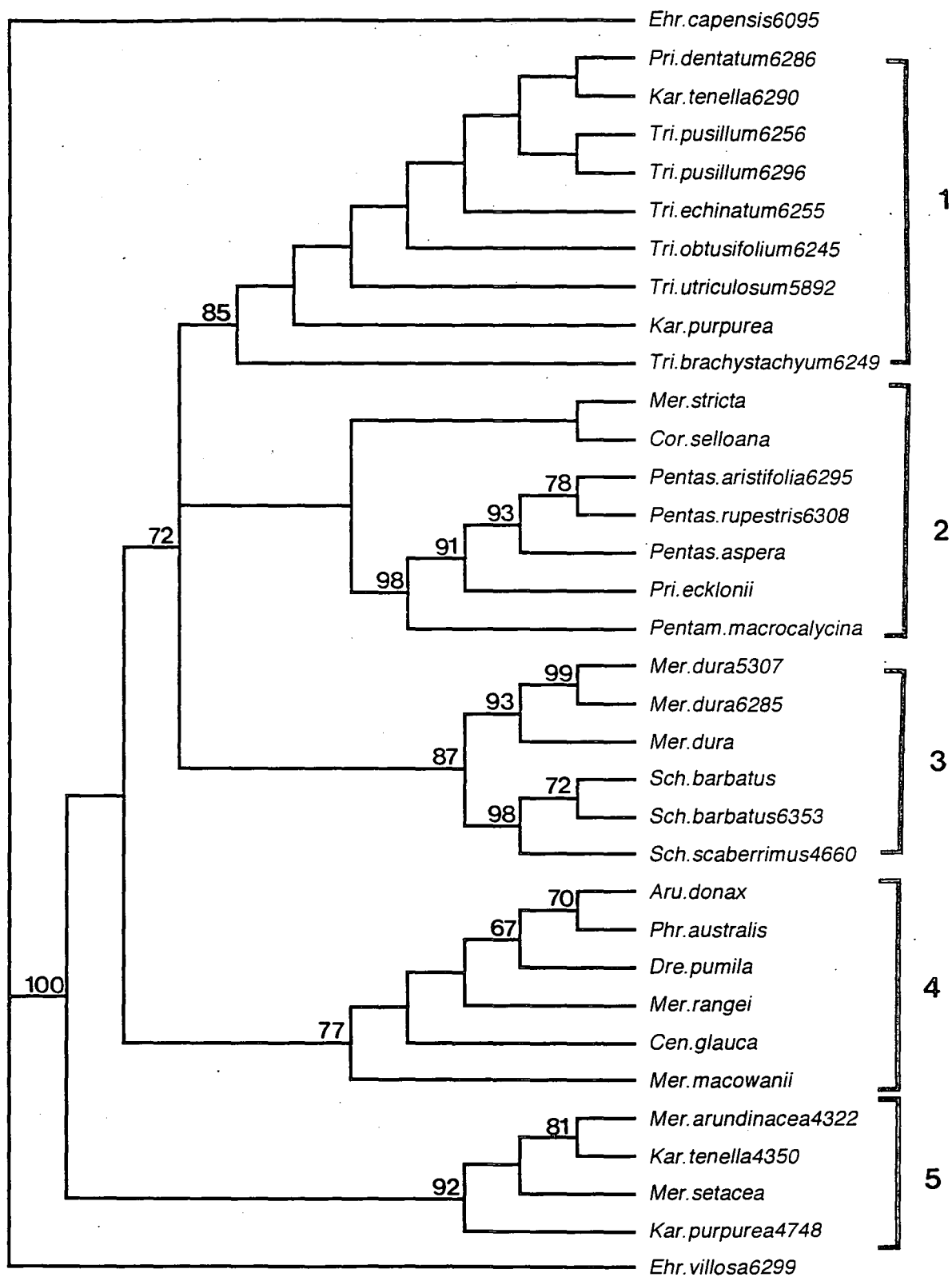


Figure 5.2. The Adams consensus tree computed from the two equally parsimonious cladograms in Figures 5.1A, B. This cladogram resolves the positions of *Merxmullera stricta* and *Cortaderia selloana*. Bootstrap values are indicated on the relevant branches. Clade numbers are referred to in the text.

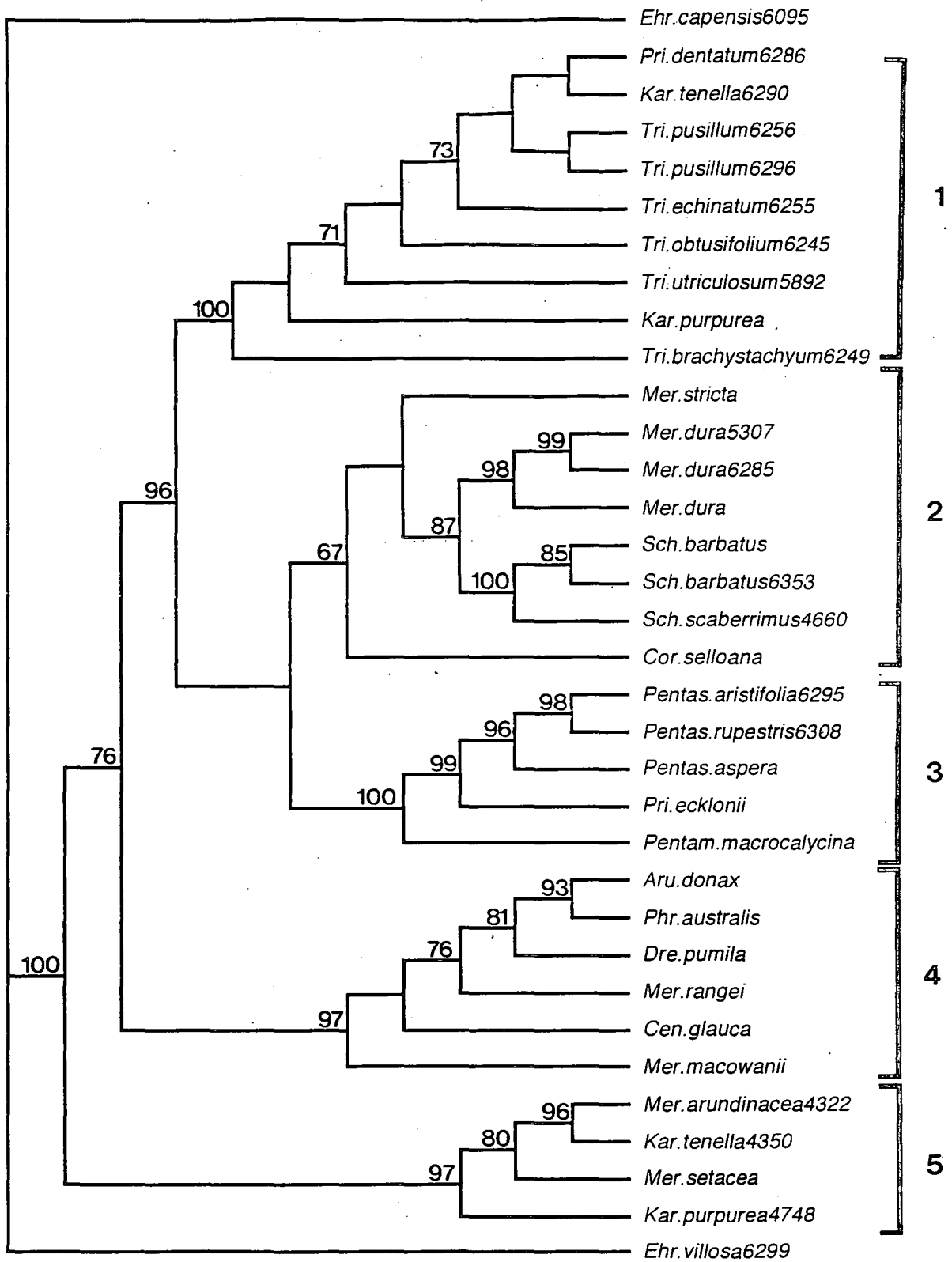


Figure 5.3 The single most parsimonious cladogram obtained after successive weighting of the data set. This tree has a length of 16996, CI of 0.67 and RI of 0.81. Bootstrap support values are indicated on the relevant branches. Clade numbers are referred to in the text.

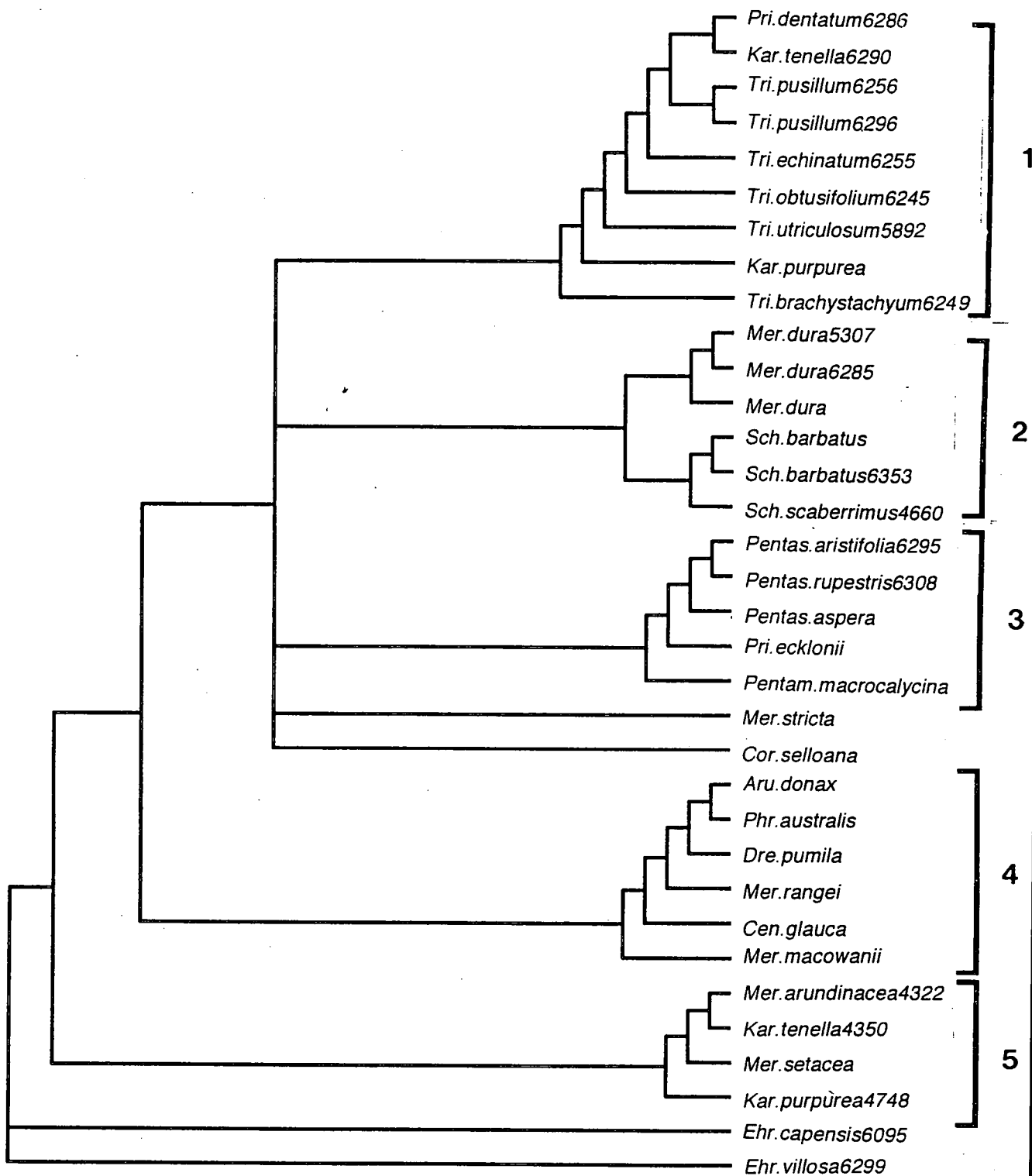


Figure 5.4 The nelsen consensus tree obtained in the mh, bb search using the HENNIG86 compute software program. The tree has a length of 582, CI of 0.55 and RI of 0.66. Clade numbers are referred to in the text.

vious chapter (DNA amplification fingerprinting), this method proved to be the less informative of the three tested. Character removal resulted in loss of resolution and was not comparable with the trees obtained from the original data set and the data set to which successive weighting was applied.

With the jackknife monophyly index, two equally parsimonious trees were computed. In the first tree 29 of the 31 groups had support values of more than 0.88. Fifteen of these had jackknife monophyly values of 1. In the second tree 29 groups had values of more than 0.88. Fifteen of these groups again had a value of 1.

With the bootstrap monophyly index analysis, two equally parsimonious trees were also computed. In the first tree 13 of the 31 groups had bootstrap support values of greater than 0.68. In the second tree only three monophyly groupings were computed, none of which had any significant bootstrap support.

5.3 Discussion

Not all DNA character types are equally informative. Some will be more informative than others. Those that undergo multiple changes obscure the original mutations and are less informative. They could blur character relations and cause underestimates of branch lengths. Characters such as the synonymous changes that are often found on third codon positions, in protein coding genes, are expected to be poor characters over long evolutionary periods, although they may be very useful at low levels of divergence (Doyle 1993). Källersjö *et al.* (1998) has, however, shown third codon positions to be phylogenetic informative in a study done on 2538 *rbcL* DNA sequences of all the major groups of plants. When these positions were omitted, loss of resolution resulted. Furthermore, fewer groups were lost in the analyses based solely on third codon positions, when compared to first and second positions only.

Likewise, transitions are thought to generally occur far more readily than transversions. This difference in probability of change can be reflected in the weight given to the transformation type (being either transversions or transitions). One extreme is to view DNA sequences as having only two states, pyrimidine versus purine, instead of four (A, C, G, T). This would ignore transitions entirely (Doyle 1993). In this study, all four nucleotides were used for analysis.

The first stage of phylogenetic analysis of DNA sequences defines the character homologies on which the whole study is based (Doyle 1993) and, therefore, it is an

absolutely critical step. Since the probabilities of occurrence of both indels and point mutations are unknown, similarities among sequences are often maximised by adding gaps almost randomly. Options as to how the gaps should be treated range from ignoring them completely, to considering them more informative than single nucleotide changes (Lloyd & Calder 1991). In this study, the indels and nucleotide substitutions were treated as equally informative although the indels represent only a third of the possible variable informative sites. Transitions and transversions were also treated equally. Cracraft and Helm-Bychowski (1991) found that when transitions and transversions are equally weighted, they could reveal a stable phylogenetic signal, even in cases in which the data are generally thought to be rather "noisy". Furthermore, they state that there is a general increase in the phylogenetic signal as determined by the bootstrap when sample size increases. The faster rate of accumulation for transitions in a sequence, however, make transitions more sensitive to multiple substitutions at a given nucleotide position, and thus less informative for phylogeny as the divergence between species increases (Mindell 1991).

5.3.1 ITS region: length, variation and GC content

The GC contents of the *ITS* region of most grasses are higher than 50%. The average of 67% found in this study, is similar to that of other grass subfamilies (Hsiao *et al.* 1995a, 1998a, b). Salinas *et al.* (1988) showed by reassociation kinetics of single-stranded DNA that grasses growing in arid regions have on average a higher GC content than plants from temperate areas. This suggests an adaptive significance of the higher GC content. Furthermore, the high GC content observed in most of the specimens studied could account for the difficulties experienced in sequencing the *5.8S* and *ITS2* region. These high GC levels can cause stronger template secondary structures, which may confound the sequencing reactions (Steane *et al.* 1999).

Although several small gaps (1-4bp) are present in the *ITS1* spacer, nucleotide substitution appears to be the main source of variability. This was the most common polymorphism observed in this spacer region. This is in agreement with other plant groups (Baldwin *et al.* 1995). The presence of larger indels is suggested by segments of sequence that do not have any homologues in the sequences of the other taxa. The transition and transversion ratios were about equal, with transitions being the more common mutation.

As can be seen from the pairwise distance analysis (Appendix U), the variation within species is the lowest. The values within certain genera, such as the outgroup

As can be seen from the pairwise distance analysis (Appendix U), the variation within species is the lowest. The values within certain genera, such as the outgroup *Ehrharta* (0.069) and the genus *Pentaschistis* (0.076) were, however, also very low. This could be attributed to the fact that only a few specimens were investigated and that the variation in these large genera are not represented by these values. Within the genera *Karoochloa* (0.419) and *Prionanthium* (0.377) the variation is very high. This is unexpected for small genera such as these. These high values are, however, corroborated on the cladograms where *Karoochloa* is polyphyletic and *Prionanthium* shares affinities with *Tribolium* and the *Pentameris-Pentaschistis* groupings. The variation is, as expected, higher between the outgroup and the ingroup taxa.

5.3.2 Phylogenetic analyses

The two equally parsimonious trees obtained in the first search are in general agreement, except for the position of *Merxmuellera stricta* and *Cortaderia selloana* and the relationships of clades 1-3 to one another. The Strict (Fig. 5.2) and Semistrict consensus trees computed put these two genera and the clades at unresolved positions in the cladograms to form polytomies. The Adams consensus tree (Fig. 5.3) resolved the relationships by grouping the two genera into a single clade in the same manner as in Fig. 5.1A. The positions of the clades were, however, not resolved.

Of these consensus tree methods, the Strict consensus is theoretically the simplest. Sokal and Rohlf (1981) have defined this consensus as the unique tree that contains only those groups that appear on all of the rival trees. The biggest advantage of the Strict consensus is the simplicity of the interpretation thereof. If a group appears on the consensus, it certainly appears on all the rival trees. Some say, however, that it may be too strict in some instances (Swofford 1991).

Bremer (1990) formally described the combinable component (Semistrict) consensus tree. Hillis (1987) basically also put the same idea forth. This tree is defined by the set of all combinable groups (i.e., each group retained in the consensus is equal to, or combinable with all groups of every rival tree). Two groups are termed "combinable" (Nelson 1979) if either:

1. They have no taxa in common (exclusion),
2. They are identical (replication),
3. One group is a proper subset (further resolved) of the other (inclusion).

When all the rival trees are fully dichotomous, the Strict and combinable component consensus methods will render similar results (Swofford 1991).

Polytomous nodes on consensus trees do not necessarily indicate simultaneous cladogenetic events (hard polytomies; Maddison 1989). They rather indicate areas of uncertain-resolution (soft polytomies) interpretation, that will efficiently allow a polytomy to be resolved in a way that is most favourable for each character considered individually (Swofford 1991).

The method of Adams (1972) predates all the other consensus methods. The Adams consensus often preserves more of the structure found in the rival trees than do Strict consensus's (Swofford 1991). According to Adams (1972), this consensus satisfies the following two conditions:

1. Any nesting found in all of the rival trees must also occur in the consensus tree.
2. Any nesting that reflects clusters of the consensus tree (this being a nesting involving the inclusion of one monophyletic grouping within a larger monophyletic group) must be found on all the rival trees.

In biological terms, a group of taxa nests within a larger one if the more recent common ancestor of the smaller group is a descendant of the most recent common ancestor of the larger group (Adams 1986). Critique against this consensus is that it might produce a consensus containing clusters that do not appear on any of the rival trees. This complicates the interpretation of Adams consensus trees, but according to Adams (1986), this must be accepted if it is agreed that the structure of a tree encompasses more information than a simple listing of its clusters.

The problem of clusters appearing on the consensus, which do not appear on any of the rival trees, was not experienced in this study. This can be attributed to the fact that only two rival trees exist. It might be a problem with a large amount of equally parsimonious trees.

The tree obtained after successive weighting (Fig. 5.3), corresponds 100% to one of the two equally parsimonious trees (Fig 5.1B) obtained from the first search. This tree places *Merxmullera stricta* and *Cortaderia selloana* at opposite positions of clade 2 and not as a single clade. The only other difference between the first search and the search after successive weighting is that the latter had a remarkable increase in the values of the descriptive statistics, CI and RI.

In the nelsen consensus tree (Fig. 5.4), the *Merxmuellera-Cortaderia* clade and clade 1-3 polytomy is unresolved as in the Strict and Semistrict consensus trees (Fig. 5.2).

As can be seen, the consensus trees obtained from PAUP and HENNIG86 respectively, are in general agreement in tree length, CI and RI values. The trees consist of five clades, as well as the two outgroup *Ehrharta* species. The positions of the clades differ in some respects between the trees, but are in general agreement with the species and genera contained within the clades.

The following conclusions can be drawn from the phylogenetic trees (See Fig. 5.4):

- Of special interest is clade 3, which consist of the genera *Prionanthium*, *Pentameris* and *Pentaschistis*. This grouping is supported by a bootstrap value of 100%, with values of 96, 98 and 99% support within the different branches in the clade. This grouping is not suprising. These genera (along with *Dregeochloa*) are the only genera with seven as a basic chromosome number. Furthermore, they are anatomically very similar. Ellis (1985c, 1986a; Ellis & Linder 1992) is of the opinion that on the basis of leaf anatomy, *Pentameris* is closely allied to *Pentaschistis*. Three *Pentaschistis* species, *P. pallescens* (Schrad.) Stapf, *P. silvatica* Adamson and *P. tortuosa* (Trin.) Stapf bear a very strong resemblance with *Pentameris thuarii* (Ellis 1985c). *Prionanthium* and *Pentaschistis*, on the other hand, are the only two genera which possess glands. *Prionanthium* is unique in possessing two gland types. In *Pentaschistis* both these gland types occur on different plants or species and are characteristic of two different species groups. On the basis of this, *Prionanthium* appears to be intermediate between the *Pentaschistis* species group represented by *P. trisetata* (Thunb.) Stapf and *P. thunbergii* (Kunth) Stapf (Ellis 1989).
- The *Prionanthium dentatum* specimen is, however, not grouped with this clade but within clade 1, which consists of *Karoochloa* and *Tribolium*. This again groups the genera *Prionanthium* and *Tribolium* together as proposed by Clayton and Renvoize (1986). It is possible that this particular specimen is distinct in the genus *Prionanthium*. In the fingerprinting chapter this specimen did, however, group with the other *P. dentatum* specimen in the phylogenetic tree with 64-74% bootstrap support.
- The genera *Karoochloa*, *Merxmuellera* and *Prionanthium* are polyphyletic according to the *ITS* data.

- According to Hsiao *et al.* (1998a), *Merxmuellera rangei* (Pilg.) Conert and *M. macowanii* are closer related to *Centropodia*, than to other *Merxmuellera* species. *Centropodia* is the only genus in the tribe with Kranz leaf anatomy and a C₄ photosynthetic pathway (Ellis 1984). This relationship is corroborated by this study.
- The species *Merxmuellera dura* forms a monophyletic clade, sister to a *Schismus* assemblage. These two genera form clade 2. Hsiao *et al.* (1995b) found that *ITS* sequence divergence between grass species were useful for inferring phylogenetic relationships only at tribal and generic levels and not at the infraspecific level in Triticeae. As can be seen from *Merxmuellera dura*, *Tribolium pusillum* and *Schismus barbatus*, *ITS* sequence data successfully resolved infraspecific relationships within these species.

According to Hsiao *et al.* (1998a), *Arundo* and *Phragmites*, despite similarities in both having erect elongated multi-noded stems, feathery terminal inflorescences and habitats, did not appear closely related on basis of *ITS* sequence data, supporting similar conclusions by Clayton and Renvoize (1986) on the basis of morphology. In this study, where none of the "foreign" (not occurring in South Africa) arundinoid genera such as *Molinia* Schrank, *Monachather* Steud., *Spartochloa* C.E.Hubb. etc. were investigated, the relationships between *Arundo* and *Phragmites* is that of sister genera in clade 4 with 93% bootstrap support. This is corroborated by the study done by Barker *et al.* (1995) in which *Arundo* and *Phragmites* were grouped together into a single clade with the *Arundo* and *Monachather* clade grouped as the sistergroup to the *Phragmites* and *Moliniopsis* Hayata clade.

When the prolamin structure of some Arundineae were investigated, Hilu and Esen (1990) found that the epitope structure and prolamin size of different arundinoids were similar in spite of the antiquity of the group. The answer might be the limited degree of speciation and radiated evolution, which is encountered in most members of the group. As stated by Renvoize (1981), the Arundinoideae generally failed to have a dramatic impact on the environment, except for a few genera, which are often dominant in their chosen environments, such as *Arundo* and *Phragmites*. *Phragmites*, however, shows great divergence in terms of prolamin structure and polypeptide profile as opposed to the rest of the subfamily. Renvoize (1986) excluded *Phragmites* from the core genera of the

Arundinoideae, placing it in his group of peripheral genera on the basis of distinctive anatomical characteristics, along which is the presence of arm cells. In 1987, Ellis supported the removal of *Phragmites* from the core genera of the Arundinoideae, to produce a more homogeneous grouping. Conert (1987), treating the Arundinoideae in a narrow sense, indicated that the reeds group, which includes *Phragmites*, is the most primitive of the arundinoid taxa. He indicated that the small size of the genera (1-3 species per genus) and their chronology indicate the great age of the group. In this study *Phragmites* did not occupy any isolated position in the Arundineae, as proposed by these prolamin studies.

According to Hsiao *et al.* (1998a), *ITS* sequence data suggest that Danthonieae is the youngest tribe in the Arundinoideae. The Arundineae include most of the reedy arundinoid genera. *Cortaderia*, however, appears in the Danthonieae clade. The large robust habit, seemingly a parallel trait between Arundineae and Danthonieae, is thus not a useful character for tribal distinction. It must be stressed that in this study the tribal delimitation of Arundineae and Danthonieae were not used. Therefore, most of the specimens in this study referred to as Arundineae are commonly known as danthonoid genera, except *Arundo* and *Phragmites*, which are known arundinoid genera.

Hsiao *et al.* (1998a) found Arundineae to be paraphyletic (*Arundo*, *Phragmites*, *Dregeochloa*) and Aristideae and Danthonieae to be monophyletic. General groupings in these tribes agreed with the results of Barker *et al.* (1995), which were based on *rpoC2* and *rbcL* data. However, in the study by Barker *et al.* (1995), the Arundinoideae were polyphyletic in relation to other subfamilies in the family-level analysis of the *rbcL* sequence data. In this study the South African Arundineae (*Arundo* and *Phragmites*) were, however, found to be monophyletic as well. When "foreign" genera are added the Arundineae do become paraphyletic, as mentioned above. Only if more specimens in these genera are investigated, can the monophyly of the Arundineae (*Arundo*, *Phragmites* as well as *Dregeochloa*) be corroborated.

The recognition of Arundineae and Danthonieae as separate tribes is supported by *rbcL* phylogeny (Barker *et al.* 1995). In this study, however, the Arundineae were found to be an integral part of the Danthonieae, nested in clade 4 with danthonoid genera. These species (*Merxmuellera macowanii*, *M. rangei* and *Centropodia glauca*) were considered by Hsiao *et al.* (1998a) to be the link between Arundineae and Danthonieae.

Most of the clades show bootstrap support of more than 65%. The only groupings which are not supported are most of the higher level relationships in clade 1, which has

more basal bootstrap support, the *Merxmuellera-Cortaderia* polytomy discussed earlier and *Centropodia glauca* and *Merxmuellera macowanii* in clade 4. The polytomy between clades 1, 2 and 3 also show good branch support with values ranging from 67 to 100% supporting each of the clades.

The values obtained by the two monophyly indices do not correlate precisely with the values obtained by PAUP. With the Bootstrap monophyly index, only clades 2 to 5 show any branch support, while clade 4 shows little basal support. No support is found at the base of the tree, as with the analysis with PAUP.

With the jackknife monophyly index, practically all the monophyletic groupings show branch support. Most of these support values range from 90% to 100% support. The only clades which are less supported with values of about 50%, depends on where the *Merxmuellera stricta* and *Cortaderia selloana* polytomy is grouped. When grouped with the *Merxmuellera dura-Schismus* clade, this assemblage shows little branch support compared to the rest of the tree. When the polytomy is grouped with the *Pentaschistis-Prionanthium-Pentameris* clade, this grouping shows very weak branch support. The grouping of these two genera is problematic. An explanation could be that *Merxmuellera stricta* is a species, which has possibly undergone hybridisation within the species itself, as well as with other closely related species. This is corroborated by the fact that the species exhibits a great amount of morphological variability (Ellis 1980b). Furthermore, cytogenetic studies have also indicated that this species might be of hybrid origin (See cytotaxonomy chapter). The probability that one or both putative parents are included in this study is very slight and this could explain why *Merxmuellera* is not grouped satisfactorily with any other groupings. In an examination of the entire *ITS* region, the position of these two genera were resolved to different clades (Hsiao *et al.* 1998a).

5.4 Conclusions

The difficulties that exist in studying morphological and molecular based phylogenies of the Arundinoideae must be an accurate representation of their complex evolutionary history. In part the problems could be explained by an extensive extinction of the core arundinoid taxa (Clayton 1978) and a subsequent adaptive radiation that may have interrupted the continuity of their phylogenetic relationships (Hsiao *et al.* 1998a).

ITS analyses have proved to be useful in the elucidation of the relationships between some representatives of the South African Arundineae. Where more than one

specimen per species was studied, the technique proved to indicate the close affinities within the species. The grouping of *Pentameris*, *Prionanthium* and *Pentaschistis* seems to be proven. This is, however, complicated by *Prionanthium* also grouping with *Tribolium*. The genera *Karoochloa*, *Merxmuellera* and *Prionanthium* seem to be polyphyletic. The position of *Arundo* and *Phragmites*, nested within the Danthonieae, has been shown in this study.

In order to obtain further resolution, the inclusion of more species per genus, and especially more specimens per species, seems essential to determine the most possible phylogenetic history of the Arundineae in South Africa.

Ultimately the following argument is applicable: gene trees may not represent species trees (Doyle 1992). No amount of resolution or confidence in any gene tree can unmistakably prove that the history of those particular genes tracks the evolutionary history of the species from which the genes were isolated (Doyle 1993).

CHAPTER 6

PHYLOGENY

6.1 Introduction

Most phylogenetic studies have as a principle objective to contribute to the discovery of the true species phylogeny underlying biological diversity (Hillis *et al.* 1994). Heritable characters of taxa are the products of this history and, therefore, are of potential value in its explanation. Therefore, different data sets are expected to converge onto the true species phylogeny for their group (Miyamoto & Cracraft 1991). In systematics, this expectation forms the basis for testing the reliability of the phylogenetic assumptions with new independent data sets. Usually those hypotheses that are supported by different lines of evidence are preferred over those that are not supported, in other words, congruent trees obtained from the analyses of independent data sets provide the greatest estimates of the true phylogeny for any group (Cracraft & Helm-Bychowski 1991).

There has been some debate on whether to combine different data sets that are informative on the phylogeny of the group of taxa being studied, or whether to treat these data sets separately (for example, Miyamoto 1985; Kluge 1989; Barrett *et al.* 1991; Swofford 1991; De Queiroz 1993; Olmstead & Sweere 1994; Miyamoto & Fitch 1995). The combination of all available data into a single total evidence analysis can be analytically advantageous (Kluge 1989; Olmstead & Sweere 1994; Linder & Crisp 1995), but in the cases where the different data sets do not track the same phylogenetic pathway the results will be misleading (Swofford 1991; Doyle 1992).

Another approach is consensus methods, in which single most parsimonious consensus cladograms are generated from each of the separate trees obtained by the separate analyses (for example DNA fingerprinting and DNA sequencing). These methods have been advocated because:

1. they give equal weight to each data set, thus reducing the effect of large data sets swamping smaller data sets (Kluge 1983),
2. they are thought to give a more conservative estimate of phylogeny (Hillis 1987).

Advocates for the combined method argues as follows:

1. giving data sets equal weights results in arbitrary differential weighting of characters (Cracraft & Mindell 1989),
2. consensus trees do not necessarily indicate the most parsimonious pattern of character change (Miyamoto 1985),
3. consensus trees can contradict combined trees, and thus, consensus methods are not necessarily conservative (Barrett *et al.* 1991).

A combined tree can be better resolved than a consensus tree because, firstly the information that resolves certain relationships may only be present in certain data sets. Secondly, where there is a conflict among characters, increasing the number of characters may allow the phylogenetic signal (synapomorphy) to assert itself over phylogenetic noise (homoplasy) (De Queiroz 1993).

Miyamoto and Fitch (1995) argued that the biological and evolutionary properties of different data sets make it probable that agreement among their resultant topologies is the result of true species phylogeny, rather than of similar (but nonphylogenetic) factors responsible for the independent histories of the character sets, or of systematic errors and model failures in phylogeny reconstruction methods. They, therefore, emphasise corroboration between independent data sets as a means to formulate phylogenetic hypotheses. This simultaneous analysis of independent data sets provides an assessment of the overall congruence of characters from all sources of data and may enhance the detection of the true phylogeny (Steane *et al.* 1999). Where data sets yield strongly supported, but conflicting cladograms it may, however, be judicious to keep the data sets separate (Steane *et al.* 1999).

The aim of this chapter is to investigate the phylogeny of the tribe Arundineae. This will be done by comparing the results obtained in the previous chapters with one another and then also to investigate whether a combined analysis of the different data sets, provides better resolution of the phylogeny of the tribe Arundineae than any of the separate analyses.

6.2 Results

Initially in this study the data sets were analysed separately to locate the sets of minimally length trees, to calculate the descriptive statistics of the trees and to determine the phylogenetic signals in each data set (See chapters four and five).

A problem that arose was that the DNA sequencing data and DNA fingerprinting data was, however, not all collected from the same specimens. The assumption was made that these data sets could be combined for a combined data analysis. Summary taxa were created for most of the species by combining data were more than one specimen per species was investigated. The data sets were unweighted, as suggested by Huelsenbeck *et al.* (1994).

The combined data matrix was only analysed with the PAUP computer program. The matrix comprised of 575 characters. This is outside the range of characters, which the HENNIG86 software can analyse.

Thirty-four equally parsimonious trees were computed from the total combined data set, with a length of 1133, CI of 0.42 and RI of 0.43. The Strict and Semistrict consensus trees (Fig. 6.1) computed were identical and differed from the Adams consensus trees computed. The Adams consensus tree (see Fig. 6.2) resolves some of the relationships in the *Pentameris-Pentaschistis* clade. Bootstrap support values calculated ranged from 71% to 97%, with no support in the upper half of the topology.

After successive weighting was applied to the data set three times, the tree stabilised. Seventeen equally most parsimonious trees, with a length of 21294, CI of 0.67 and RI of 0.74, was computed. The Strict and Semistrict consensus trees were identical and also correspond with the Strict and Semistrict consensus trees computed during the first heuristic search (see Fig. 6.1). These consensus trees differed from the Adams consensus tree, which resolves the higher level relationships in the *Pentameris-Pentaschistis* clade as with the first heuristic search (Fig. 6.2). Bootstrap support values calculated, supported most of the groupings on the tree with values ranging from 69% to 100%.

Due to the fact that HENNIG86 could not be run, the Random Cladistics software program could also not be used for analyses.

6.3 Discussion

6.3.1 Combined analysis

The consensus cladograms obtained in the analysis of the equally weighted combined data set, is in agreement with the results obtained in the DNA sequencing analysis. The tree is well resolved in its branches, except for the trichotomy in the *Penta-*

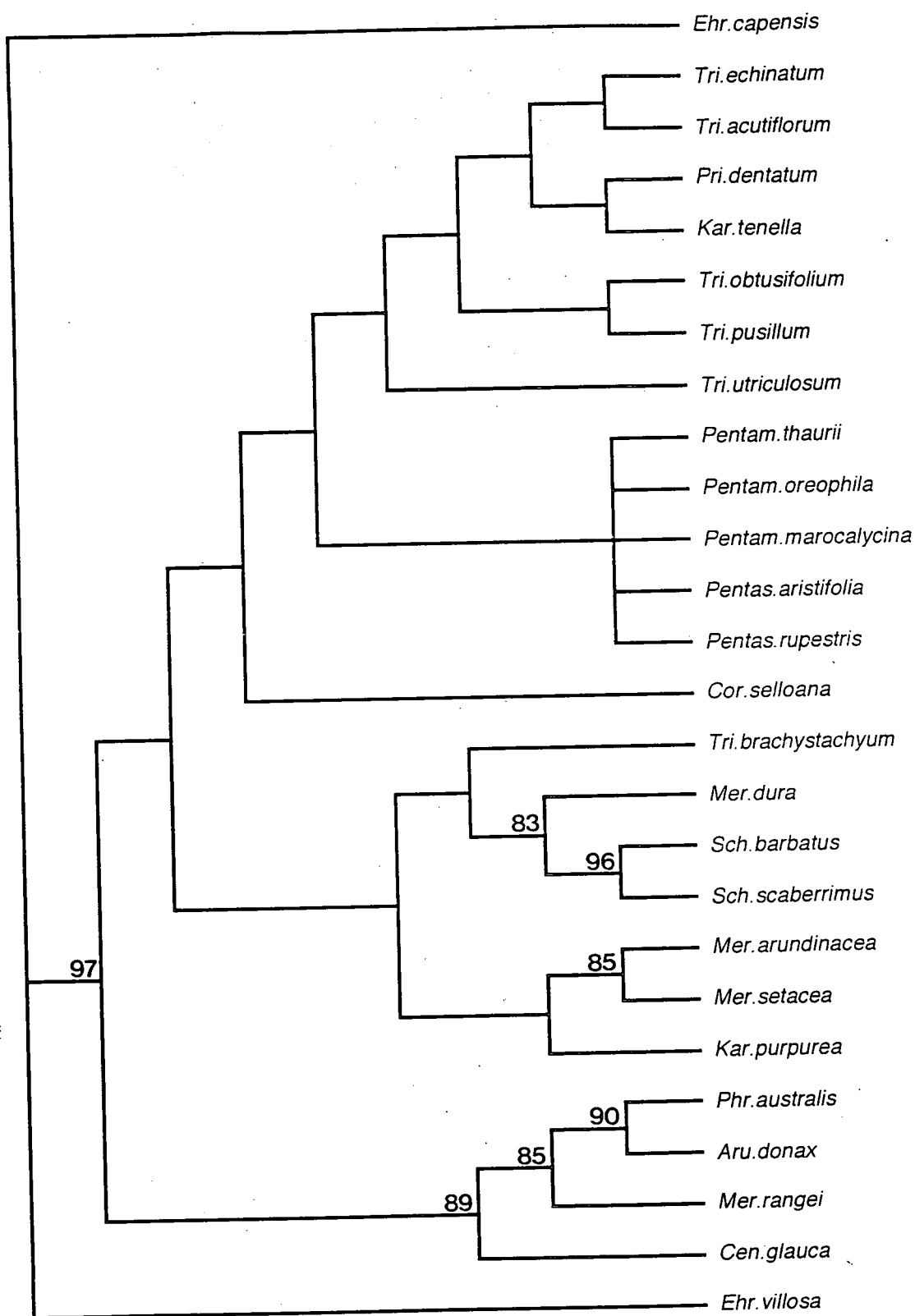


Figure 6.1 The Strict consensus tree computed from 34 equally most parsimonious trees obtained by a heuristic search of the combined data set. The trees have a length of 1133, CI of 0.42 and RI of 0.43. The Strict and Semistrict consensus trees were identical. Bootstrap support values are indicated on the relevant branches.

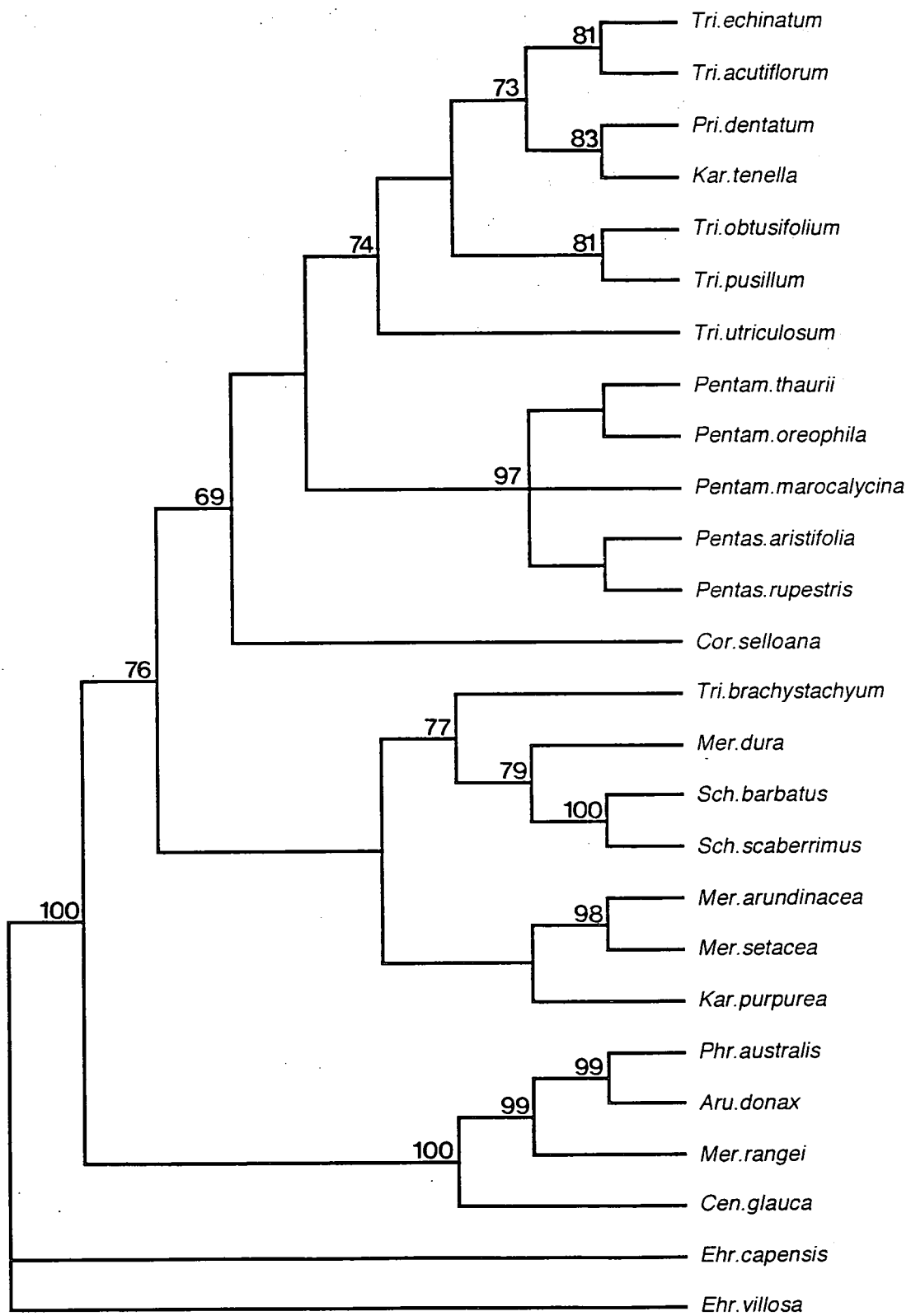


Figure 6.2 The Adams consensus tree computed from 17 equally most parsimonious trees, with a length of 21294, CI of 0.67 and RI of 0.74, after successive weighting was applied to the combined data set. Bootstrap support values are indicated on the relevant branches.

meris-Pentaschistis clade and at the base of the tree a trichotomy is evident at the outgroup positions.

Bootstrap support values, support only some of the groupings. These are mostly only the clades containing the genera *Pentaschistis*, *Merxmuellera* and *Schismus*. There is little support for any of the species of *Pentameris*, *Karoochloa* or *Tribolium*.

After successive weighting, a marked increase in the consistency index and the retention index was observed. The Strict, Semistrict and Adams consensus trees were identical in topology to the trees obtained in the search of the equally weighted combined data set (Fig. 6.1 + 6.2).

More bootstrap support is evident in this search when compared to the first search. Most of the clades show support of more than 69%. Only *Tribolium utriculosum*, *K. purpurea* and the *Pentameris-Pentaschistis* clade show no support.

From the cladograms the following can be deduced:

- *Tribolium* forms a largely uniform grouping including *Prionanthium dentatum* and *Karoochloa tenella*. Linder and Davidse (1997) reported on a more distant relationship between *Tribolium* and *Karoochloa*. This is not corroborated by this study. Furthermore, only *Tribolium brachystachyum* falls outside the large *Tribolium* grouping. The reason could be the proposed hybrid origin of this species. This is evident from cytogenetic studies (Spies *et al.* 1992). Linder and Davidse (1997) supported the hybrid origin and proposed that the putative parents could be *T. uniolae* and *T. hispidum*. The position of *T. brachystachyum* is, however, not understood. Once again the *Prionanthium-Tribolium* relationship, as proposed by Clayton and Renvoize (1986), can be deduced.
- *Pentameris* and *Pentaschistis* once again form a closely-knit unit. In the absence of more *Prionanthium* specimens the relationships between these three genera cannot be corroborated from the combined analysis.
- The genera *Merxmuellera* and *Karoochloa* are scattered over the cladogram and the polyphyly of these genera are proposed again. For the genus *Merxmuellera*, morphology and anatomy confirm this: species within the genus *Merxmuellera* show substantial morphological and anatomical diversity (Barker & Ellis 1991; Ellis 1980 a, b, 1981a, b, 1982a, b, 1983). Despite the morphological diversity this genus was long considered to be a natural unit. Molecular data has, however, recently shown, as in this study, that this is not the case and that the genus is

probably polyphyletic (Barker 1995a; Barker *et al.* in press; Hsiao *et al.* 1998a). *Merxmuellera arundinacea* and *M. setacea* N.P.Barker are the only species in the genus grouped together into a single clade, with 100% bootstrap support. *Merxmuellera setacea* is quite distinct from all other members of the genus, in that it has basally thickened and swollen sheath bases covering the rhizome and axillary buds (Barker & Ellis 1991). Three other *Merxmuellera* species with swollen basal parts are *M. rufa* (Nees) Conert, *M. lupulina* (Thunb.) Conert and *M. decora*. In these species, however, the basal sheaths are densely villous and unthickened (Barker & Ellis 1991). Unfortunately, these species were not investigated in this study. *Merxmuellera setacea* also has a distinct floral morphology. The only other species in the genus with a somewhat similar arrangement of hairs, is *M. dura*. These similarities are, however, not supported by the vascular bundle arrangement (Barker & Ellis 1991). From the cladogram (Fig. 6.2) can be seen that *M. dura* and *M. setacea* form sisterspecies relationships. A similar vascular bundle arrangement occurs in most species of *Pentameris*, especially *P. macrocalycina* and *P. oreophila*, as well as many species of *Pentaschistis*. Relevant examples are *P. pyrophila* Linder, *P. eriostoma* and *P. curvifolia* and its close allies (Barker & Ellis 1991). From the cladogram these similarities are not evident in the relevant species.

- As was the case with the sequencing data, *Arundo* and *Phragmites* are grouped together with a bootstrap confidence value of 90%-99%.
- The combined analysis, as was inferred from the sequencing data, indicates that the genus (comprising only of the species *S. barbatus* and *S. scaberrimus* in this study) forms a monophyletic grouping. This grouping indicates close affinities with *Merxmuellera dura*, as was also found in the DNA sequencing study.
- The genus *Centropodia* shows close affinities with the *Arundo-Phragmites* clade. The position of *Cortaderia* was not well resolved in the sequencing study, but grouped with a bootstrap support value of 69% within the upper portion of the cladogram, in this combined analysis.
- Amalgamation of the data sets in a study by Steane *et al.* (1999) resulted in an increase in resolution of unresolved regions of the Semistrict consensus of separate data sets. In regions where the two data sets agreed, the combined data yielded fully resolved clades with increased bootstrap support. There were some regions in which the independent data sets did not agree, but the signal from the combined

data set yielded fully results congruent with those from either one or the other data set. In this study, when the combined analysis is compared with each of the separate analyses, resolution is lost in some parts of the cladogram (as in the *Pentameris-Pentaschistis* clade), whereas other parts showed an increase in resolution (as with *Cortaderia selloana*).

- Potential hybridisation is a continuing problem in phylogenetic analyses, and the uncertainty concerning its frequency complicates the interpretation of cladograms (Kellogg & Watson 1993). Hybridisation has been postulated to be a major evolutionary force in the angiosperms. For example, Stebbins (1950) suggested that most plant genera creating classification problems are of hybrid origin. Because hybridisation introduces a reticulate pattern into a phylogenetic tree, it is not easily accommodated by any analytical method that assumes strict divergence (Funk 1981; Kellogg 1989). Hybrids have been shown (Funk 1981, 1985; McDade 1990, 1992) not to create any predictable pattern in a cladogram and are, thus, not easily detectable.
- Many of the problematic taxa encountered during the study may be of a hybrid origin. This is especially evident from chromosomal studies such as those done on *Merxmullera stricta* (see cytotaxonomy chapter) and *Tribolium brachystachyum* (Spies *et al.* 1992; Visser & Spies 1994d). Furthermore, the great number of polyploid chromosome numbers and polyploid complexes which exist in the grasses, indicate that hybridisation has been a major evolutionary driving force. It is, however, as mentioned earlier, not always easy to identify these taxa.

According to Sanderson and Donoghue (1989), parsimony algorithms assign characters scored as unknown or missing, to whichever state is most parsimonious, given the position of the taxon in the tree based on known characters. Since this coding can never increase inconsistency, whereas if these unknowns were replaced by definite scores homoplasy might be introduced, data sets with more unknowns will tend to have higher consistencies than those which might have more definite character scores (Sanderson & Donoghue 1989). This is corroborated by Nixon and Davis (1991), who state that taxa with many missing data may tend to be misplaced in the phylogenetic analysis, in part because extensive missing data implies many more character state combinations than actually occur. Therefore, levels of homoplasy and actual lengths of trees will tend to be

underestimated because of the hidden homoplasy created by variation within the terminal specimens. This might be evident in this study, in which many taxa contain missing data, either for DNA sequencing or DNA fingerprinting characters.

The number of missing data was inadvertently increased by the creation of summary taxa for species in which more than one specimen was available. Summary taxa may have more variation and, consequently, more missing data. Therefore, their cladistic positions may be misleading (Nixon & Davis 1991).

Lastly, as suggested by Huelsenbeck *et al.* (1994), the data sets were unweighted. Although it did not seem to have a large effect on the results obtained in the combined analysis, the implication of this is that the larger data set (the DNA sequencing data set in this case) might overpower the effects of the smaller data set (DNA fingerprinting in this case).

6.3.2 Cytotaxonomy, DNA fingerprinting and DNA sequencing – a final assessment

Combining data sets for phylogenetic analysis assumes firstly, that the same history is true for each character set and secondly, that the methods of tree reconstruction chosen are equipped to handle the differences in their evolutionary rules. The existence of strongly supported but conflicting trees is evidence that either one, or both of these assumptions for combining data sets, have been violated. It follows then that the different data sets should not be combined, but rather be kept separate when they strongly support conflicting trees (Miyamoto & Fitch 1995).

The trees obtained with the separate analyses are not conflicting in the strict sense of the word, but are not congruent due to the fact that the DNA fingerprinting analysis did not show any higher taxonomic level resolution above species level (Fig. 6.3 versus Fig. 6.4).

Furthermore, due to the fact that the chromosomal parameters such as basic chromosome number, polyploid level, chromosomal abnormalities etc. could not be used to construct a cladogram, the use of a combined method is not advocated in this study.

When investigating the results obtained with the different techniques implemented in this study (cytotaxonomy, DNA fingerprinting and DNA sequencing), the following can be deduced, especially concerning the relationship between *Prionanthium* and *Tribolium* on the one hand, and *Pentameris*, *Pentaschistis* and *Prionanthium* on the other.

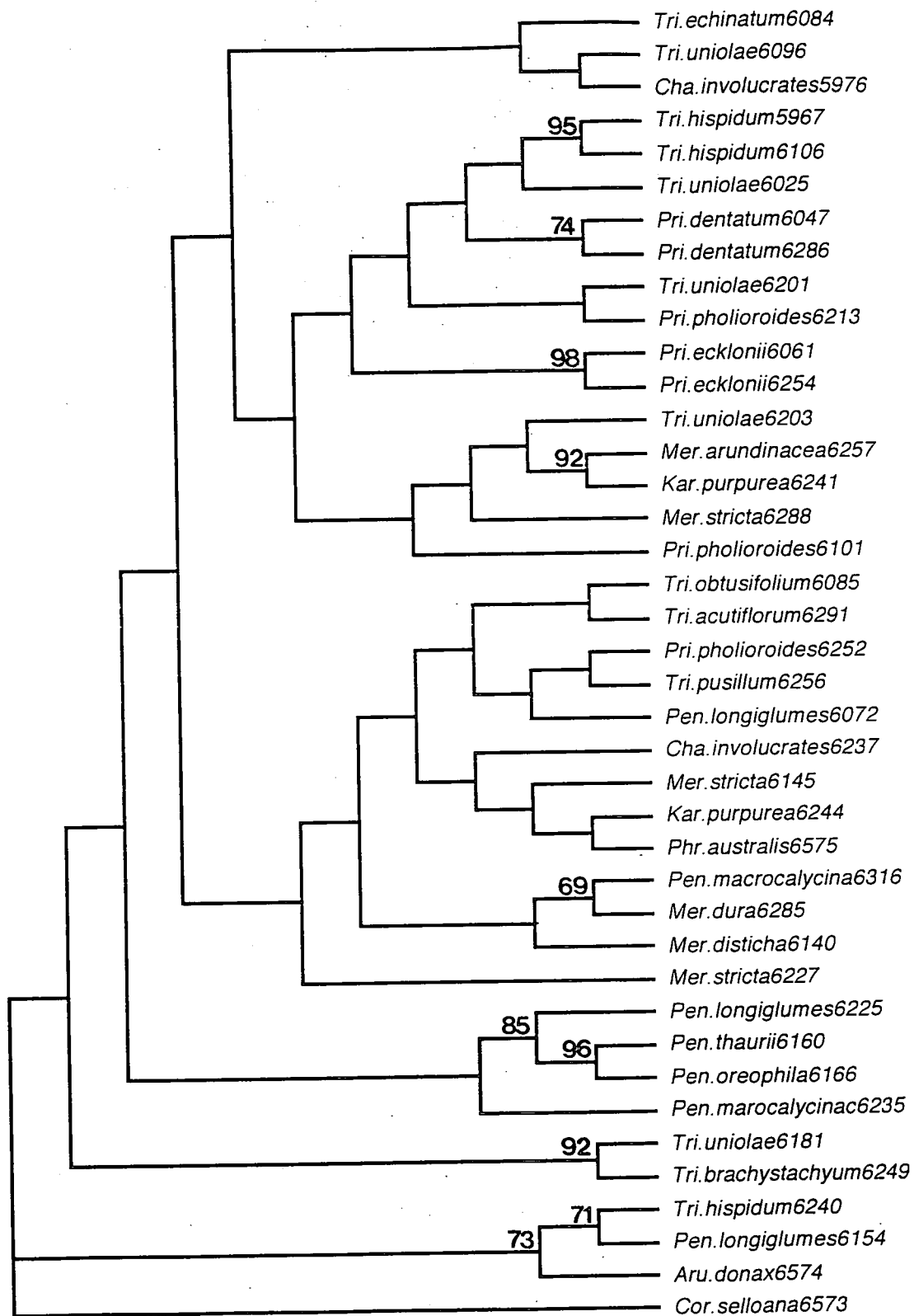


Figure 6.3 The single most parsimonious tree with length of length of 9977, CI of 0.18 and RI of 0.45, computed after successive weighting was applied to the DNA fingerprinting data set. Bootstrap support values are indicated on the relevant branches.

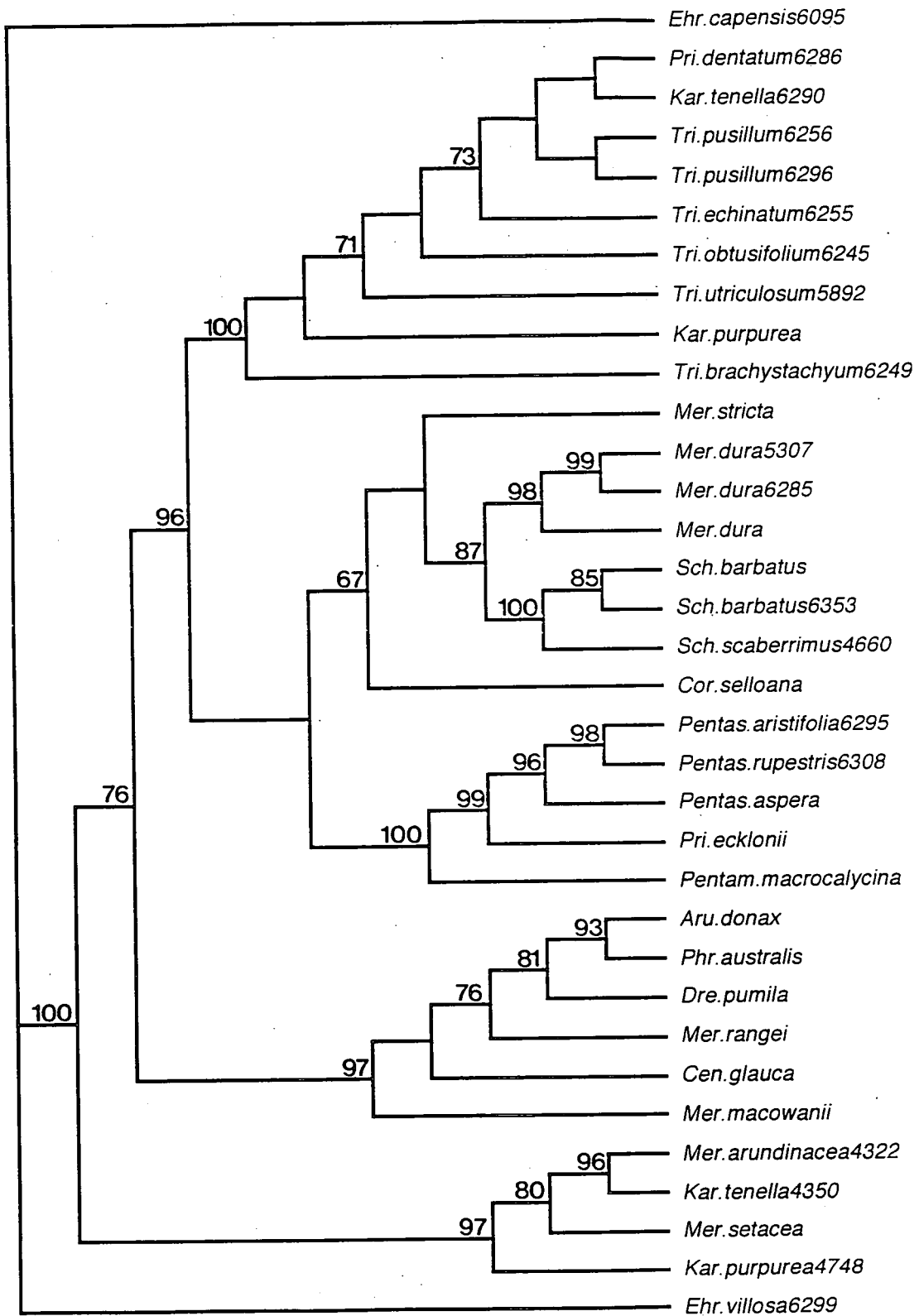


Figure 6.4 The single most parsimonious cladogram obtained after successive weighting of the sequencing data set. This tree has a length of 16996, CI of 0.67 and RI of 0.81. Bootstrap support values are indicated on the relevant branches.

Probably the most cytogenetic, morphological, anatomical and phylogenetic research the past decade in the Arundineae, has been on the genus *Tribolium* (Renvoize 1985; Spies *et al.* 1992; Visser & Spies 1994a-e; Linder & Davidse 1997).

This genus was traditionally classified into two genera, namely *Lasiochloa* and *Plagiochloa*. These genera comprised of the following species: *Plagiochloa uniolae* (L.f.) Adamson & Sprague (= *Tribolium uniolae*), *P. acutiflora* (Nees) Adamson & Sprague (= *T. acutiflorum*), *P. oblitera* (Hemsl.) Adamson & Sprague (= *T. obliterum*), *P. glomerata* (Stapf) Adamson & Sprague (= *T. obliterum*), *P. brachystachya* (Nees) Adamson & Sprague (= *T. brachystachyum*), *P. alternans* (Nees) Adamson & Sprague (= *T. alternans*) and *P. ciliaris* (Stapf) Adamson & Sprague (= *T. ciliare*). The genus *Lasiochloa* comprised of the species *L. utriculosa* Nees (= *T. utriculosum*), *L. echinata* (Thunb.) Adamson (= *T. echinatum*), *L. longifolia* (Schrad.) Kunth (= *T. hispidum*) and *L. obtusifolia* Nees (= *T. obtusifolium*) (Renvoize 1985). These two genera are closely related and although the more extreme species were sufficiently distinct to merit generic separation, they were bridged by a number of intermediate species which made generic division doubtful and, therefore, the genera were combined into the genus *Tribolium* as it is known today (Renvoize 1985).

Visser and Spies (1994a) also commented on the great morphological diversity in *Tribolium*. In a phylogenetic analysis, using 20 morphological characters, they divided the genus into three morphological groupings. The first group consists of the species *T. ciliare*, *T. echinatum*, *T. hispidum* and *T. utriculosum*. These are all annual species with the exception of *T. hispidum*. This section was named *Tribolium* and corresponded with the former genus *Lasiochloa*, except for the species *T. ciliare* (in this morphological grouping) and *T. obtusifolium* (in the genus *Lasiochloa*) (Visser & Spies 1994a). The second group consisted of *T. uniolae*, *T. brachystachyum*, *T. alternans* and *T. amplexum*. This section was termed *Uniolae* and contains perennial, mostly tufted plants. The last grouping contained the species *T. acutiflorum*, *T. glomeratum sensu* Davidse, *T. obliterum* and *T. obtusifolium* (Nees) Renvoize and was named *Acutiflorae*. These species are perennial and mostly stoloniferous, except for *T. acutiflorum* (Visser & Spies 1994a). Unique in Danthoniaceae due to its mode of dispersal of the inflorescence, is the species *T. pusillum*. At maturity the culm disarticulates at the uppermost node, complete with the inflorescence and modified upper sheath, and this whole structure acts as a dispersal unit (Chippindall 1955; Clayton & Renvoize 1986). According to Ellis (1988a), the leaf anatomy of *T. pusillum* resembles that of *T. utriculosum* and *T. echinatum* very closely. All three species always have prominent cushion-based macrohairs. Chippindall (1955) and Clayton and

Renvoize (1986) suggested that *T. utriculosum* and *T. pusillum* are closely related due to the fact that both have tubercle-based hairs, as well as capitate hairs on the lemmas and glumes. In *T. echinatum* the hairs of the glumes are slender and tapering. In *T. utriculosum* the inflorescence is partly enclosed in the uppermost leaf sheath, a condition developed further in *T. pusillum*.

A study of the chromosome numbers by Visser and Spies (1994c, d, e) revealed that the section *Tribolium* is usually diploid, with some tetraploid *T. hispidum* specimens (Visser & Spies 1994c). The section *Acutiflorae* is usually tetraploid with *T. glomeratum* being the only hexaploid species (Visser & Spies 1994e). The section *Uniolae* represents the widest polyploid range, from mostly tetraploid (diploidy rare) and hexaploid specimens (Visser & Spies 1994d).

The genus has a basic chromosome number of $x = 6$. *Tribolium ciliare*, *T. pusillum* and *T. utriculosum* are diploid species, *T. acutiflorum* and *T. brachystachyum* are tetraploid species, *T. obtusifolium* is a hexaploid species, *T. uniolae*, and *T. hispidum* exhibit from diploid to hexaploid behaviour, *T. echinatum* is diploid and tetraploid and *T. obliterum* contains tetraploid and hexaploid specimens (Spies *et al.* 1992; Visser & Spies 1994c, d, e). The species *T. hispidum* and *T. uniolae* are the species in the genus with the widest distribution range.

These marked differences in polyploid level can be mainly attributed to the distribution ranges of the different species and also the overlapping of some of these distributional ranges. Spies *et al.* (1992) suggested that *T. uniolae* and *T. brachystachyum* undergo hybridisation and introgression. Especially in the area that overlaps between the two species (*T. uniolae* has the wider distribution, which encompasses the restricted distribution area of *T. brachystachyum*), there are many morphological intermediate plants. Also most of the *T. uniolae* specimens with meiotic abnormalities occur in this hybridisation area. Furthermore, for example, the perennial *T. hispidum* is especially variable and hybridisation and introgression with the related diploid annual *T. echinatum* was suggested (Spies *et al.* 1992; Visser & Spies 1994c). Visser and Spies (1994c) also proposed combining these two species into a single *T. echinatum* hybrid swarm.

According to Visser and Spies (1994c), occasional hybridisation between *T. hispidum* and *T. utriculosum* is also possible. Visser and Spies (1994d) combined *T. alternans*, *T. amplexum* and *T. uniolae* into a single hybrid swarm on the basis of extensive morphological similarities, which makes separation of these three species nearly impossible. Linder and Davidse (1997) now unite these three species under the species *T.*

uniolae in the most recent classification. In the section *Acutiflorae*, Visser and Spies (1994e) combined the species *T. glomeratum*, *T. oblitterum* and *T. obtusifolium* into a hybrid swarm. Spies *et al.* (1992), on the basis of meiotic chromosome behaviour, stated that the combination of *Lasiochloa* and *Plagiochloa* seemed justified.

Linder and Davidse (1997) agreed with the three groupings suggested by Visser and Spies (1994a). They, however, found all three groupings to be monophyletic as opposed to Visser and Spies (1994c), who found the section *Tribolium* to be paraphyletic. According to Linder and Davidse (1997), the species in the section *Uniolae* are separated altitudinally, by soil type and by different flowering times. The section *Acutiflorae* contains three allotopic, but broadly sympatric, species, which often co-occur in the same localities. In the section *Tribolium*, the species are quite distinct. *Tribolium hispidum* and *T. echinatum* share the same general distribution patterns and they often co-occur. To the north the species are replaced by *T. utriculosum*. *Tribolium pusillum* and *T. ciliare* have more limited distributional ranges to the north and south, respectively (Linder & Davidse 1997). *Tribolium ciliare*, *T. pusillum* and *T. utriculosum* are specialists, which have speciated into unique habitats. *Tribolium echinatum* and *T. hispidum* (the other two species in the section *Tribolium*) are probably the stock from which these species evolved. This has, however, yet to be proven. In the section *Uniolae* altitudinal differentiation may have been important in the evolution of the section and in *Acutiflorae* probably soil-type differentiation (Linder & Davidse 1997).

In this study, investigating the genus *Tribolium*, the following was observed:

- With the DNA fingerprinting analysis (Fig. 6.3), no definite boundary can be drawn between the three informal groupings suggested by Visser and Spies (1994a). *Tribolium obtusifolium* and *T. acutiflorum* (section *Acutiflorae*) are grouped together. Species comprising of the section *Uniolae*, mostly *T. uniolae* and *T. brachystachyum*, are scattered all over the cladogram. The only close relationship in this section is between *T. uniolae*, Spies 6181 and *T. brachystachyum*, Spies 6249. These two specimens are, however, removed from the rest of the group higher up in the tree. The section *Tribolium* is also scattered across the tree. Affinities between the groups are indicated by various close affinities across the informal sections. The groupings in the tree are clearly not monophyletic and the topology of the genus is not very robust. This is in congruence with the findings by Linder and Davidse (1997). They

accounted the lack of robustness in the genus firstly to the absence of strong synapomorphies for the sections *Tribolium* and *Acutiflorae*, which suggests that the section *Uniolae* is imbedded deep within the first two. Secondly, several unique characters in the genus do not have congruent distributions within the genus (Linder & Davidse 1997).

- With the DNA sequencing analysis (Fig. 6.4), only six specimens representative of all three groupings, but comprising mostly of section *Tribolium*, were studied. These specimens were grouped together into a single clade (clade 1). Close relationships in section *Tribolium* are evident from these results. The monophyly of the genus is indicated by the sequencing results, but due to the fact that only six specimens were investigated, the relationships within the groupings and between the different species cannot be examined. Linder and Davidse (1997) proposed a distant relationship to *Karroochloa*. This is corroborated by this study. In the combined analysis (Fig. 6.2), however, *Karroochloa* shows affinities with *Tribolium*, with *Karroochloa tenella* grouping in the *Tribolium* clade.
- The three informal groupings within the genus, as proposed by Visser and Spies (1994a), appear justified, although hybridisation, polyploidy, morphological overlapping and merging occur widespread within the genus and make the boundaries between species very narrow.

The genus *Prionanthium* consists of three annual species. This genus is almost unique in the possession of glands, presumably secretory, on the glumes of all three species (Davidse 1988). The other genus in which multicellular, secretory glands also occur in about half of the species, is the genus *Pentaschistis*. Their occurrence in this genus is on glumes, pedicels and the leaves of several species (Linder *et al.* 1990). The function of these glands in *Prionanthium* is unknown, whereas in *Pentaschistis* it may be an anti-herbivore mechanism (Davidse 1988). The glands in *Pentaschistis* and *Prionanthium* may be analogous to glands recorded from other genera. Within these two genera, however, they appear to have differentiated *de novo* from the epidermal tissues (Linder *et al.* 1990). The basic chromosome number of this genus is $x = 7$ (Davidse 1988; Visser & Spies 1994e). Only diploid specimens have been recorded in the genus.

Within the genus the three species can be readily distinguished from one another. *Prionanthium dentatum* is unique in the genus in possessing macrohairs, but resembles *P. ecklonii* very closely in leaf anatomy. Several other features also indicate a closer relationship between these two species than to *P. pholioroides* (Ellis 1989).

The relationship between *Prionanthium* and *Tribolium* has been based on the following observations: In *T. uniolae* the inflorescence is reduced to a distichous spike, while in the other species the inflorescence is a compact panicle. This is a remarkable development, paralleled only by *Prionanthium*. In *Tribolium* the callus of the spikelets is very short and glabrous. The only other occurrence of glabrous calli in the South African danthonoid grasses is in *Prionanthium*. They also share unlobed lemmas without setae or awns (Linder & Davidse 1997). These characters are virtually unique in the Danthoneae. These similarities, however, do not unite the genera and Linder and Davidse (1997) suggest only a distant relationship, with extensive convergence having occurred between the two genera.

Spikelet characters that point to a relationship between *Prionanthium* and *Pentaschistis* are the following: the occurrence of well differentiated multicellular glands, two florets per spikelet, a small rachilla extension above the upper floret and small paleas. This is opposed to the many differences in spikelet characteristics (Davidse 1988).

In this study the following was observed:

- With the DNA fingerprinting analysis (Fig. 6.3), the close relationship within the genus is evident. *Prionanthium dentatum* and *P. ecklonii* were grouped closely together as sisterspecies. *Prionanthium pholioroides* was more variable but still grouped closely with the other two species. One specimen of *P. pholioroides* (Spies 6252) grouped with *Tribolium pusillum*. As stated previously, this relationship is proposed as being only distant.
- With the DNA sequencing analysis (Fig. 6.4), *Prionanthium ecklonii* groups with *Pentaschistis*. A *Prionanthium dentatum* (Spies 6286) specimen, however, grouped within clade 1 with *Tribolium*. This specimen is also problematic during the combined analysis (Fig. 6.2) in its grouping. In the light of the small number of specimens in this genus that has been sequenced, affinities cannot be unequivocally accepted or rejected.
- The DNA fingerprinting analysis has confirmed the species boundaries within the genus. As previously mentioned, this technique, however, cannot

satisfactorily be used at generic levels. Although affinities with *Tribolium* do exist, accumulated evidence indicates a closer affinity with *Pentaschistis* and *Pentameris*.

The genus *Pentaschistis* is another large genus in the tribe Arundineae, which has been the subject of many studies (Spies & Du Plessis 1988; Linder & Ellis 1990b; Du Plessis & Spies 1992; Ellis & Linder 1992; Spies *et al.* 1994a; Klopper *et al.* 1998), especially at morphological, cytogenetic and phylogenetic level. This genus consists of 68 species (Linder & Ellis 1990b), of which 57 are indigenous and 40 are endemic to South Africa (Gibbs Russell *et al.* 1990).

Although the genus *Pentaschistis* is one of the Arundineae investigated, this genus will only be investigated minimally in this study due to the fact that a phylogenetic analysis of the entire genus is being conducted in our laboratories at the moment. Therefore, discussion of this genus will be briefly. This genus has two basic chromosome numbers, namely $x = 7$ and $x = 13$ (Spies & Du Plessis 1988; Du Plessis & Spies 1992; Spies *et al.* 1994a; Klopper *et al.* 1998). In the species with $x = 7$, the ploidy levels range from diploid (mostly), to 13-ploid. In the species with $x = 13$, ploidy levels range from diploid (mostly) to heptaploid ($7x$) (Klopper *et al.* 1998). This indicates that $x = 7$ represents an older polyploid complex than $x = 13$ and supports the secondary origin of $x = 13$.

In this study, *Pentaschistis* was only investigated by the DNA sequencing of a few specimens (Fig. 6.4). These three specimens (*P. aristifolia*, *P. aspera* and *P. rupestris*) all grouped together into a clade, together with *Prionanthium* and *Pentameris*. In the combined analysis, basically the same observations were made. No concrete conclusions can be made from the limited number of specimens investigated in this study. All of the specimens studied do, however, indicate affinities with *Prionanthium* and *Pentameris*.

Although the genus *Pentameris* has been investigated extensively at anatomical level (Ellis 1985b, c, 1986a; Barker 1986; Barker 1993), the genus has only until recently been cytogenetically investigated (Barker 1993; in the present study).

This genus was originally described to contain five species, namely *P. dregeana*, *P. longiglumes*, *P. macrocalycina*, *P. obtusifolia* and *P. thurii* by Palisot de Beauvois (1812). Renvoize (1981) places *Pentameris* in his core group of the arundinoid grasses – a group, which possesses finger-like microhairs.

Ellis (1985c) noted that *P. thuarii* differs substantially from the other four species and that *P. thuarii* shows affinities with several *Pentaschistis* species such as *P. pallescens*, *P. silvatica* and *P. tortuosa*. This relationship relates only to the anatomical features of the leaf blades, but it is believed that these anatomical resemblances reflect the natural relationships of this group of species. Of particular note are the distinctive micro-hairs shared by *Pentameris thuarii* and these *Pentaschistis* species, in which the basal cells are much longer than the very short, tapering apical cells (Ellis 1985b).

The heavily lignified, thick, setaceous leaves of *Pentameris longiglumes*, *P. macrocalycina* and *P. obtusifolia* are strongly reminiscent of the members of the genus *Merxmuellera* and these species show a definite anatomical resemblance to this genus (Ellis 1985c). The ecological, as well as vegetative and spikelet morphology, of *Pentameris macrocalycina* and *P. obtusifolia* are very similar and they appear to be closely allied (Ellis 1985d). These three species appear to form a natural grouping; best accorded generic status apart from *P. thuarii* and *P. dregeana* (Ellis 1985d).

On the basis of unusual leaf blade anatomy (Ellis 1985d; Barker 1990) and fruit morphology (Barker 1986, 1989, 1990, 1995b), however, *P. obtusifolia* was removed from *Pentameris* to the genus *Pseudopentameris*.

The leaf anatomy of *Pentameris dregeana* differs considerably from that *P. macrocalycina* and *P. obtusifolia* and, according to Ellis (1986a), it appears unlikely that morphology would indicate a close relationship. Atypical *P. dregeana* specimens studied by Ellis (1986a), seemed to indicate a link between *P. dregeana* and *Pentaschistis colorata* (Steud.) Stapf. These specimens indicate an anatomical gradation of *Pentameris dregeana* into the *Pentaschistis colorata* species complex. Ellis (1986a) states that it is clear that the affinities of *Pentameris dregeana* are closer to some species currently placed in *Pentaschistis*, than they are to any of the *Pentameris* species.

In a biosystematic study done by Barker (1993) five new species were added to the genus: *P. glacialis* N.P.Barker, *P. hirtiglumes*, *P. oreophila*, *P. swartbergensis* N.P.Barker and *P. uniflora* N.P.Barker. One new subspecies, *P. longiglumes* subsp. *gymnocolea* N.P.Barker, was also described.

Ellis (1985c, 1986a; Linder & Ellis 1992), on the basis of leaf anatomical studies, is of the opinion that *Pentameris* is closely allied to *Pentaschistis*.

Stapf (1900) separated *Pentameris* from *Pentaschistis* on the basis of fruit morphology, namely the crustaceous pericarp and free seed. According to him, the structure of the ovary is "... so alike in the five species of this genus that it is very probable

that they agree in the peculiarities of the ripe fruit which is known only in *P. thuarii*". Furthermore, Stapf stated that "... there is in *Pentaschists* no approach to the characteristic structure of the ovary and the fruit of *Pentameris*."

A phylogenetic analysis conducted by Barker (1993) identified *Pentameris* as a monophyletic genus. This is confirmed by fruit and ovary characters.

On a cytogenetic level the basic chromosome number for this genus has been proven to be $x = 7$. Diploid specimens were observed in *P. thuarii* and *P. oreophila*, with a hexaploid specimen found in *P. macrocalycina*.

In this study the following was observed:

- With the DNA fingerprinting analysis (Fig. 6.3), monophyly in the genus was not found. Four specimens (representing three species, namely *P. longiglumes*, *P. oreophila* and *P. thuarii*) formed a monophyletic grouping. *Pentameris macrocalycina* was, however, totally removed from this clade as well as two *P. longiglumes* specimens. These specimens were not classified down to subspecies level and may represent subspecies or great genetic variation within the species.
- With the DNA sequencing analysis (Fig. 6.4), only a *P. macrocalycina* was investigated. This species grouped with three *Pentaschistis* species and *Prionanthium ecklonii* with a bootstrap confidence of 99%. With the combined analysis (Fig. 6.2), the same was observed.
- The boundaries within this genus remain problematic. *Pentameris thuarii*, the type species, supposedly has closer affinities within *Pentaschistis*. This study did not confirm this. Although the most *Pentameris* specimens formed a monophyletic clade (with 85-96% bootstrap support within the clade), some specimens remain problematic. The lack of sequencing data could not corroborate relationships within the genus.

6.4 Conclusions

Whether to combine different data matrices, or whether determining the consensus tree from different analyses in a combined data analysis, remains the question. The combined analysis in this study was no better or worse than any of the separate analyses. This could be attributed to the fact that although both DNA fingerprinting and DNA

sequencing resolved species relationships, only the latter resolved generic relationships as well.

The close relationship between *Pentaschistis* and *Pentameris* seems evident from this and previous chapters. The position of the genus *Prionanthium* remains problematic. It is grouped either with *Pentameris-Pentaschistis* or with *Tribolium*. In the absence of any more studied specimens, these relationships cannot unequivocally be rejected or supported. We are, however of the opinion that *Prionanthium*, *Pentameris* and *Pentaschistis* are closely related, as has been corroborated by cytogenetic studies, morphology, anatomy and in part by DNA sequencing and DNA fingerprinting analysis, in this study. Whether these three genera deserve subtribal rank, depends on investigating the genera more thoroughly by means of DNA sequencing, but on basis of these preliminary results, a subtribal classification for these genera is proposed.

The close relationship between *Arundo* and *Phragmites* is evident from DNA sequencing and the combined analysis again. These members of the Arundineae are nested firmly within the rest of the Danthoneae.

Only when more species per genus and more specimens per species are investigated with the help of DNA sequencing, will many of the still unanswered questions be resolved. We, however, hope to have laid some stepping stones in the unraveling of the correct phylogenetic history and tribal classification for the South African Arundineae.

CHAPTER 7

SUMMARY

This study dealt with the phylogenetic history of some representatives of the South African Arundineae. Arundineae Dumort. is one of the tribes in the grass (Poaceae) subfamily Arundinoideae Tateoka. This is known to be a heterogeneous assemblage and a taxonomically difficult group. Three techniques were used to investigate the relationships within the tribe Arundineae, namely cytogenetics, DNA amplification fingerprinting and DNA sequencing of the *ITS* region of the nuclear DNA.

The genera *Pentameris* P.Beauv., *Pentaschistis* (Nees) Spach. and *Prionanthium* Desv. are the only three genera in the tribe with seven as a basic chromosome number (excluding the genus *Dregeochloa* Conert, which was not investigated in this study). A close affinity between these genera is, thus, recognised. This relationship is corroborated by the DNA fingerprinting and DNA sequencing data. One *Prionanthium dentatum* (L.f.) Henr. specimen was, however problematic. This specimen grouped with *Tribolium* Desv. Clayton and Renvoize (1986) proposed this relationship, but in the light of various studies, as in this study, *Prionanthium* is removed from this closely related genus. Therefore, the recognition of subtribes in Arundineae, is proposed.

The genera *Arundo* L. and *Phragmites* Adans. were grouped together. This is unexpected, because according to Clayton and Renvoize (1986), these genera are not as closely related, as their similarity in habit would suggest. In this study these two genera, known as arundinoid genera, are a monophyletic grouping nested within the rest of the genera, known as danthonoid genera.

The genera *Karroochloa* Conert & Türrpe, *Merxmuellera* Conert and *Prionanthium* were shown to be polyphyletic with the sequencing data.

DNA amplification fingerprinting was helpful in resolving species relationships, but failed to elucidate most of the generic relationships in the study. DNA sequencing was the more informative of the two techniques. The combined analysis was helpful in that some resolution was gained, while other was lost. Due to large amounts of missing data and the inability of DNA fingerprinting to resolve generic relationships, the combined analysis could not be used to its fullest capacity.

PAUP and HENNIG86 were both used and both software programs gave the same results. In general, the trees created with PAUP were usually shorter. Both software programs can be used, but the ease of PAUP makes it the preferred analysis package.

Successive weighting usually resulted in a tree with better CI and RI values, compared to a search conducted on the equally weighted data set. The topologies of the trees, however, remained the same.

Adams consensus trees were the only consensus trees, which were usually the best (or most) resolved when compared to Strict and Semistrict consensus trees.

The present tribal classification for the Arundineae seems justified. However, a subdivision of the tribe Arundineae, to accommodate the clustering of the genera *Pentameris*, *Pentaschistis* and *Prionanthium* into a subtribe, may be justified. The position of *Arundo* and *Phragmites* is monophyletic if only the South African representatives are investigated. Some confusion still exists within the tribe and DNA sequencing studies of more specimens per species and more species per genus is advised.

Keywords: Arundineae, Arundinoideae, cladistics, cytogenetics, DAFs, DNA sequencing, *ITS* region, phylogeny, Poaceae, tribal classification.

CHAPTER 8

OPSOMMING

In hierdie studie is 'die filogenetiese ontwikkeling van sekere verteenwoordigers van die Suid Afikaanse Arundineae ondersoek. Arundineae Dumort. is een van die tribusse in die grassubfamilie, Arundinoideae Tateoka. Hierdie subfamilie is bekend as a heterogeniese groepering en is 'n taksonomiese moeilike groep. Drie tegnieke is gebruik om die verwantskappe in die tribus te ondersoek, naamlik sitogenetika, DNA-amplifiserings vingerafdrukke en DNA-nukleotiedvolgordebepaling van die *ITS* gebied van die kern-DNA.

Die genera *Pentameris* P.Beauv., *Pentaschistis* (Nees) Spach. en *Prionanthium* Desv. is die enigste drie genera in die tribus met 'n basiese chromosoomgetal van sewe (uitsluitend die genus *Dregeochloa* Conert, wat nie in hierdie studie ondersoek is nie). Die genera is naverwant. Hierdie verwantskap word ondersteun deur die DNA-vingerafdrukke en die DNA-nukleotiedvolgordebepaling. 'Een *Prionanthium dentatum* (L.f.) eksemplaar het egter afgewyk van die verwagte resultate. Die eksemplaar toon affiniteite met *Tribolium* Desv. In 1986 is hierdie verwantskap deur Clayton en Renvoize voorgestel, maar in die lig van verskeie studies, soos in hierdie betrokke studie, word *Prionanthium* verwyder van hierdie naverwante genus. Die erkenning van 'n subtribus vir hierdie genera word dus voorgestel.

Die genera *Arundo* L. en *Phragmites* Adans. word in hierdie studie saamge-groepeer. Hierdie verwantskap is onverwags, aangesien Clayton en Renvoize (1986) hierdie genera nie as naverwant beskou het nie, ten spyte van hulle eenderse habitate. In hierdie studie vorm die twee genera, bekend as arundinoid genera, 'n monofiletiese groepering wat tussen die res van die genera, bekend as danthonoid genera, gegroepeer is.

Die genera *Karroochloa* Conert & Türpe, *Merxmuellera* Conert en *Prionanthium* is deur middel van DNA-nukleotiedvolgordebepaling, as polifiletiese genera uitgewys.

DNA-amplifiserings vingerafdrukke kon gebruik word in die bepaling van die verwantskappe tussen spesies, maar kon nie daarin slaag om verwantskappe op genusvlak op te los nie. DNA-nukleotiedvolgordebepaling was die tegniek wat die verwantskappe die beste kon aantoon op spesie-, sowel as genusvlak. Die gekombineerde analise kon sommige van die verwantskappe beter verklaar, maar het verminderde resoluësie op ander

gebiede getoon. As gevolg van die groot hoeveelhede onbekende data en ook die onvermoë van die DNA-vingerafdrukke om verwantskappe op genusvlak uit te klaar, kon die gekombineerde analise nie tot die maksimum beproef word nie.

PAUP en HENNIG86 is albei gebruik en albei programme het min of meer dieselfde resultate gelewer. In die algemeen is die kladogramme wat met PAUP bereken is korter. Albei programme kan gebruik word, maar die gemak van PAUP maak dit verkieslik.

Opeenvolgende gewigtoekenning het gewoonlik die kladogram met die beste CI en RI waardes tot gevolg gehad, in vergelyking met die kladogramme verkry vanaf 'n datamatriks waarin dieselfde gewig aan die data toegeken is. Die topologie van die bome het egter feitlik altyd eenders gebly.

Die Adams konsensuskladogram was gewoonlik die enigste kladogram wat die beste resolusie getoon het, in vergelyking met die "Strict" en "Semistrict" konsensuskladogramme.

Die huidige tribus klassifikasie vir die Arundineae blyk korrek te wees. 'n Onderverdeling van die tribus Arundineae, om die genera *Pentameris*, *Pentaschistis* en *Prionanthium* in 'n subtribus saam te voeg, mag gewens wees. Die posisie van *Arundo* en *Phragmites* is monofileties, indien slegs die Suid Afrikaanse verteenwoordigers ondersoek word. Verwarring is egter nogsteeds teenwoordig in die tribus en DNA-nukleotiedvolgordebepaling van meer eksemplare per spesie, en meer spesies per genus, is nodig.

Sleutelwoorde: Arundineae, Arundinoideae, DAFs, DNA-nukleotiedvolgordebepaling, filogenie, *ITS*-gebied, kladistiek, Poaceae, sitogenetika, tribus klassifikasie.

CHAPTER 9

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Appendix A. Table of specimens investigated cytogenetically, and the various results obtained for each of them. x = basic chromosome number, B = number of B-chromosomes per cell, II, IV = bivalents and/or multivalents present, U = range of univalents per cell, M = number of micronuclei per cell, L = number of laggards per cell.

Species	Voucher number	Polyploid level	x	B	II IV	U	M	L
<i>Centropodia glauca</i>	Spies 5706	6x	6	-	II	-	-	-
<i>Chaetobromus involucretes</i> subsp. <i>dregeanus</i>	Spies 5691	2x	6	-	II	-	-	-
<i>Karooochloa purpurea</i>	Spies 2473	4x	6	3-5	II, IV	-	-	-
<i>K. purpurea</i>	Spies 2477	2x	6	-	II	-	-	-
<i>Merxmuellera cincta</i>	Spies 3504	6x	6	-	II	-	-	-
<i>M. decora</i>	Spies 3465	8x	6	-	II	-	-	-
<i>M. stricta</i>	Spies 3140	8x	6	-	II	5-9	-	7-27
<i>M. stricta</i>	Spies 6288	4x	6	5	II	-	-	2-5
<i>Pentameris thuarii</i>	Spies 6160	2x	7	-	II	-	-	-
<i>P. oreophila</i>	Spies 6166	2x	7	-	II	-	-	-
<i>P. macrocalycina</i>	Spies 3644	6x	7	-	II, IV	-	-	-
<i>P. macrantha</i>	Spies 3431	2x	6	-	II	-	-	-
<i>Schismus barbatus</i>	Spies 6596	2x	6	-	II	-	-	-
<i>S. barbatus</i>	Davidse 34033	2x	6	-	II	-	-	-
<i>Stypeiochloa gynoglossa</i>	Spies 1485	4x	6	-	II, IV	-	-	-
<i>S. gynoglossa</i>	Spies 2642	4x	6	-	II, IV	-	-	-
<i>S. gynoglossa</i>	Saayman 79	8x	6	-	II	-	1	6
<i>Tribolium acutiflorum</i>	Spies 3866	4x	6	-	II	-	-	-
<i>T. hispidum</i>	Spies 3509	4x	6	-	II	-	-	-
<i>T. pusillum</i>	Davidse 34022	* 2x	6	-	II	-	-	-

Appendix B. Data set of fragments absent (0) or present (1) for each of the studied specimens, using DAF primer 1. Question marks indicate missing data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
<i>Spies 6084</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 5967</i>	0	1	0	0	0	0	1	0	0	0	0	0	1	1	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0
<i>Spies 6106</i>	1	0	0	0	0	1	1	1	0	0	0	1	0	1	1	0	1	0	0	1	1	1	0	1	0	1	0	0	0
<i>Spies 6240</i>	1	0	0	1	1	1	1	1	1	1	1	1	1	0	0	1	0	0	0	1	1	1	1	1	1	0	0	1	1
<i>Spies 6025</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
<i>Spies 6096</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>Spies 6203</i>	1	1	0	0	0	0	0	0	1	0	1	1	0	1	0	1	1	0	0	0	1	1	1	1	1	0	0	0	1
<i>Spies 6085</i>	0	1	1	1	1	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0
<i>Spies 6201</i>	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>Spies 6291</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6181</i>	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	0	0	0	0	0	0	1	1	0
<i>Spies 6249</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 5976</i>	1	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>Spies 6237</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	0
<i>Spies 6047</i>	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
<i>Spies 6286</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0
<i>Spies 6061</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6254</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6101</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>Spies 6213</i>	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	1	1
<i>Spies 6252</i>	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	1	1
<i>Spies 6256</i>	1	1	0	0	0	1	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	0	1	0	1	1	1	1	0
<i>Spies 6072</i>	0	0	1	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1
<i>Spies 6154</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6225</i>	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1
<i>Spies 6160</i>	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	1	0	1	0	1	1
<i>Spies 6166</i>	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	1	1	0
<i>Spies 6235</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	1
<i>Spies 6316</i>	0	1	0	0	1	0	1	0	1	1	0	0	0	1	0	0	1	1	0	0	1	0	1	0	1	1	1	1	1
<i>Spies 6140</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
<i>Spies 6285</i>	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	0
<i>Spies 6227</i>	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1	0	0
<i>Spies 6145</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6288</i>	0	1	1	0	0	1	0	0	0	1	1	1	1	0	1	0	1	0	0	0	0	1	1	0	0	1	0	0	1
<i>Spies 6257</i>	1	0	1	1	0	1	1	0	1	0	1	1	1	0	0	0	1	0	0	0	1	0	1	1	1	1	1	1	0
<i>Spies 6241</i>	1	0	1	1	1	1	1	0	0	1	0	1	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0
<i>Spies 6244</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6575</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6574</i>	1	1	0	1	1	1	1	1	1	1	0	1	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	0
<i>Spies 6573</i>	0	1	1	0	1	0	1	1	0	1	0	0	1	0	1	1	1	0	0	0	0	1	0	0	1	0	0	0	0

Appendix C. Data set of fragments absent (0) or present (1) for each of the studied specimens, using DAF primer 2. Question marks indicate missing data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
<i>Spies 6084</i>	0	0	0	0	0	0	0	1	0	1	0	1	0	0	1	0	1	0	0	0
<i>Spies 5967</i>	0	1	0	1	0	0	0	0	1	0	0	1	1	0	1	1	0	0	0	1
<i>Spies 6106</i>	1	1	0	1	0	1	0	1	1	0	0	1	1	0	1	0	1	1	0	1
<i>Spies 6240</i>	1	1	0	1	0	0	1	0	1	1	1	0	1	1	0	1	0	0	0	0
<i>Spies 6025</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6096</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6203</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6085</i>	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
<i>Spies 6201</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6291</i>	0	1	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0
<i>Spies 6181</i>	1	0	0	0	1	1	0	0	0	1	1	0	1	1	0	0	1	0	1	0
<i>Spies 6249</i>	0	1	0	0	1	0	0	1	0	1	0	1	1	1	1	0	1	0	0	1
<i>Spies 5976</i>	0	1	0	0	0	0	0	1	0	1	0	0	1	0	1	0	1	0	1	1
<i>Spies 6237</i>	0	1	0	0	1	0	1	0	1	0	1	1	1	1	1	1	0	1	0	1
<i>Spies 6047</i>	1	1	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0	1
<i>Spies 6286</i>	1	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1
<i>Spies 6061</i>	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	0	1	0	0	1
<i>Spies 6254</i>	0	0	1	0	1	1	1	0	1	0	0	0	1	0	0	0	0	0	0	1
<i>Spies 6101</i>	0	1	0	1	1	0	1	1	0	1	1	0	0	0	0	0	0	0	0	1
<i>Spies 6213</i>	1	1	0	0	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1
<i>Spies 6252</i>	1	1	1	0	1	0	0	0	1	0	1	1	0	1	1	1	1	0	1	0
<i>Spies 6256</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6072</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6154</i>	1	1	0	1	0	0	1	0	0	1	0	0	1	1	0	0	1	0	1	0
<i>Spies 6225</i>	0	0	0	0	1	0	0	1	0	1	0	0	1	1	0	0	1	0	1	0
<i>Spies 6160</i>	1	0	0	1	1	0	0	1	0	0	0	0	1	0	1	1	0	0	0	0
<i>Spies 6166</i>	1	0	1	0	0	0	0	1	0	0	0	0	1	0	1	1	0	1	0	1
<i>Spies 6235</i>	0	1	0	1	1	0	1	0	1	1	0	0	1	0	1	1	0	0	0	1
<i>Spies 6316</i>	1	1	0	1	0	0	1	0	0	1	0	0	1	0	1	1	1	0	0	0
<i>Spies 6140</i>	0	0	0	0	0	0	1	0	1	0	1	0	1	1	0	0	0	0	0	0
<i>Spies 6285</i>	0	1	0	0	1	1	0	1	1	0	1	0	1	1	0	0	0	0	0	0
<i>Spies 6227</i>	1	1	0	0	1	0	1	1	1	0	1	1	0	1	1	0	1	1	0	1
<i>Spies 6145</i>	0	0	0	0	1	0	1	1	0	0	1	0	0	1	1	1	0	0	0	0
<i>Spies 6288</i>	0	1	1	0	0	0	0	1	1	1	0	0	1	0	1	0	1	0	0	0
<i>Spies 6257</i>	1	0	1	0	1	1	0	0	0	1	1	0	1	1	0	0	1	1	0	0
<i>Spies 6241</i>	1	1	1	0	1	0	0	1	0	1	1	0	0	1	1	0	0	1	0	0
<i>Spies 6244</i>	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	1	1	0
<i>Spies 6575</i>	1	1	0	0	1	0	0	1	0	0	1	1	0	1	0	1	1	1	0	0
<i>Spies 6574</i>	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
<i>Spies 6573</i>	1	1	0	1	1	0	1	0	1	0	0	1	0	1	0	1	0	0	1	1

Appendix C (continued). Data set of fragments absent (0) or present (1) for each of the studied specimens, using DAF primer 2.

Question marks indicate missing data.

	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
<i>Spies 6084</i>	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
<i>Spies 5967</i>	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0
<i>Spies 6106</i>	1	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0
<i>Spies 6240</i>	0	0	0	1	0	1	1	0	0	1	0	1	1	0	0	0
<i>Spies 6025</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6096</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6203</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6085</i>	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1
<i>Spies 6201</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6291</i>	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
<i>Spies 6181</i>	0	1	1	0	0	0	1	0	0	1	0	0	0	1	1	0
<i>Spies 6249</i>	1	1	1	0	0	0	1	0	0	1	1	0	1	0	1	1
<i>Spies 5976</i>	1	1	1	0	1	1	0	1	0	1	0	0	0	1	1	0
<i>Spies 6237</i>	0	1	0	0	1	0	0	1	0	1	0	0	0	1	1	1
<i>Spies 6047</i>	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0
<i>Spies 6286</i>	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>Spies 6061</i>	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0
<i>Spies 6254</i>	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
<i>Spies 6101</i>	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
<i>Spies 6213</i>	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0
<i>Spies 6252</i>	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6256</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6072</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6154</i>	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
<i>Spies 6225</i>	0	0	1	0	0	0	1	0	0	0	0	0	1	0	1	1
<i>Spies 6160</i>	0	1	0	0	1	0	0	0	1	0	0	0	1	0	1	1
<i>Spies 6166</i>	1	0	0	0	1	1	0	0	1	0	0	0	1	0	1	1
<i>Spies 6235</i>	0	1	0	1	0	0	0	0	0	0	0	0	1	0	1	1
<i>Spies 6316</i>	0	0	1	0	1	0	0	1	0	0	1	0	0	0	1	1
<i>Spies 6140</i>	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1	1
<i>Spies 6285</i>	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	1
<i>Spies 6227</i>	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	1
<i>Spies 6145</i>	0	0	1	0	1	1	0	1	0	0	1	0	0	0	1	1
<i>Spies 6288</i>	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
<i>Spies 6257</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6241</i>	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
<i>Spies 6244</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6575</i>	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6574</i>	1	1	1	1	1	1	1	1	1	0	0	0	1	1	0	1
<i>Spies 6573</i>	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0

Appendix D. Data set of fragments absent (0) or present (1) for each of the studied specimens, using DAF primer 3. Question marks indicate missing data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
<i>Spies 6084</i>	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 5967</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6106</i>	1	0	1	0	1	1	0	1	0	1	0	1	1	1	0	1	0	1	1	0	0	1	1
<i>Spies 6240</i>	0	1	0	1	0	1	0	0	0	1	1	1	0	0	1	0	0	1	1	0	0	0	0
<i>Spies 6025</i>	0	1	0	1	0	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	1	1	0
<i>Spies 6096</i>	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6203</i>	0	1	1	0	1	0	1	1	0	1	1	0	0	1	0	1	0	1	1	0	1	0	0
<i>Spies 6085</i>	1	0	1	0	1	0	0	1	1	0	1	0	1	0	0	1	0	1	0	1	0	0	0
<i>Spies 6201</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6291</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6181</i>	1	0	1	0	1	0	0	1	0	1	0	1	0	0	0	1	0	0	1	1	0	1	0
<i>Spies 6249</i>	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0
<i>Spies 5976</i>	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6237</i>	0	0	1	0	1	1	0	1	1	1	1	1	1	0	1	1	0	1	0	1	0	0	0
<i>Spies 6047</i>	0	0	1	1	1	1	1	0	0	1	0	1	0	1	1	0	0	1	0	1	0	1	0
<i>Spies 6286</i>	0	0	1	0	1	0	1	0	1	1	1	1	0	1	0	0	0	0	0	1	0	0	1
<i>Spies 6061</i>	0	0	0	0	1	1	1	0	0	1	1	1	0	1	0	0	0	0	0	1	0	0	1
<i>Spies 6254</i>	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0
<i>Spies 6101</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6213</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6252</i>	1	0	1	0	1	0	1	1	0	1	1	0	0	1	0	1	0	1	1	0	0	0	1
<i>Spies 6256</i>	0	1	1	0	0	0	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	0	0
<i>Spies 6072</i>	1	1	1	1	1	0	0	1	1	1	1	1	1	0	0	1	0	0	1	0	1	0	0
<i>Spies 6154</i>	0	0	0	1	0	1	1	0	0	1	1	1	1	1	0	0	0	1	0	1	1	0	0
<i>Spies 6225</i>	0	0	1	0	1	0	0	1	0	1	0	0	1	1	0	0	0	1	1	0	1	0	1
<i>Spies 6160</i>	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1	0	1	1	1	1	1	1
<i>Spies 6166</i>	0	1	0	0	1	0	0	0	1	0	1	0	1	0	0	0	0	1	1	0	1	0	1
<i>Spies 6235</i>	0	1	1	0	1	0	0	0	1	1	1	1	0	1	1	1	1	1	0	0	1	1	1
<i>Spies 6316</i>	0	0	1	0	1	0	1	0	0	1	1	1	1	1	0	0	0	1	0	1	0	0	0
<i>Spies 6140</i>	0	0	0	0	1	0	1	0	0	1	1	0	1	0	1	1	1	0	0	0	0	1	1
<i>Spies 6285</i>	0	0	0	0	1	0	1	0	0	1	0	1	0	1	0	1	0	1	0	1	0	1	1
<i>Spies 6227</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6145</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6288</i>	0	0	1	0	1	0	1	0	1	1	1	1	0	1	0	0	0	1	1	0	1	0	0
<i>Spies 6257</i>	1	0	1	0	1	0	1	0	0	1	0	0	1	0	1	0	0	1	0	1	0	0	1
<i>Spies 6241</i>	1	0	1	1	1	0	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	1	1
<i>Spies 6244</i>	0	1	1	1	1	1	0	1	0	1	0	1	1	1	1	0	0	0	1	0	0	1	0
<i>Spies 6575</i>	1	1	0	1	0	1	1	0	1	1	0	?	0	0	?	1	0	0	1	0	0	?	0
<i>Spies 6574</i>	0	0	?	0	1	1	0	0	1	0	1	0	0	1	0	1	0	1	0	?	0	0	?
<i>Spies 6573</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?

Appendix D (continued). Data set of fragments absent (0) or present (1) for each of the studied specimens using DAF primer 3.

Question marks indicate missing data.

	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
<i>Spies 6084</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	0	0
<i>Spies 5967</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6106</i>	0	1	0	1	0	0	1	0	1	0	1	0	1	0	0	1	1	0	1	1	0	0	0
<i>Spies 6240</i>	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6025</i>	1	0	0	1	0	1	0	0	1	0	0	0	1	0	0	1	1	0	1	1	0	0	0
<i>Spies 6096</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6203</i>	1	0	0	0	0	1	0	0	0	1	0	1	1	0	0	1	1	0	0	0	0	0	0
<i>Spies 6085</i>	1	0	1	0	0	0	1	0	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0
<i>Spies 6201</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6291</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6181</i>	1	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0
<i>Spies 6249</i>	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
<i>Spies 5976</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
<i>Spies 6237</i>	0	0	1	1	0	0	1	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0
<i>Spies 6047</i>	1	1	0	0	0	1	1	0	0	1	0	1	0	0	0	1	0	0	1	1	0	0	0
<i>Spies 6286</i>	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1	1	0	1	0
<i>Spies 6061</i>	0	1	1	1	0	0	0	1	1	0	0	1	1	1	1	1	0	1	1	0	0	1	1
<i>Spies 6254</i>	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
<i>Spies 6101</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6213</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6252</i>	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
<i>Spies 6256</i>	0	0	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0
<i>Spies 6072</i>	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>Spies 6154</i>	1	0	0	0	1	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0
<i>Spies 6225</i>	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6160</i>	0	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	0
<i>Spies 6166</i>	0	1	0	1	0	0	0	1	1	0	0	1	0	1	1	0	0	1	1	0	0	0	0
<i>Spies 6235</i>	1	0	0	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6316</i>	0	0	1	0	0	0	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0
<i>Spies 6140</i>	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0
<i>Spies 6285</i>	1	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0
<i>Spies 6227</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6145</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6288</i>	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6257</i>	0	0	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6241</i>	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
<i>Spies 6244</i>	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6575</i>	0	1	0	0	1	0	0	1	0	0	0	?	0	0	0	0	0	?	0	0	0	0	0
<i>Spies 6574</i>	0	0	1	0	0	0	1	0	0	0	0	?	0	0	0	0	0	0	0	?	0	0	0
<i>Spies 6573</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?

Appendix E. Data set of fragments absent (0) or present (1) for each of the studied specimens, using DAF primer 4. Question marks indicate missing data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
<i>Spies 6084</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 5967</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6106</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6240</i>	1	1	0	1	1	0	0	1	0	0	0	0	1	1	0	1	0	0	0	1
<i>Spies 6025</i>	1	0	1	0	1	1	0	1	1	0	1	0	1	1	0	1	0	1	0	0
<i>Spies 6096</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6203</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6085</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6201</i>	1	1	0	0	0	1	1	1	0	1	1	1	0	1	1	1	1	1	0	1
<i>Spies 6291</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6181</i>	1	1	1	1	1	1	1	1	1	0	0	0	1	0	1	0	0	1	0	0
<i>Spies 6249</i>	0	1	0	1	0	1	0	1	1	1	0	0	1	0	1	0	1	0	0	0
<i>Spies 5976</i>	0	1	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0
<i>Spies 6237</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
<i>Spies 6047</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6286</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6061</i>	1	1	1	0	0	1	0	1	1	1	0	1	0	0	1	0	0	1	0	1
<i>Spies 6254</i>	1	1	1	0	0	1	0	1	1	1	0	1	0	0	1	1	0	0	0	0
<i>Spies 6101</i>	1	0	1	1	0	1	0	1	1	1	0	1	0	0	1	1	0	1	0	1
<i>Spies 6213</i>	1	1	0	1	1	1	1	1	1	0	1	1	0	1	0	1	0	1	0	1
<i>Spies 6252</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6256</i>	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
<i>Spies 6072</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6154</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6225</i>	1	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	1	0
<i>Spies 6160</i>	1	0	0	0	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0
<i>Spies 6166</i>	1	1	1	1	0	1	1	1	1	1	1	0	1	0	0	0	0	1	0	0
<i>Spies 6235</i>	0	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0
<i>Spies 6316</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6140</i>	0	0	1	0	0	1	0	1	1	0	1	0	0	1	0	0	0	0	0	0
<i>Spies 6285</i>	0	1	1	1	1	1	1	1	0	1	0	1	0	0	0	0	0	1	0	1
<i>Spies 6227</i>	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	1
<i>Spies 6145</i>	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
<i>Spies 6288</i>	1	1	0	1	1	1	0	1	0	1	1	1	0	0	1	1	0	1	1	1
<i>Spies 6257</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6241</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6244</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6575</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6574</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6573</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?

Appendix E (continued). Data set of fragments absent (0) or present (1) for each of the studied specimens, using DAF primer 4.

Question marks indicate missing data.

	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
<i>Spies 6084</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 5967</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6106</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6240</i>	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0
<i>Spies 6025</i>	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6096</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6203</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6085</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6201</i>	1	1	1	1	1	1	1	0	1	1	0	1	0	1	1	0	0	0	0
<i>Spies 6291</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6181</i>	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6249</i>	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
<i>Spies 5976</i>	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	0	1	0	0
<i>Spies 6237</i>	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6047</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6286</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6061</i>	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1
<i>Spies 6254</i>	0	1	1	1	1	0	0	0	0	0	0	0	0	1	0	0	1	0	1
<i>Spies 6101</i>	1	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6213</i>	0	1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
<i>Spies 6252</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6256</i>	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
<i>Spies 6072</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6154</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6225</i>	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0
<i>Spies 6160</i>	0	1	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
<i>Spies 6166</i>	0	1	0	1	1	1	0	1	0	1	1	1	0	1	0	0	1	0	0
<i>Spies 6235</i>	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6316</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6140</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6285</i>	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6227</i>	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6145</i>	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6288</i>	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6257</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6241</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6244</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6575</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6574</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6573</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?

Appendix F. Data set of fragments absent (0) or present (1) for each of the studied specimens, using DAF primer 5. Question marks indicate missing data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
<i>Spies 6084</i>	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
<i>Spies 5967</i>	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
<i>Spies 6106</i>	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
<i>Spies 6240</i>	0	1	1	0	1	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0
<i>Spies 6025</i>	0	0	1	0	1	0	1	1	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0
<i>Spies 6096</i>	1	0	0	1	1	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0	0	1	1
<i>Spies 6203</i>	1	0	1	1	1	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0
<i>Spies 6085</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6201</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6291</i>	0	1	1	0	1	1	0	1	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0
<i>Spies 6181</i>	1	0	1	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	1	0	1	1	1
<i>Spies 6249</i>	0	1	0	1	1	0	1	0	1	1	1	1	0	0	1	1	0	1	0	1	0	1	0
<i>Spies 5976</i>	0	1	0	0	1	0	1	0	1	0	1	0	1	1	1	0	0	0	0	0	1	1	1
<i>Spies 6237</i>	0	1	0	0	1	0	1	0	1	0	1	0	1	1	1	0	0	0	0	1	0	1	0
<i>Spies 6047</i>	1	0	1	0	1	1	0	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1
<i>Spies 6286</i>	1	0	1	0	1	0	1	0	1	0	0	0	0	1	0	1	0	0	0	1	0	0	1
<i>Spies 6061</i>	1	0	1	0	1	1	1	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1
<i>Spies 6254</i>	1	0	1	0	1	1	0	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1
<i>Spies 6101</i>	1	0	1	0	1	1	0	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
<i>Spies 6213</i>	0	0	1	0	0	0	0	1	0	1	1	1	1	0	0	1	0	0	0	0	0	1	1
<i>Spies 6252</i>	1	0	1	1	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	0	0	1
<i>Spies 6256</i>	1	0	1	1	0	0	1	0	0	0	1	1	0	0	1	1	0	0	1	0	0	1	1
<i>Spies 6072</i>	1	0	1	1	1	0	0	0	1	0	1	0	0	0	1	0	0	0	1	1	1	1	0
<i>Spies 6154</i>	0	0	1	1	0	1	0	0	0	1	1	0	1	1	1	1	0	1	1	0	0	0	0
<i>Spies 6225</i>	1	0	1	0	1	0	1	1	1	0	1	0	1	0	1	0	0	0	1	0	1	0	1
<i>Spies 6160</i>	1	0	0	1	0	1	0	1	1	0	1	1	1	1	1	1	0	1	1	0	1	0	0
<i>Spies 6166</i>	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	0	0	0	0	0	1	0
<i>Spies 6235</i>	1	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1
<i>Spies 6316</i>	1	0	1	0	1	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	1
<i>Spies 6140</i>	0	1	0	1	1	1	1	0	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0
<i>Spies 6285</i>	1	1	0	1	0	1	1	0	1	0	0	0	1	0	0	0	1	1	0	0	0	0	1
<i>Spies 6227</i>	0	1	1	0	1	0	0	0	1	0	1	0	0	1	1	1	1	0	0	0	0	1	1
<i>Spies 6145</i>	1	1	1	1	1	1	0	1	1	1	1	0	0	1	0	1	0	0	0	0	1	1	1
<i>Spies 6288</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6257</i>	0	1	0	1	1	0	0	1	1	1	0	0	1	1	1	1	0	1	0	0	1	1	0
<i>Spies 6241</i>	1	1	1	1	1	0	0	1	1	1	0	0	1	1	0	1	0	1	0	0	0	1	1
<i>Spies 6244</i>	0	1	1	1	1	0	1	1	0	1	0	1	0	1	0	1	0	0	0	0	1	1	0
<i>Spies 6575</i>	1	1	1	1	1	0	1	0	0	1	0	1	0	1	0	1	0	1	0	1	1	0	1
<i>Spies 6574</i>	0	1	1	1	1	0	1	0	1	1	1	1	0	1	0	1	0	1	0	0	0	1	0
<i>Spies 6573</i>	1	0	1	0	1	0	1	0	1	0	1	1	0	1	1	1	0	1	0	0	0	1	0

Appendix F (continued). Data set of fragments absent (0) or present (1) for each of the studied specimens, using DAF primer 5.

Question marks indicate missing data.

	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
<i>Spies 6084</i>	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
<i>Spies 5967</i>	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1
<i>Spies 6106</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0
<i>Spies 6240</i>	0	0	0	0	0	0	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0
<i>Spies 6025</i>	1	1	1	0	0	0	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0
<i>Spies 6096</i>	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
<i>Spies 6203</i>	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
<i>Spies 6085</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6201</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6291</i>	0	0	1	1	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	0
<i>Spies 6181</i>	1	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0
<i>Spies 6249</i>	1	0	1	0	1	1	1	0	0	0	0	1	1	0	0	1	1	1	0	1	0	1	0
<i>Spies 5976</i>	0	1	0	0	1	0	1	0	1	0	1	0	1	0	0	0	1	0	0	1	0	0	0
<i>Spies 6237</i>	1	0	1	0	1	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0
<i>Spies 6047</i>	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6286</i>	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
<i>Spies 6061</i>	0	0	0	1	1	0	0	0	0	1	0	1	0	1	1	0	0	0	0	0	0	1	0
<i>Spies 6254</i>	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6101</i>	1	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6213</i>	0	0	0	1	1	1	0	0	0	0	1	1	1	0	1	0	0	0	1	1	0	0	0
<i>Spies 6252</i>	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6256</i>	1	0	1	1	0	0	0	1	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0
<i>Spies 6072</i>	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	1	0	0
<i>Spies 6154</i>	1	1	1	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0
<i>Spies 6225</i>	0	0	1	1	1	0	0	0	0	1	0	0	1	1	0	0	0	1	1	1	0	0	0
<i>Spies 6160</i>	0	1	1	0	1	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0
<i>Spies 6166</i>	1	0	0	1	1	0	0	0	0	1	0	0	0	1	0	0	1	1	0	0	0	0	1
<i>Spies 6235</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6316</i>	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
<i>Spies 6140</i>	0	1	1	0	1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0
<i>Spies 6285</i>	1	1	0	1	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
<i>Spies 6227</i>	0	1	1	0	1	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	0	0
<i>Spies 6145</i>	1	1	1	0	1	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0
<i>Spies 6288</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6257</i>	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
<i>Spies 6241</i>	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0
<i>Spies 6244</i>	1	1	1	0	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
<i>Spies 6575</i>	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6574</i>	1	1	1	0	1	0	0	0	1	1	0	0	0	0	0	1	0	0	0	1	0	0	0
<i>Spies 6573</i>	1	0	1	0	1	0	0	1	0	0	0	0	0	1	1	1	0	1	1	0	0	0	0

Appendix G. Data set of fragments absent (0) or present (1) for each of the studied specimens, using DAF primer 6. Question marks indicate missing data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		
<i>Spies 6084</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?		
<i>Spies 5967</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?		
<i>Spies 6106</i>	1	1	0	1	0	1	0	1	0	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	?	0	0	?		
<i>Spies 6240</i>	1	1	0	1	1	1	0	1	1	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	
<i>Spies 6025</i>	0	1	0	0	0	0	1	0	1	1	0	1	1	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	
<i>Spies 6096</i>	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	0	1	1	
<i>Spies 6203</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
<i>Spies 6085</i>	0	1	0	0	0	1	1	1	0	0	0	1	1	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	
<i>Spies 6201</i>	0	1	0	0	1	0	1	0	1	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	1	0	1	0	0	1
<i>Spies 6291</i>	0	1	0	1	0	1	0	1	0	0	1	1	1	1	1	1	1	0	0	1	0	1	0	1	0	1	0	0	0	0	1	
<i>Spies 6181</i>	1	1	0	1	0	1	0	1	0	0	1	1	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	
<i>Spies 6249</i>	0	1	0	1	0	1	0	1	0	0	0	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	
<i>Spies 5976</i>	1	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
<i>Spies 6237</i>	1	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	
<i>Spies 6047</i>	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	
<i>Spies 6286</i>	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	
<i>Spies 6061</i>	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	
<i>Spies 6254</i>	0	0	0	1	1	1	0	0	0	0	1	1	1	0	0	0	1	0	0	0	0	0	1	0	1	0	1	1	0	0	1	
<i>Spies 6101</i>	1	0	0	1	0	1	0	1	0	1	0	0	1	0	0	1	0	1	0	0	0	1	0	0	1	0	0	1	0	1	0	0
<i>Spies 6213</i>	1	0	0	1	0	0	0	0	1	1	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	
<i>Spies 6252</i>	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	
<i>Spies 6256</i>	0	1	0	1	0	1	0	1	0	1	1	1	1	0	0	1	0	0	1	0	0	0	1	0	1	0	1	0	0	0	1	0
<i>Spies 6072</i>	1	0	0	1	0	0	0	1	0	1	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6154</i>	0	1	0	1	0	1	0	0	1	0	1	1	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	1	
<i>Spies 6225</i>	1	0	0	1	0	0	1	0	0	0	1	1	1	0	0	1	0	0	1	0	0	1	1	1	1	1	1	0	1	1	0	
<i>Spies 6160</i>	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	
<i>Spies 6166</i>	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>Spies 6235</i>	1	0	0	1	0	1	0	1	0	1	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	
<i>Spies 6316</i>	1	0	0	1	0	0	1	0	1	0	1	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	
<i>Spies 6140</i>	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	
<i>Spies 6285</i>	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	
<i>Spies 6227</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
<i>Spies 6145</i>	0	0	1	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	
<i>Spies 6288</i>	0	1	1	0	0	0	0	1	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	1	0	1	0	1	0	1	
<i>Spies 6257</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
<i>Spies 6241</i>	1	0	0	1	0	0	0	1	0	0	0	1	1	0	1	0	1	1	1	1	0	1	1	0	?	1	0	1	0	?		
<i>Spies 6244</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
<i>Spies 6575</i>	0	0	1	0	1	0	1	1	1	0	1	0	1	1	1	1	1	0	0	0	1	0	0	0	?	0	?	0	0	?		
<i>Spies 6574</i>	1	1	0	1	0	1	0	1	1	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	?	1	0	0	?	0	
<i>Spies 6573</i>	0	1	1	1	0	0	0	1	1	0	0	1	1	1	1	1	1	0	0	1	0	1	0	0	?	0	0	1	0	0		

Appendix H. Data set of fragments absent (0) or present (1) for each of the studied specimens, using DAF primer 9. Question marks indicate missing data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
<i>Spies 6084</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 5967</i>	0	1	0	1	1	1	0	0	1	1	1	1	0	0	1	0	0	0	1	1
<i>Spies 6106</i>	0	1	1	1	0	0	1	1	1	1	0	1	0	1	0	1	0	1	1	1
<i>Spies 6240</i>	1	0	1	0	1	1	0	0	1	1	1	0	1	0	1	0	1	0	1	1
<i>Spies 6025</i>	0	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	1	0	0	0
<i>Spies 6096</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6203</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6085</i>	1	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	1
<i>Spies 6201</i>	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1	0	1	1	1	1
<i>Spies 6291</i>	1	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	1	0	1
<i>Spies 6181</i>	0	0	0	0	0	1	0	1	1	1	1	0	1	1	1	1	1	1	0	1
<i>Spies 6249</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 5976</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6237</i>	1	1	1	0	1	0	1	0	1	1	0	0	1	0	1	0	1	0	0	1
<i>Spies 6047</i>	1	1	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	1	0
<i>Spies 6286</i>	1	1	0	0	0	1	0	1	0	1	0	0	0	1	0	0	0	1	1	1
<i>Spies 6061</i>	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	0
<i>Spies 6254</i>	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	0
<i>Spies 6101</i>	0	0	1	0	0	1	1	1	0	1	1	1	1	0	1	1	0	0	1	0
<i>Spies 6213</i>	1	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	1	0	1
<i>Spies 6252</i>	1	0	1	0	1	0	1	1	1	1	1	0	1	0	1	0	0	0	0	0
<i>Spies 6256</i>	1	0	1	0	1	1	0	1	1	1	1	1	1	1	1	0	0	1	0	1
<i>Spies 6072</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6154</i>	1	0	1	0	1	1	0	1	0	1	1	0	1	1	1	0	1	0	1	0
<i>Spies 6225</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6160</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6166</i>	1	1	0	1	1	1	1	1	1	1	1	0	0	0	0	1	0	0	1	1
<i>Spies 6235</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6316</i>	1	1	1	1	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0
<i>Spies 6140</i>	0	1	1	1	0	1	0	0	0	1	0	1	0	0	1	0	1	0	0	0
<i>Spies 6285</i>	1	1	0	1	0	1	0	1	0	0	0	1	0	0	1	0	1	0	0	0
<i>Spies 6227</i>	1	1	1	0	1	1	0	1	0	0	0	1	0	1	0	1	0	1	0	0
<i>Spies 6145</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6288</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6257</i>	1	1	1	0	1	1	1	0	0	1	1	0	1	0	0	1	0	0	1	0
<i>Spies 6241</i>	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
<i>Spies 6244</i>	1	1	1	1	1	0	1	0	1	0	1	1	1	0	0	0	1	1	0	1
<i>Spies 6575</i>	1	0	1	1	0	1	1	0	0	0	1	0	1	0	1	0	1	0	1	1
<i>Spies 6574</i>	1	1	1	1	1	1	0	0	0	1	1	0	0	0	0	1	1	0	0	1
<i>Spies 6573</i>	0	0	0	1	1	1	0	1	0	0	1	0	0	1	0	1	0	1	0	1

Appendix H (continued). Data set of fragments absent (0) or present (1) for each of the studied specimens, using DAF primer 9.

Question marks indicate missing data.

	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
<i>Spies 6084</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 5967</i>	1	1	1	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0
<i>Spies 6106</i>	1	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	0	0	0
<i>Spies 6240</i>	0	0	1	1	1	1	1	0	0	1	1	0	0	1	1	0	1	0	0
<i>Spies 6025</i>	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	0	0	1
<i>Spies 6096</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6203</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6085</i>	0	0	1	0	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0
<i>Spies 6201</i>	0	1	1	1	1	1	1	0	0	1	1	0	1	1	1	0	0	1	0
<i>Spies 6291</i>	0	0	1	0	0	1	0	0	1	1	0	0	0	1	1	1	1	0	0
<i>Spies 6181</i>	0	0	1	0	0	1	0	0	1	1	1	1	1	1	0	0	0	0	0
<i>Spies 6249</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 5976</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6237</i>	1	0	1	0	0	1	0	0	0	1	1	1	1	0	1	0	0	0	0
<i>Spies 6047</i>	1	0	1	0	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0
<i>Spies 6286</i>	1	1	1	0	1	1	0	0	1	1	1	1	1	1	1	0	0	1	0
<i>Spies 6061</i>	1	1	1	0	0	1	0	0	1	1	1	1	1	1	1	0	0	1	0
<i>Spies 6254</i>	1	1	1	0	0	1	0	0	1	1	1	1	1	1	0	0	0	1	0
<i>Spies 6101</i>	1	0	0	0	1	0	0	0	1	1	1	1	1	0	0	0	0	1	0
<i>Spies 6213</i>	0	1	1	1	0	1	1	0	0	0	1	1	1	1	0	0	0	0	1
<i>Spies 6252</i>	0	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	0	1
<i>Spies 6256</i>	0	1	0	1	1	0	1	0	0	1	1	0	0	1	1	0	1	0	1
<i>Spies 6072</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6154</i>	0	0	1	1	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0
<i>Spies 6225</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6160</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6166</i>	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
<i>Spies 6235</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6316</i>	1	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0
<i>Spies 6140</i>	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0
<i>Spies 6285</i>	1	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	1
<i>Spies 6227</i>	1	0	0	0	1	0	0	1	1	1	0	1	1	1	0	1	0	0	1
<i>Spies 6145</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6288</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6257</i>	1	0	0	0	1	0	1	0	1	1	1	0	1	0	0	0	0	0	1
<i>Spies 6241</i>	1	0	1	0	1	0	1	1	0	1	1	1	0	1	0	1	0	0	1
<i>Spies 6244</i>	1	0	1	1	1	0	0	1	0	0	0	1	0	0	1	1	0	0	1
<i>Spies 6575</i>	0	0	0	1	1	1	0	0	0	1	0	1	0	0	1	1	0	0	0
<i>Spies 6574</i>	1	0	1	1	0	1	0	0	1	1	0	1	1	1	1	0	1	0	0
<i>Spies 6573</i>	1	1	1	0	0	1	0	0	1	1	0	0	0	1	0	0	0	1	0

Appendix I. Data set of fragments absent (0) or present (1) for each of the studied specimens, using DAF primer 11. Question marks indicate missing data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
<i>Spies 6084</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 5967</i>	0	0	0	1	0	1	0	1	1	0	0	0	1	1	1	0	1	0	1	0	0	0	0	0	0	0
<i>Spies 6106</i>	0	0	1	1	0	1	0	1	0	0	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0
<i>Spies 6240</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6025</i>	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6096</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6203</i>	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
<i>Spies 6085</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6201</i>	0	1	0	0	1	1	1	1	0	1	1	1	1	1	0	0	1	0	1	0	0	0	0	1	0	0
<i>Spies 6291</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6181</i>	1	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
<i>Spies 6249</i>	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0
<i>Spies 5976</i>	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6237</i>	0	0	1	1	1	1	0	1	0	0	1	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0
<i>Spies 6047</i>	0	0	1	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	1	0
<i>Spies 6286</i>	0	1	0	1	1	1	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6061</i>	0	0	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
<i>Spies 6254</i>	0	0	0	0	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6101</i>	1	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
<i>Spies 6213</i>	0	0	0	0	1	1	0	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0
<i>Spies 6252</i>	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6256</i>	1	0	0	1	1	1	0	0	0	1	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>Spies 6072</i>	1	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
<i>Spies 6154</i>	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
<i>Spies 6225</i>	1	0	1	1	0	1	0	0	0	0	1	0	1	1	0	1	0	1	1	0	1	0	0	0	0	0
<i>Spies 6160</i>	0	0	0	1	1	1	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0
<i>Spies 6166</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6235</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6316</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6140</i>	1	0	0	0	1	0	0	1	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0
<i>Spies 6285</i>	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>Spies 6227</i>	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6145</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6288</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6257</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6241</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6244</i>	1	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6575</i>	0	1	0	1	1	1	0	1	0	0	1	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0
<i>Spies 6574</i>	1	1	0	1	1	1	0	1	1	0	1	0	1	1	1	0	0	1	1	1	0	0	0	0	0	0
<i>Spies 6573</i>	1	0	1	1	1	1	0	1	1	1	1	0	0	0	1	0	1	1	1	0	0	1	0	0	1	0

Appendix J. Data set of fragments absent (0) or present (1) for each of the studied specimens, using DAF primer 12. Question marks indicate missing data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
<i>Spies 6084</i>	0	1	0	0	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	1
<i>Spies 5967</i>	1	0	0	0	1	1	1	0	0	1	1	0	1	0	1	1	1	1	0	0	0	1	0
<i>Spies 6106</i>	1	1	1	1	0	1	1	0	0	1	0	0	1	0	1	1	0	1	0	1	1	0	1
<i>Spies 6240</i>	1	0	1	0	0	1	0	0	0	0	0	1	0	0	1	1	0	1	0	1	0	1	1
<i>Spies 6025</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6096</i>	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
<i>Spies 6203</i>	1	0	0	1	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0	1	0	0	1
<i>Spies 6085</i>	0	0	0	1	0	1	0	0	0	1	1	1	0	1	0	1	0	1	0	0	0	0	1
<i>Spies 6201</i>	1	0	1	1	1	0	0	1	0	1	1	1	0	1	0	1	1	0	1	1	0	1	1
<i>Spies 6291</i>	0	0	1	0	1	1	0	1	0	0	1	0	0	0	1	0	1	0	0	0	0	0	1
<i>Spies 6181</i>	1	1	0	1	0	1	1	0	1	1	1	1	0	1	1	1	0	0	1	1	1	0	1
<i>Spies 6249</i>	0	0	1	1	0	1	1	0	1	0	1	1	0	1	1	1	0	1	1	1	1	0	0
<i>Spies 5976</i>	0	1	1	0	1	1	0	1	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0
<i>Spies 6237</i>	1	1	0	1	0	1	1	0	0	0	0	1	0	1	1	1	0	1	0	1	1	1	1
<i>Spies 6047</i>	1	0	1	0	1	1	0	0	0	1	1	0	0	1	1	1	1	1	1	0	0	1	0
<i>Spies 6286</i>	1	1	0	0	1	0	1	1	0	1	1	0	1	1	0	1	0	0	0	1	0	1	0
<i>Spies 6061</i>	0	0	0	1	0	1	0	0	0	1	0	1	0	0	1	1	0	1	0	1	0	1	0
<i>Spies 6254</i>	0	0	0	1	0	1	0	0	0	1	0	1	0	0	1	1	0	1	0	1	0	1	0
<i>Spies 6101</i>	1	0	1	0	1	1	1	0	0	0	1	0	1	0	1	1	0	1	0	0	1	1	0
<i>Spies 6213</i>	1	1	0	1	0	1	0	1	0	0	0	1	0	1	0	0	0	1	0	0	1	1	0
<i>Spies 6252</i>	1	0	1	0	1	1	1	1	0	1	1	1	0	1	1	1	0	1	0	1	0	1	0
<i>Spies 6256</i>	1	0	0	0	1	1	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	1	0
<i>Spies 6072</i>	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	1	1
<i>Spies 6154</i>	1	0	1	0	0	1	1	0	0	1	1	1	1	0	1	1	1	0	1	1	0	1	0
<i>Spies 6225</i>	1	0	1	0	0	1	1	0	0	1	1	1	1	0	1	1	1	0	1	1	0	1	0
<i>Spies 6160</i>	0	0	0	1	0	1	0	1	0	1	0	1	0	1	0	1	1	0	1	0	1	1	0
<i>Spies 6166</i>	0	1	0	0	1	0	0	0	0	1	1	1	0	1	0	1	0	0	1	1	0	1	0
<i>Spies 6235</i>	1	0	0	0	0	1	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	1	0
<i>Spies 6316</i>	1	1	0	1	0	0	1	0	0	1	1	1	0	1	1	0	0	1	1	1	1	0	1
<i>Spies 6140</i>	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	1	1	1	1	0	0
<i>Spies 6285</i>	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1	0	0	1	1	0	1	1	1
<i>Spies 6227</i>	1	1	1	0	1	1	1	1	0	0	1	1	0	0	1	1	0	1	1	1	0	1	1
<i>Spies 6145</i>	0	1	0	0	1	0	1	0	0	0	1	1	0	1	0	1	0	0	1	0	0	0	1
<i>Spies 6288</i>	0	1	0	1	0	1	1	1	0	1	1	1	0	1	1	1	0	0	1	1	0	1	0
<i>Spies 6257</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6241</i>	0	1	0	0	0	0	1	0	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0
<i>Spies 6244</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6575</i>	0	0	0	1	0	1	0	1	0	0	1	1	0	1	1	0	1	0	1	0	1	0	1
<i>Spies 6574</i>	1	1	1	0	0	1	1	0	0	1	0	1	0	0	1	1	0	1	1	0	1	0	1
<i>Spies 6573</i>	1	0	0	1	1	0	0	0	0	0	1	0	1	1	0	1	1	1	0	0	0	0	0

Appendix J (continued). Data set of fragments absent (0) or present (1) for each of the studied specimens, using DAF primer 12.

Question marks indicate missing data.

	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
<i>Spies 6084</i>	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
<i>Spies 5967</i>	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
<i>Spies 6106</i>	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6240</i>	0	1	1	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0	0	0
<i>Spies 6025</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6096</i>	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0
<i>Spies 6203</i>	0	1	0	0	1	0	1	1	0	0	1	1	0	1	1	0	0	1	0	1
<i>Spies 6085</i>	0	0	1	0	1	0	1	0	0	0	1	0	0	0	1	0	0	0	0	1
<i>Spies 6201</i>	1	0	0	1	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	1
<i>Spies 6291</i>	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6181</i>	1	0	1	1	1	1	1	1	1	0	0	0	0	1	0	0	0	0	0	1
<i>Spies 6249</i>	1	1	0	1	0	1	0	1	1	0	0	0	0	1	0	0	0	0	0	1
<i>Spies 5976</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
<i>Spies 6237</i>	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	1	1
<i>Spies 6047</i>	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
<i>Spies 6286</i>	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6061</i>	0	1	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
<i>Spies 6254</i>	0	1	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
<i>Spies 6101</i>	0	1	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6213</i>	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0
<i>Spies 6252</i>	0	0	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
<i>Spies 6256</i>	0	0	0	0	1	1	0	0	0	0	1	0	0	0	1	0	0	0	0	1
<i>Spies 6072</i>	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0
<i>Spies 6154</i>	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>Spies 6225</i>	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0
<i>Spies 6160</i>	1	1	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
<i>Spies 6166</i>	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1
<i>Spies 6235</i>	1	1	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0
<i>Spies 6316</i>	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spies 6140</i>	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0
<i>Spies 6285</i>	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
<i>Spies 6227</i>	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
<i>Spies 6145</i>	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
<i>Spies 6288</i>	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1
<i>Spies 6257</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6241</i>	0	1	0	1	0	0	1	0	0	0	1	1	0	0	0	?	0	0	0	1
<i>Spies 6244</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Spies 6575</i>	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
<i>Spies 6574</i>	1	1	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0
<i>Spies 6573</i>	1	1	0	1	0	0	1	1	0	0	0	1	0	0	0	0	1	0	0	0

Appendix K. Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 1.

Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies 6084</i>	<i>Spies 5967</i>	<i>Spies 6106</i>	<i>Spies 6240</i>	<i>Spies 6025</i>	<i>Spies 6096</i>	<i>Spies 6203</i>	<i>Spies 6085</i>	<i>Spies 6201</i>	<i>Spies 6291</i>	<i>Spies 6181</i>	<i>Spies 6249</i>	<i>Spies 5976</i>	<i>Spies 6237</i>	<i>Spies 6047</i>	<i>Spies 6286</i>	<i>Spies 6061</i>	<i>Spies 6254</i>	<i>Spies 6101</i>	<i>Spies 6213</i>
<i>Spies 6084</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 5967</i>	—	—	0.73	0.29	0.18	0.20	0.36	0.19	0.00	—	0.33	—	0.29	0.00	0.36	0.00	—	—	0.00	0.24
<i>Spies 6106</i>	—	0.32	—	0.56	0.27	0.14	0.54	0.32	0.13	—	0.36	—	0.33	0.22	0.13	0.13	—	—	0.14	0.29
<i>Spies 6240</i>	—	1.25	0.58	—	0.19	0.10	0.63	0.58	0.19	—	0.36	—	0.33	0.00	0.19	0.09	—	—	0.60	0.44
<i>Spies 6025</i>	—	1.70	1.32	1.66	—	0.67	0.27	0.00	0.50	—	0.00	—	0.29	0.29	0.00	0.40	—	—	0.67	0.00
<i>Spies 6096</i>	—	1.61	1.95	2.30	0.41	—	0.00	0.00	0.00	—	0.00	—	0.00	0.00	0.00	0.00	—	—	0.00	0.00
<i>Spies 6203</i>	—	1.01	0.62	0.47	1.32	#	—	0.32	0.27	—	0.27	—	0.33	0.33	0.13	0.25	—	—	0.14	0.57
<i>Spies 6085</i>	—	1.66	1.14	0.54	#	#	1.14	—	0.14	—	0.38	—	0.24	0.12	0.29	0.00	—	—	0.00	0.60
<i>Spies 6201</i>	—	#	2.01	1.66	0.69	#	1.32	1.95	—	0.00	—	0.29	0.29	0.00	0.40	—	—	0.67	0.20	
<i>Spies 6291</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6181</i>	—	1.10	1.01	1.03	#	#	1.30	0.97	#	—	—	—	0.29	0.14	0.00	0.00	—	—	0.00	0.24
<i>Spies 6249</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 5976</i>	—	1.25	1.10	1.10	1.25	#	1.10	1.45	1.25	—	1.25	—	—	0.20	0.29	0.25	—	—	0.33	0.15
<i>Spies 6237</i>	—	#	1.50	#	1.25	#	1.10	2.14	1.25	—	1.95	—	1.61	—	0.00	0.75	—	—	0.33	0.31
<i>Spies 6047</i>	—	1.01	2.01	1.66	#	#	2.01	1.25	#	—	#	—	1.25	#	—	0.00	—	—	0.00	0.40
<i>Spies 6286</i>	—	#	2.08	2.40	0.92	#	1.39	#	0.92	—	#	—	1.39	0.29	#	—	—	—	0.50	0.00
<i>Spies 6061</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6254</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6101</i>	—	#	1.95	0.51	0.41	#	1.95	#	0.41	—	#	—	1.10	1.10	#	0.69	—	—	—	0.00
<i>Spies 6213</i>	—	1.45	1.25	0.81	#	#	0.56	0.51	1.61	—	1.45	—	1.87	1.18	0.82	#	—	—	#	—
<i>Spies 6252</i>	—	2.20	1.30	0.24	1.70	#	0.79	0.97	1.70	—	1.10	—	0.85	0.56	1.70	0.69	—	—	1.61	1.04
<i>Spies 6256</i>	—	0.62	0.63	0.59	#	#	0.76	0.73	2.25	—	0.77	—	1.30	1.30	1.56	1.61	—	—	#	0.92
<i>Spies 6072</i>	—	1.75	0.99	0.86	2.08	#	0.81	0.62	1.39	—	1.34	—	1.56	0.64	2.08	1.04	—	—	2.01	0.79
<i>Spies 6154</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6225</i>	—	1.56	0.83	0.73	1.79	#	0.83	1.30	1.79	—	1.15	—	0.92	0.41	1.79	0.77	—	—	1.70	0.81
<i>Spies 6160</i>	—	1.15	1.06	0.73	1.79	#	1.06	1.70	1.79	—	0.86	—	0.92	0.92	#	1.18	—	—	1.70	1.50
<i>Spies 6166</i>	—	#	1.56	0.92	1.39	#	0.86	1.50	1.39	—	0.92	—	1.01	0.61	#	0.81	—	—	1.25	1.25
<i>Spies 6235</i>	—	#	2.14	1.75	1.10	#	1.45	2.08	1.10	—	1.87	—	1.50	0.81	#	0.56	—	—	0.92	1.79
<i>Spies 6316</i>	—	0.83	0.81	0.72	2.08	#	0.66	0.62	1.39	—	1.06	—	0.86	1.15	1.39	2.14	—	—	2.01	0.61
<i>Spies 6140</i>	—	#	1.95	2.30	0.41	#	1.95	#	0.41	—	#	—	1.10	1.10	#	0.69	—	—	0.00	#
<i>Spies 6285</i>	—	0.86	0.52	0.23	2.56	#	0.43	0.41	1.87	—	0.86	—	1.06	1.06	1.87	1.50	—	—	2.53	0.69
<i>Spies 6227</i>	—	1.04	0.97	0.99	#	#	0.97	1.20	#	—	0.19	—	0.77	0.77	1.61	1.70	—	—	#	1.39
<i>Spies 6145</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6288</i>	—	1.01	0.77	0.83	2.01	#	0.77	0.73	2.01	—	1.30	—	1.50	1.50	1.32	1.39	—	—	1.95	1.25
<i>Spies 6257</i>	—	0.96	0.63	0.33	2.25	2.20	0.51	0.59	2.25	—	0.96	—	1.70	1.01	2.25	1.61	—	—	#	0.73
<i>Spies 6241</i>	—	0.88	0.41	0.26	1.70	2.35	0.61	0.58	2.40	—	0.48	—	1.14	0.92	2.40	1.75	—	—	2.35	0.85
<i>Spies 6244</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6575</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6574</i>	—	0.79	0.46	0.19	1.79	2.44	0.46	0.44	1.79	—	0.95	—	0.99	0.99	1.79	1.43	—	—	2.44	0.63
<i>Spies 6573</i>	—	0.74	0.92	0.79	1.25	#	1.44	0.88	1.95	—	0.97	—	1.04	1.45	1.25	1.32	—	—	1.87	1.61

Appendix K (continued). Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 1. Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies 6252</i>	<i>Spies 6256</i>	<i>Spies 6072</i>	<i>Spies 6154</i>	<i>Spies 6225</i>	<i>Spies 6160</i>	<i>Spies 6166</i>	<i>Spies 6235</i>	<i>Spies 6316</i>	<i>Spies 6140</i>	<i>Spies 6285</i>	<i>Spies 6227</i>	<i>Spies 6145</i>	<i>Spies 6288</i>	<i>Spies 6257</i>	<i>Spies 6241</i>	<i>Spies 6244</i>	<i>Spies 6575</i>	<i>Spies 6574</i>	<i>Spies 6573</i>
<i>Spies 6084</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 5967</i>	<i>0.11</i>	<i>0.54</i>	<i>0.17</i>	—	<i>0.21</i>	<i>0.32</i>	<i>0.00</i>	<i>0.00</i>	<i>0.43</i>	<i>0.00</i>	<i>0.42</i>	<i>0.35</i>	—	<i>0.36</i>	<i>0.38</i>	<i>0.41</i>	—	—	<i>0.45</i>	<i>0.48</i>
<i>Spies 6106</i>	<i>0.27</i>	<i>0.53</i>	<i>0.37</i>	—	<i>0.43</i>	<i>0.34</i>	<i>0.21</i>	<i>0.12</i>	<i>0.44</i>	<i>0.14</i>	<i>0.59</i>	<i>0.38</i>	—	<i>0.46</i>	<i>0.53</i>	<i>0.67</i>	—	—	<i>0.63</i>	<i>0.40</i>
<i>Spies 6240</i>	<i>0.79</i>	<i>0.56</i>	<i>0.42</i>	—	<i>0.48</i>	<i>0.48</i>	<i>0.40</i>	<i>0.17</i>	<i>0.48</i>	<i>0.10</i>	<i>0.79</i>	<i>0.37</i>	—	<i>0.44</i>	<i>0.72</i>	<i>0.77</i>	—	—	<i>0.83</i>	<i>0.45</i>
<i>Spies 6025</i>	<i>0.18</i>	<i>0.00</i>	<i>0.13</i>	—	<i>0.17</i>	<i>0.17</i>	<i>0.25</i>	<i>0.33</i>	<i>0.13</i>	<i>0.67</i>	<i>0.08</i>	<i>0.00</i>	—	<i>0.13</i>	<i>0.11</i>	<i>0.18</i>	—	—	<i>0.17</i>	<i>0.29</i>
<i>Spies 6096</i>	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>	—	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>	—	<i>0.00</i>	<i>0.11</i>	<i>0.10</i>	—	—	<i>0.09</i>	<i>0.00</i>
<i>Spies 6203</i>	<i>0.45</i>	<i>0.47</i>	<i>0.44</i>	—	<i>0.43</i>	<i>0.35</i>	<i>0.42</i>	<i>0.24</i>	<i>0.52</i>	<i>0.14</i>	<i>0.65</i>	<i>0.38</i>	—	<i>0.46</i>	<i>0.60</i>	<i>0.55</i>	—	—	<i>0.63</i>	<i>0.32</i>
<i>Spies 6085</i>	<i>0.38</i>	<i>0.48</i>	<i>0.54</i>	—	<i>0.27</i>	<i>0.18</i>	<i>0.22</i>	<i>0.13</i>	<i>0.54</i>	<i>0.00</i>	<i>0.67</i>	<i>0.30</i>	—	<i>0.48</i>	<i>0.55</i>	<i>0.56</i>	—	—	<i>0.65</i>	<i>0.42</i>
<i>Spies 6201</i>	<i>0.18</i>	<i>0.11</i>	<i>0.25</i>	—	<i>0.17</i>	<i>0.17</i>	<i>0.25</i>	<i>0.33</i>	<i>0.25</i>	<i>0.67</i>	<i>0.15</i>	<i>0.00</i>	—	<i>0.13</i>	<i>0.11</i>	<i>0.09</i>	—	—	<i>0.17</i>	<i>0.14</i>
<i>Spies 6291</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6181</i>	<i>0.33</i>	<i>0.46</i>	<i>0.26</i>	—	<i>0.32</i>	<i>0.42</i>	<i>0.40</i>	<i>0.15</i>	<i>0.35</i>	<i>0.00</i>	<i>0.42</i>	<i>0.82</i>	—	<i>0.27</i>	<i>0.38</i>	<i>0.62</i>	—	—	<i>0.39</i>	<i>0.38</i>
<i>Spies 6249</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 5976</i>	<i>0.43</i>	<i>0.27</i>	<i>0.21</i>	—	<i>0.40</i>	<i>0.40</i>	<i>0.36</i>	<i>0.22</i>	<i>0.42</i>	<i>0.33</i>	<i>0.34</i>	<i>0.46</i>	—	<i>0.22</i>	<i>0.18</i>	<i>0.32</i>	—	—	<i>0.37</i>	<i>0.35</i>
<i>Spies 6237</i>	<i>0.57</i>	<i>0.27</i>	<i>0.53</i>	—	<i>0.67</i>	<i>0.40</i>	<i>0.55</i>	<i>0.44</i>	<i>0.32</i>	<i>0.33</i>	<i>0.34</i>	<i>0.46</i>	—	<i>0.22</i>	<i>0.36</i>	<i>0.40</i>	—	—	<i>0.37</i>	<i>0.24</i>
<i>Spies 6047</i>	<i>0.18</i>	<i>0.21</i>	<i>0.13</i>	—	<i>0.17</i>	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>	<i>0.25</i>	<i>0.00</i>	<i>0.15</i>	<i>0.20</i>	—	<i>0.27</i>	<i>0.11</i>	<i>0.09</i>	—	—	<i>0.17</i>	<i>0.29</i>
<i>Spies 6286</i>	<i>0.50</i>	<i>0.20</i>	<i>0.35</i>	—	<i>0.46</i>	<i>0.31</i>	<i>0.44</i>	<i>0.57</i>	<i>0.12</i>	<i>0.50</i>	<i>0.22</i>	<i>0.18</i>	—	<i>0.25</i>	<i>0.20</i>	<i>0.17</i>	—	—	<i>0.24</i>	<i>0.27</i>
<i>Spies 6061</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6254</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6101</i>	<i>0.20</i>	<i>0.00</i>	<i>0.13</i>	—	<i>0.18</i>	<i>0.18</i>	<i>0.29</i>	<i>0.40</i>	<i>0.13</i>	<i>1.00</i>	<i>0.08</i>	<i>0.00</i>	—	<i>0.14</i>	<i>0.00</i>	<i>0.10</i>	—	—	<i>0.09</i>	<i>0.15</i>
<i>Spies 6213</i>	<i>0.35</i>	<i>0.40</i>	<i>0.45</i>	—	<i>0.44</i>	<i>0.22</i>	<i>0.29</i>	<i>0.17</i>	<i>0.55</i>	<i>0.00</i>	<i>0.50</i>	<i>0.25</i>	—	<i>0.29</i>	<i>0.48</i>	<i>0.43</i>	—	—	<i>0.53</i>	<i>0.20</i>
<i>Spies 6252</i>	—	<i>0.54</i>	<i>0.52</i>	—	<i>0.74</i>	<i>0.53</i>	<i>0.67</i>	<i>0.62</i>	<i>0.43</i>	<i>0.20</i>	<i>0.48</i>	<i>0.71</i>	—	<i>0.45</i>	<i>0.46</i>	<i>0.41</i>	—	—	<i>0.52</i>	<i>0.29</i>
<i>Spies 6256</i>	<i>0.62</i>	—	<i>0.58</i>	—	<i>0.52</i>	<i>0.59</i>	<i>0.35</i>	<i>0.19</i>	<i>0.45</i>	<i>0.00</i>	<i>0.73</i>	<i>0.56</i>	—	<i>0.47</i>	<i>0.65</i>	<i>0.54</i>	—	—	<i>0.67</i>	<i>0.41</i>
<i>Spies 6072</i>	<i>0.65</i>	<i>0.54</i>	—	—	<i>0.75</i>	<i>0.50</i>	<i>0.40</i>	<i>0.44</i>	<i>0.57</i>	<i>0.13</i>	<i>0.68</i>	<i>0.45</i>	—	<i>0.52</i>	<i>0.77</i>	<i>0.59</i>	—	—	<i>0.61</i>	<i>0.31</i>
<i>Spies 6154</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6225</i>	<i>0.31</i>	<i>0.66</i>	<i>0.29</i>	—	—	<i>0.60</i>	<i>0.63</i>	<i>0.57</i>	<i>0.58</i>	<i>0.18</i>	<i>0.53</i>	<i>0.56</i>	—	<i>0.35</i>	<i>0.59</i>	<i>0.53</i>	—	—	<i>0.56</i>	<i>0.27</i>
<i>Spies 6160</i>	<i>0.64</i>	<i>0.52</i>	<i>0.69</i>	—	<i>0.51</i>	—	<i>0.63</i>	<i>0.43</i>	<i>0.42</i>	<i>0.18</i>	<i>0.53</i>	<i>0.44</i>	—	<i>0.35</i>	<i>0.52</i>	<i>0.40</i>	—	—	<i>0.50</i>	<i>0.45</i>
<i>Spies 6166</i>	<i>0.41</i>	<i>1.06</i>	<i>0.92</i>	—	<i>0.47</i>	<i>0.47</i>	—	<i>0.40</i>	<i>0.40</i>	<i>0.29</i>	<i>0.33</i>	<i>0.43</i>	—	<i>0.21</i>	<i>0.35</i>	<i>0.38</i>	—	—	<i>0.36</i>	<i>0.11</i>
<i>Spies 6235</i>	<i>0.49</i>	<i>1.66</i>	<i>0.81</i>	—	<i>0.56</i>	<i>0.85</i>	<i>0.92</i>	—	<i>0.33</i>	<i>0.40</i>	<i>0.21</i>	<i>0.17</i>	—	<i>0.24</i>	<i>0.19</i>	<i>0.17</i>	—	—	<i>0.23</i>	<i>0.25</i>
<i>Spies 6316</i>	<i>0.83</i>	<i>0.79</i>	<i>0.56</i>	—	<i>0.54</i>	<i>0.88</i>	<i>0.92</i>	<i>1.10</i>	—	<i>0.13</i>	<i>0.63</i>	<i>0.36</i>	—	<i>0.44</i>	<i>0.45</i>	<i>0.53</i>	—	—	<i>0.61</i>	<i>0.46</i>
<i>Spies 6140</i>	<i>1.61</i>	#	<i>2.01</i>	—	<i>1.70</i>	<i>1.70</i>	<i>1.25</i>	<i>0.92</i>	<i>2.01</i>	—	<i>0.08</i>	<i>0.00</i>	—	<i>0.14</i>	<i>0.00</i>	<i>0.10</i>	—	—	<i>0.09</i>	<i>0.15</i>
<i>Spies 6285</i>	<i>0.73</i>	<i>0.31</i>	<i>0.38</i>	—	<i>0.64</i>	<i>0.64</i>	<i>1.10</i>	<i>1.54</i>	<i>0.46</i>	<i>2.53</i>	—	<i>0.50</i>	—	<i>0.65</i>	<i>0.78</i>	<i>0.77</i>	—	—	<i>0.91</i>	<i>0.67</i>
<i>Spies 6227</i>	<i>0.35</i>	<i>0.58</i>	<i>0.79</i>	—	<i>0.59</i>	<i>0.81</i>	<i>0.85</i>	<i>1.79</i>	<i>1.01</i>	#	<i>0.69</i>	—	—	<i>0.38</i>	<i>0.56</i>	<i>0.43</i>	—	—	<i>0.53</i>	<i>0.40</i>
<i>Spies 6145</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6288</i>	<i>0.79</i>	<i>0.76</i>	<i>0.66</i>	—	<i>1.06</i>	<i>1.06</i>	<i>1.58</i>	<i>1.45</i>	<i>0.81</i>	<i>1.95</i>	<i>0.43</i>	<i>0.97</i>	—	—	<i>0.53</i>	<i>0.55</i>	—	—	<i>0.57</i>	<i>0.56</i>
<i>Spies 6257</i>	<i>0.77</i>	<i>0.44</i>	<i>0.26</i>	—	<i>0.52</i>	<i>0.66</i>	<i>1.06</i>	<i>1.66</i>	<i>0.79</i>	#	<i>0.25</i>	<i>0.58</i>	—	<i>0.83</i>	—	<i>0.70</i>	—	—	<i>0.77</i>	<i>0.34</i>
<i>Spies 6241</i>	<i>0.88</i>	<i>0.62</i>	<i>0.53</i>	—	<i>0.63</i>	<i>0.92</i>	<i>0.96</i>	<i>1.79</i>	<i>0.64</i>	<i>2.35</i>	<i>0.26</i>	<i>0.85</i>	—	<i>0.61</i>	<i>0.35</i>	—	—	—	<i>0.81</i>	<i>0.50</i>
<i>Spies 6244</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6575</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6574</i>	<i>0.66</i>	<i>0.41</i>	<i>0.49</i>	—	<i>0.58</i>	<i>0.69</i>	<i>1.03</i>	<i>1.47</i>	<i>0.49</i>	<i>2.44</i>	<i>0.09</i>	<i>0.63</i>	—	<i>0.56</i>	<i>0.26</i>	<i>0.21</i>	—	—	—	<i>0.65</i>
<i>Spies 6573</i>	<i>1.25</i>	<i>0.88</i>	<i>1.18</i>	—	<i>1.30</i>	<i>0.79</i>	<i>2.20</i>	<i>1.39</i>	<i>0.77</i>	<i>1.87</i>	<i>0.41</i>	<i>0.92</i>	—	<i>0.58</i>	<i>1.06</i>	<i>0.69</i>	—	—	<i>0.44</i>	—

Appendix L. Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 2.

Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies 6084</i>	<i>Spies 5967</i>	<i>Spies 6106</i>	<i>Spies 6240</i>	<i>Spies 6025</i>	<i>Spies 6096</i>	<i>Spies 6203</i>	<i>Spies 6085</i>	<i>Spies 6201</i>	<i>Spies 6291</i>	<i>Spies 6181</i>	<i>Spies 6249</i>	<i>Spies 5976</i>	<i>Spies 6237</i>	<i>Spies 6047</i>	<i>Spies 6286</i>	<i>Spies 6061</i>	<i>Spies 6254</i>	<i>Spies 6101</i>	<i>Spies 6213</i>
<i>Spies 6084</i>		<i>0.25</i>	<i>0.42</i>	<i>0.17</i>	—	—	—	<i>0.00</i>	—	<i>0.31</i>	<i>0.18</i>	<i>0.38</i>	<i>0.42</i>	<i>0.23</i>	<i>0.22</i>	<i>0.27</i>	<i>0.22</i>	<i>0.00</i>	<i>0.24</i>	<i>0.11</i>
<i>Spies 5967</i>	1.39		<i>0.77</i>	<i>0.40</i>	—	—	—	<i>0.29</i>	—	<i>0.27</i>	<i>0.25</i>	<i>0.50</i>	<i>0.54</i>	<i>0.57</i>	<i>0.30</i>	<i>0.35</i>	<i>0.50</i>	<i>0.53</i>	<i>0.42</i>	<i>0.40</i>
<i>Spies 6106</i>	0.88	0.26		<i>0.08</i>	—	—	—	<i>0.18</i>	—	<i>0.17</i>	<i>0.38</i>	<i>0.50</i>	<i>0.59</i>	<i>0.44</i>	<i>0.43</i>	<i>0.32</i>	<i>0.43</i>	<i>0.44</i>	<i>0.37</i>	<i>0.43</i>
<i>Spies 6240</i>	1.75	0.92	2.80		—	—	—	<i>0.29</i>	—	<i>0.55</i>	<i>0.45</i>	<i>0.40</i>	<i>0.30</i>	<i>0.46</i>	<i>0.44</i>	<i>0.33</i>	<i>0.22</i>	<i>0.31</i>	<i>0.46</i>	<i>0.37</i>
<i>Spies 6025</i>	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6096</i>	—	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6203</i>	—	—	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6085</i>	#	1.25	1.70	1.25	—	—	—		—	<i>0.18</i>	<i>0.00</i>	<i>0.08</i>	<i>0.09</i>	<i>0.25</i>	<i>0.00</i>	<i>0.00</i>	<i>0.13</i>	<i>0.13</i>	<i>0.00</i>	<i>0.13</i>
<i>Spies 6201</i>	—	—	—	—	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6291</i>	1.18	1.32	1.75	0.61	—	—	—	1.70	—		<i>0.29</i>	<i>0.24</i>	<i>0.17</i>	<i>0.32</i>	<i>0.35</i>	<i>0.29</i>	<i>0.00</i>	<i>0.00</i>	<i>0.38</i>	<i>0.24</i>
<i>Spies 6181</i>	1.70	1.39	0.98	0.79	—	—	—	#	—	1.25		<i>0.59</i>	<i>0.56</i>	<i>0.47</i>	<i>0.46</i>	<i>0.26</i>	<i>0.31</i>	<i>0.40</i>	<i>0.40</i>	<i>0.38</i>
<i>Spies 6249</i>	0.96	0.69	0.69	0.92	—	—	—	2.48	—	1.43	0.53		<i>0.67</i>	<i>0.58</i>	<i>0.40</i>	<i>0.37</i>	<i>0.40</i>	<i>0.41</i>	<i>0.48</i>	<i>0.33</i>
<i>Spies 5976</i>	0.88	0.62	0.53	1.19	—	—	—	2.40	—	1.75	0.58	0.41		<i>0.56</i>	<i>0.43</i>	<i>0.48</i>	<i>0.50</i>	<i>0.30</i>	<i>0.37</i>	<i>0.21</i>
<i>Spies 6237</i>	1.47	0.56	0.81	0.78	—	—	—	1.39	—	1.14	0.75	0.55	0.59		<i>0.40</i>	<i>0.37</i>	<i>0.47</i>	<i>0.34</i>	<i>0.34</i>	<i>0.47</i>
<i>Spies 6047</i>	1.50	1.20	0.85	0.81	—	—	—	#	—	1.04	0.77	0.92	0.85	0.92		<i>0.63</i>	<i>0.27</i>	<i>0.38</i>	<i>0.48</i>	<i>0.55</i>
<i>Spies 6286</i>	1.32	1.04	1.14	1.10	—	—	—	#	—	1.25	1.34	0.99	0.73	0.99	0.46		<i>0.42</i>	<i>0.33</i>	<i>0.44</i>	<i>0.32</i>
<i>Spies 6061</i>	1.50	0.69	0.85	1.50	—	—	—	2.08	—	#	1.18	0.92	0.69	0.76	1.30	0.86		<i>0.76</i>	<i>0.38</i>	<i>0.36</i>
<i>Spies 6254</i>	#	0.64	0.81	1.18	—	—	—	2.01	—	#	0.92	0.88	1.22	1.06	0.97	1.10	0.27		<i>0.50</i>	<i>0.48</i>
<i>Spies 6101</i>	1.45	0.86	0.99	0.77	—	—	—	#	—	0.98	0.92	0.73	0.99	1.06	0.74	0.81	0.97	0.69		<i>0.50</i>
<i>Spies 6213</i>	2.20	0.92	0.85	0.99	—	—	—	2.08	—	1.45	0.96	1.10	1.54	0.76	0.61	1.15	1.01	0.74	0.74	
<i>Spies 6252</i>	1.20	0.79	0.92	1.06	—	—	—	1.50	—	0.86	0.69	0.83	1.10	0.58	1.39	1.25	1.10	1.34	1.34	0.69
<i>Spies 6256</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6072</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6154</i>	1.50	1.20	0.85	0.41	—	—	—	2.08	—	1.04	0.77	1.10	0.85	1.32	1.30	0.96	1.30	1.66	0.97	1.70
<i>Spies 6225</i>	1.15	1.66	1.29	1.03	—	—	—	2.14	—	1.50	0.41	0.34	0.73	1.35	1.75	2.30	1.06	1.01	0.79	2.44
<i>Spies 6160</i>	1.61	1.01	1.10	1.06	—	—	—	1.10	—	2.25	1.03	0.69	0.92	0.69	2.48	1.66	1.39	1.75	1.34	1.10
<i>Spies 6166</i>	1.70	0.88	0.69	1.13	—	—	—	0.92	—	2.35	1.61	0.75	0.69	0.75	1.18	1.34	0.96	1.14	1.83	1.18
<i>Spies 6235</i>	1.70	0.54	0.98	0.54	—	—	—	1.20	—	1.66	1.10	0.53	0.83	0.44	1.47	0.83	0.96	0.92	0.73	1.18
<i>Spies 6316</i>	1.30	0.88	0.98	0.79	—	—	—	1.61	—	0.97	0.92	0.75	0.83	0.53	1.18	1.06	0.63	1.83	0.92	1.18
<i>Spies 6140</i>	#	1.56	1.50	0.62	—	—	—	0.63	—	1.39	0.92	1.06	1.50	0.73	1.66	2.20	1.25	0.92	1.20	0.97
<i>Spies 6285</i>	2.25	1.25	0.88	0.85	—	—	—	1.04	—	1.10	0.81	0.79	1.06	0.66	1.34	2.30	1.34	1.01	1.01	0.83
<i>Spies 6227</i>	1.10	0.96	0.53	0.86	—	—	—	1.30	—	1.34	0.98	0.49	0.89	0.41	1.03	1.14	1.03	1.22	0.81	0.69
<i>Spies 6145</i>	1.66	1.34	1.35	1.10	—	—	—	1.15	—	1.20	1.06	0.72	0.79	0.50	1.83	1.70	0.92	1.89	0.88	1.43
<i>Spies 6288</i>	1.04	1.15	0.99	0.62	—	—	—	0.92	—	0.47	1.43	1.29	1.22	1.06	0.97	1.50	1.66	1.61	0.92	1.25
<i>Spies 6257</i>	1.45	2.25	0.99	0.96	—	—	—	#	—	0.98	0.45	1.06	1.50	1.06	0.74	1.50	0.97	0.92	1.20	0.97
<i>Spies 6241</i>	1.15	1.25	0.73	0.85	—	—	—	2.14	—	0.81	0.81	0.79	0.88	0.95	0.83	1.20	1.34	1.30	0.61	1.06
<i>Spies 6244</i>	1.25	1.39	1.10	1.34	—	—	—	1.79	—	0.77	1.30	1.47	1.10	1.18	1.10	2.01	2.20	#	1.04	1.50
<i>Spies 6575</i>	1.20	0.79	0.76	1.06	—	—	—	2.20	—	0.86	0.85	0.69	0.92	0.69	0.88	1.25	0.88	1.34	0.83	0.69
<i>Spies 6574</i>	1.18	0.62	0.43	0.54	—	—	—	1.31	—	1.34	0.59	0.47	0.49	0.47	0.77	0.92	0.67	0.74	0.74	0.67
<i>Spies 6573</i>	2.30	0.45	0.76	0.59	—	—	—	1.50	—	1.15	0.85	0.83	1.32	0.69	1.10	0.97	0.88	0.65	0.50	0.54

Appendix L (continued). Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 2. Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies</i> 6252	<i>Spies</i> 6256	<i>Spies</i> 6072	<i>Spies</i> 6154	<i>Spies</i> 6225	<i>Spies</i> 6160	<i>Spies</i> 6166	<i>Spies</i> 6235	<i>Spies</i> 6316	<i>Spies</i> 6140	<i>Spies</i> 6285	<i>Spies</i> 6227	<i>Spies</i> 6145	<i>Spies</i> 6288	<i>Spies</i> 6257	<i>Spies</i> 6241	<i>Spies</i> 6244	<i>Spies</i> 6575	<i>Spies</i> 6574	<i>Spies</i> 6573
<i>Spies</i> 6084	0.30	—	—	0.22	0.32	0.20	0.18	0.18	0.27	0.00	0.11	0.33	0.19	0.35	0.24	0.32	0.29	0.30	0.31	0.10
<i>Spies</i> 5967	0.45	—	—	0.30	0.19	0.35	0.42	0.58	0.42	0.21	0.29	0.38	0.26	0.32	0.11	0.29	0.25	0.45	0.54	0.64
<i>Spies</i> 6106	0.40	—	—	0.43	0.28	0.33	0.50	0.38	0.38	0.22	0.41	0.59	0.26	0.37	0.37	0.48	0.33	0.47	0.65	0.47
<i>Spies</i> 6240	0.34	—	—	0.67	0.36	0.34	0.32	0.58	0.45	0.54	0.43	0.42	0.33	0.54	0.38	0.43	0.26	0.34	0.58	0.55
<i>Spies</i> 6025	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6096	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6203	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6085	0.22	—	—	0.13	0.12	0.33	0.40	0.30	0.20	0.53	0.35	0.27	0.32	0.40	0.00	0.12	0.17	0.11	0.27	0.22
<i>Spies</i> 6201	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6291	0.42	—	—	0.35	0.22	0.11	0.10	0.19	0.38	0.25	0.33	0.26	0.30	0.63	0.38	0.44	0.46	0.42	0.26	0.32
<i>Spies</i> 6181	0.50	—	—	0.46	0.67	0.36	0.20	0.33	0.40	0.40	0.44	0.38	0.34	0.24	0.64	0.44	0.27	0.43	0.55	0.43
<i>Spies</i> 6249	0.44	—	—	0.33	0.71	0.50	0.47	0.59	0.47	0.34	0.45	0.61	0.48	0.28	0.34	0.45	0.23	0.50	0.63	0.44
<i>Spies</i> 5976	0.33	—	—	0.43	0.48	0.40	0.50	0.44	0.44	0.22	0.34	0.41	0.45	0.30	0.22	0.41	0.33	0.40	0.61	0.27
<i>Spies</i> 6237	0.56	—	—	0.27	0.26	0.50	0.47	0.65	0.59	0.48	0.52	0.67	0.61	0.34	0.34	0.39	0.31	0.50	0.63	0.50
<i>Spies</i> 6047	0.25	—	—	0.27	0.17	0.08	0.31	0.23	0.31	0.19	0.26	0.36	0.16	0.38	0.48	0.43	0.33	0.42	0.47	0.33
<i>Spies</i> 6286	0.29	—	—	0.42	0.10	0.19	0.26	0.43	0.35	0.11	0.10	0.32	0.18	0.22	0.22	0.30	0.13	0.29	0.40	0.38
<i>Spies</i> 6061	0.33	—	—	0.27	0.35	0.25	0.38	0.39	0.38	0.29	0.26	0.36	0.40	0.19	0.38	0.26	0.11	0.42	0.51	0.42
<i>Spies</i> 6254	0.26	—	—	0.19	0.36	0.17	0.32	0.40	0.16	0.40	0.36	0.30	0.25	0.20	0.40	0.27	0.00	0.26	0.48	0.52
<i>Spies</i> 6101	0.26	—	—	0.38	0.45	0.26	0.16	0.48	0.40	0.30	0.36	0.44	0.42	0.40	0.30	0.55	0.35	0.43	0.48	0.61
<i>Spies</i> 6213	0.50	—	—	0.18	0.09	0.33	0.31	0.31	0.31	0.38	0.43	0.50	0.24	0.29	0.38	0.35	0.22	0.50	0.51	0.58
<i>Spies</i> 6252	—	—	—	0.42	0.32	0.38	0.29	0.43	0.50	0.26	0.40	0.60	0.37	0.52	0.52	0.56	0.50	0.62	0.58	0.62
<i>Spies</i> 6256	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6072	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6154	0.88	—	—	—	0.43	0.25	0.23	0.46	0.46	0.38	0.35	0.43	0.24	0.38	0.48	0.43	0.33	0.33	0.51	0.50
<i>Spies</i> 6225	1.14	—	—	0.83	—	0.49	0.37	0.44	0.44	0.45	0.50	0.41	0.46	0.27	0.45	0.42	0.32	0.40	0.50	0.40
<i>Spies</i> 6160	0.96	—	—	1.39	0.73	—	0.71	0.64	0.50	0.35	0.40	0.40	0.52	0.17	0.26	0.32	0.20	0.38	0.53	0.31
<i>Spies</i> 6166	1.25	—	—	1.47	0.99	0.34	—	0.47	0.40	0.40	0.37	0.50	0.48	0.40	0.32	0.44	0.27	0.43	0.60	0.21
<i>Spies</i> 6235	0.85	—	—	0.77	0.81	0.44	0.76	—	0.47	0.40	0.44	0.50	0.41	0.32	0.24	0.30	0.18	0.21	0.60	0.50
<i>Spies</i> 6316	0.69	—	—	0.77	0.81	0.69	0.92	0.76	—	0.40	0.44	0.63	0.76	0.32	0.32	0.44	0.36	0.57	0.55	0.50
<i>Spies</i> 6140	1.34	—	—	0.97	0.79	1.06	0.92	0.92	0.92	—	0.64	0.52	0.50	0.40	0.30	0.27	0.12	0.17	0.43	0.35
<i>Spies</i> 6285	0.92	—	—	1.06	0.69	0.92	0.99	0.81	0.81	0.45	—	0.69	0.62	0.45	0.45	0.50	0.32	0.40	0.45	0.32
<i>Spies</i> 6227	0.51	—	—	0.85	0.88	0.92	0.69	0.69	0.47	0.66	0.37	—	0.65	0.52	0.44	0.62	0.42	0.60	0.61	0.53
<i>Spies</i> 6145	0.99	—	—	1.43	0.77	0.66	0.73	0.88	0.28	0.69	0.49	0.44	—	0.42	0.25	0.46	0.29	0.52	0.52	0.37
<i>Spies</i> 6288	0.65	—	—	0.97	1.30	1.75	0.92	1.14	1.14	0.92	0.79	0.66	0.88	—	0.60	0.73	0.59	0.52	0.48	0.35
<i>Spies</i> 6257	0.65	—	—	0.74	0.79	1.34	1.14	1.43	1.14	1.20	0.79	0.81	1.39	0.51	—	0.64	0.35	0.52	0.48	0.26
<i>Spies</i> 6241	0.58	—	—	0.83	0.88	1.14	0.81	1.22	0.81	1.30	0.69	0.48	0.77	0.32	0.45	—	0.42	0.64	0.55	0.40
<i>Spies</i> 6244	0.69	—	—	1.10	1.15	1.61	1.30	1.70	1.01	2.14	1.15	0.88	1.25	0.53	1.04	0.86	—	0.60	0.36	0.30
<i>Spies</i> 6575	0.49	—	—	1.10	0.92	0.96	0.85	1.54	0.56	1.75	0.92	0.51	0.66	0.65	0.65	0.45	0.55	—	0.58	0.54
<i>Spies</i> 6574	0.55	—	—	0.67	0.69	0.63	0.52	0.52	0.59	0.85	0.79	0.49	0.65	0.74	0.74	0.61	1.02	0.55	—	0.58
<i>Spies</i> 6573	0.49	—	—	0.69	0.92	1.18	1.54	0.69	0.69	1.06	1.14	0.63	0.99	1.06	1.34	0.92	1.20	0.62	0.55	—

Appendix M. Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 3.

Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies 6084</i>	<i>Spies 5967</i>	<i>Spies 6106</i>	<i>Spies 6240</i>	<i>Spies 6025</i>	<i>Spies 6096</i>	<i>Spies 6203</i>	<i>Spies 6085</i>	<i>Spies 6201</i>	<i>Spies 6291</i>	<i>Spies 6181</i>	<i>Spies 6249</i>	<i>Spies 5976</i>	<i>Spies 6237</i>	<i>Spies 6047</i>	<i>Spies 6286</i>	<i>Spies 6061</i>	<i>Spies 6254</i>	<i>Spies 6101</i>	<i>Spies 6213</i>
<i>Spies 6084</i>	—	—	0.26	0.12	0.32	0.20	0.23	0.31	—	—	0.09	0.13	0.18	0.22	0.21	0.33	0.27	0.35	—	—
<i>Spies 5967</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6106</i>	1.35	—	—	0.47	0.57	0.15	0.65	0.60	—	—	0.72	0.48	0.29	0.73	0.71	0.59	0.60	0.41	—	—
<i>Spies 6240</i>	2.14	—	0.75	—	0.57	0.31	0.55	0.34	—	—	0.32	0.32	0.43	0.53	0.58	0.37	0.42	0.50	—	—
<i>Spies 6025</i>	1.14	—	0.56	0.56	—	0.19	0.59	0.32	—	—	0.42	0.44	0.36	0.53	0.62	0.40	0.44	0.43	—	—
<i>Spies 6096</i>	1.61	—	1.91	1.18	1.66	—	0.27	0.18	—	—	0.22	0.17	0.29	0.26	0.08	0.20	0.15	0.46	—	—
<i>Spies 6203</i>	1.47	—	0.43	0.59	0.52	1.30	—	0.53	—	—	0.59	0.50	0.43	0.67	0.75	0.61	0.52	0.78	—	—
<i>Spies 6085</i>	1.18	—	0.50	1.13	1.13	1.70	0.64	—	—	—	0.65	0.21	0.26	0.72	0.35	0.39	0.43	0.55	—	—
<i>Spies 6201</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6291</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6181</i>	2.40	—	0.33	1.14	0.86	1.50	0.53	0.44	—	—	—	0.75	0.21	0.69	0.50	0.38	0.42	0.48	—	—
<i>Spies 6249</i>	2.08	—	0.72	1.15	0.81	1.79	0.69	1.54	—	—	0.29	—	0.15	0.48	0.47	0.38	0.44	0.42	—	—
<i>Spies 5976</i>	1.70	—	1.25	0.85	1.01	1.25	0.83	1.34	—	—	1.56	1.87	—	0.42	0.40	0.38	0.30	0.57	—	—
<i>Spies 6237</i>	1.50	—	0.32	0.63	0.64	1.34	0.41	0.33	—	—	0.38	0.73	0.88	—	0.63	0.54	0.65	0.53	—	—
<i>Spies 6047</i>	1.54	—	0.34	0.54	0.49	2.48	0.29	1.05	—	—	0.69	0.76	0.92	0.46	—	0.79	0.55	0.52	—	—
<i>Spies 6286</i>	1.10	—	0.54	0.99	0.92	1.61	0.49	0.94	—	—	0.98	0.96	0.97	0.62	0.24	—	0.80	0.52	—	—
<i>Spies 6061</i>	1.32	—	0.52	0.86	0.82	1.87	0.65	0.85	—	—	0.86	0.83	1.22	0.43	0.61	0.22	—	0.55	—	—
<i>Spies 6254</i>	1.04	—	0.89	0.69	0.85	0.77	0.28	0.59	—	—	0.73	0.86	0.56	0.63	0.66	0.66	0.61	—	—	—
<i>Spies 6101</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6213</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6252</i>	1.70	—	0.41	0.45	1.42	1.10	0.13	0.53	—	—	0.41	0.88	0.86	0.56	0.59	0.47	0.46	0.33	—	—
<i>Spies 6256</i>	1.70	—	0.57	0.45	1.01	1.50	0.27	0.53	—	—	0.41	0.69	1.15	0.38	0.59	0.58	0.38	0.45	—	—
<i>Spies 6072</i>	1.43	—	0.74	0.85	0.69	1.25	0.52	0.62	—	—	0.50	0.81	1.30	0.38	1.36	0.92	0.72	0.44	—	—
<i>Spies 6154</i>	2.44	—	0.69	0.49	0.53	1.56	0.38	0.92	—	—	0.66	0.73	1.20	0.33	0.43	0.72	0.49	0.62	—	—
<i>Spies 6225</i>	2.30	—	0.35	0.50	0.95	1.39	0.29	0.69	—	—	0.85	1.70	1.45	0.72	0.53	0.63	0.81	0.36	—	—
<i>Spies 6160</i>	1.98	—	0.30	0.69	0.80	1.43	0.46	0.62	—	—	0.43	1.13	1.18	0.34	0.43	0.67	0.41	0.06	—	—
<i>Spies 6166</i>	1.43	—	0.74	0.85	0.94	2.35	0.97	0.72	—	—	1.70	1.91	2.40	0.86	1.36	0.78	0.40	0.69	—	—
<i>Spies 6235</i>	1.91	—	0.61	0.51	0.64	1.75	0.41	0.57	—	—	0.56	0.88	1.79	0.36	0.62	0.62	0.58	0.22	—	—
<i>Spies 6316</i>	1.34	—	0.69	0.96	1.04	1.56	0.66	0.46	—	—	0.78	0.92	1.20	0.41	0.52	0.50	0.57	1.18	—	—
<i>Spies 6140</i>	1.01	—	0.89	1.14	0.61	1.50	0.64	1.04	—	—	1.32	1.10	1.15	0.92	0.59	0.69	1.00	0.92	—	—
<i>Spies 6285</i>	1.43	—	0.56	0.85	0.94	2.35	0.35	0.52	—	—	0.61	0.81	2.40	0.86	0.33	0.38	0.38	0.56	—	—
<i>Spies 6227</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6145</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6288</i>	1.66	—	0.64	0.54	1.16	1.45	0.24	0.72	—	—	0.59	0.65	1.10	0.53	0.38	0.18	0.62	0.54	—	—
<i>Spies 6257</i>	2.40	—	0.57	0.58	0.86	2.20	0.35	0.64	—	—	0.76	0.88	1.15	0.30	0.33	0.47	0.38	0.58	—	—
<i>Spies 6241</i>	1.66	—	0.46	0.69	0.69	1.04	0.72	0.41	—	—	0.37	0.83	1.50	0.35	1.07	0.79	0.84	0.54	—	—
<i>Spies 6244</i>	2.48	—	0.62	0.66	0.78	1.61	0.49	1.50	—	—	0.58	0.62	1.25	0.52	0.46	0.75	0.80	0.99	—	—
<i>Spies 6575</i>	1.79	—	0.62	0.20	0.78	2.30	0.49	0.94	—	—	0.58	0.62	1.25	0.62	0.38	0.64	0.51	0.41	—	—
<i>Spies 6574</i>	2.35	—	0.64	0.88	2.08	2.14	0.50	0.50	—	—	0.73	1.06	0.81	0.27	0.56	0.34	0.43	0.69	—	—
<i>Spies 6573</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Appendix M (continued). Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 3. Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	Spies 6252	Spies 6256	Spies 6072	Spies 6154	Spies 6225	Spies 6160	Spies 6166	Spies 6235	Spies 6316	Spies 6140	Spies 6285	Spies 6227	Spies 6145	Spies 6288	Spies 6257	Spies 6241	Spies 6244	Spies 6575	Spies 6574	Spies 6573
Spies 6084	0.18	0.18	0.24	0.09	0.10	0.14	0.24	0.15	0.26	0.36	0.24	—	—	0.19	0.09	0.19	0.08	0.17	0.10	—
Spies 5967	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Spies 6106	0.67	0.56	0.48	0.50	0.70	0.74	0.48	0.55	0.50	0.41	0.57	—	—	0.53	0.56	0.63	0.54	0.54	0.53	—
Spies 6240	0.64	0.64	0.43	0.62	0.61	0.50	0.43	0.60	0.38	0.32	0.43	—	—	0.58	0.56	0.50	0.52	0.81	0.42	—
Spies 6025	0.24	0.36	0.50	0.59	0.39	0.45	0.39	0.53	0.35	0.55	0.39	—	—	0.31	0.42	0.50	0.46	0.46	0.13	—
Spies 6096	0.33	0.22	0.29	0.21	0.25	0.24	0.10	0.17	0.21	0.22	0.10	—	—	0.24	0.11	0.35	0.20	0.10	0.12	—
Spies 6203	0.88	0.76	0.59	0.69	0.75	0.63	0.38	0.67	0.51	0.53	0.70	—	—	0.79	0.71	0.48	0.61	0.61	0.61	—
Spies 6085	0.59	0.59	0.54	0.40	0.50	0.54	0.49	0.56	0.63	0.35	0.59	—	—	0.48	0.53	0.67	0.22	0.39	0.61	—
Spies 6201	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Spies 6291	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Spies 6181	0.67	0.67	0.61	0.52	0.43	0.65	0.18	0.57	0.45	0.27	0.55	—	—	0.55	0.47	0.69	0.56	0.58	0.48	—
Spies 6249	0.42	0.50	0.44	0.48	0.18	0.32	0.15	0.41	0.40	0.33	0.44	—	—	0.52	0.42	0.43	0.54	0.54	0.35	—
Spies 5976	0.42	0.32	0.27	0.90	0.24	0.31	0.09	0.17	0.30	0.32	0.09	—	—	0.33	0.32	0.22	0.29	0.29	0.44	—
Spies 6237	0.57	0.69	0.68	0.72	0.48	0.71	0.42	0.70	0.67	0.40	0.42	—	—	0.59	0.74	0.71	0.59	0.54	0.76	—
Spies 6047	0.56	0.56	0.26	0.65	0.59	0.65	0.26	0.54	0.59	0.56	0.72	—	—	0.69	0.72	0.34	0.63	0.68	0.57	—
Spies 6286	0.63	0.56	0.40	0.48	0.53	0.51	0.46	0.54	0.61	0.50	0.69	—	—	0.84	0.63	0.45	0.47	0.53	0.71	—
Spies 6061	0.63	0.66	0.49	0.62	0.44	0.67	0.63	0.56	0.59	0.37	0.68	—	—	0.54	0.68	0.43	0.45	0.60	0.65	—
Spies 6254	0.72	0.64	0.64	0.54	0.70	0.94	0.50	0.80	0.31	0.40	0.57	—	—	0.58	0.56	0.58	0.37	0.67	0.50	—
Spies 6101	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Spies 6213	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Spies 6252	—	0.93	0.67	0.58	0.93	0.76	0.48	0.74	0.45	0.47	0.73	—	—	0.83	0.93	0.76	0.63	0.88	0.76	—
Spies 6256	0.07	—	0.67	0.58	0.71	0.59	0.30	0.69	0.52	0.27	0.79	—	—	0.62	0.80	0.55	0.56	0.75	0.76	—
Spies 6072	0.41	0.41	—	0.47	0.52	0.55	0.56	0.63	0.47	0.36	0.33	—	—	0.56	0.61	0.69	0.69	0.63	0.50	—
Spies 6154	0.54	0.54	0.75	—	0.41	0.68	0.35	0.61	0.66	0.26	0.47	—	—	0.60	0.52	0.53	0.61	0.61	0.53	—
Spies 6225	0.07	0.34	0.66	0.88	—	0.69	0.68	0.67	0.41	0.43	0.65	—	—	0.74	0.86	0.74	0.47	0.53	0.52	—
Spies 6160	0.28	0.52	0.60	0.38	0.38	—	0.60	0.71	0.42	0.27	0.55	—	—	0.61	0.54	0.67	0.56	0.67	0.67	—
Spies 6166	0.72	1.19	0.59	1.04	0.54	0.51	—	0.63	0.35	0.24	0.39	—	—	0.50	0.48	0.50	0.34	0.46	0.44	—
Spies 6235	0.30	0.38	0.46	0.49	0.41	0.34	0.46	—	0.44	0.46	0.63	—	—	0.71	0.69	0.76	0.59	0.70	0.65	—
Spies 6316	0.79	0.66	0.75	0.58	0.88	0.86	1.04	0.81	—	0.52	0.53	—	—	0.67	0.52	0.40	0.42	0.18	0.53	—
Spies 6140	0.76	1.32	1.01	1.35	0.85	1.31	1.42	0.78	0.66	—	0.55	—	—	0.34	0.53	0.41	0.38	0.38	0.28	—
Spies 6285	0.32	0.24	1.10	0.75	0.44	0.60	0.94	0.46	0.64	0.61	—	—	—	0.69	0.87	0.44	0.40	0.63	0.58	—
Spies 6227	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Spies 6145	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Spies 6288	0.19	0.48	0.58	0.51	0.30	0.49	0.69	0.35	0.41	1.06	0.37	—	—	—	0.62	0.50	0.58	0.65	0.79	—
Spies 6257	0.07	0.22	0.50	0.66	0.15	0.62	0.72	0.38	0.66	0.63	0.41	—	—	0.48	—	0.69	0.56	0.75	0.76	—
Spies 6241	0.28	0.59	0.37	0.63	0.30	0.41	0.69	0.27	0.92	0.88	0.83	—	—	0.69	0.37	—	0.52	0.58	0.50	—
Spies 6244	0.47	0.58	0.38	0.50	0.76	0.57	1.07	0.52	0.86	0.98	0.92	—	—	0.54	0.58	0.66	—	0.76	0.45	—
Spies 6575	0.13	0.29	0.46	0.50	0.63	0.41	0.78	0.35	1.70	0.98	0.46	—	—	0.44	0.29	0.54	0.27	—	0.52	—
Spies 6574	0.28	0.28	0.69	0.63	0.66	0.41	0.83	0.44	0.63	1.29	0.58	—	—	0.24	0.28	0.69	0.79	0.66	—	—
Spies 6573	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Appendix N. Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 4.

Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies 6084</i>	<i>Spies 5967</i>	<i>Spies 6106</i>	<i>Spies 6240</i>	<i>Spies 6025</i>	<i>Spies 6096</i>	<i>Spies 6203</i>	<i>Spies 6085</i>	<i>Spies 6201</i>	<i>Spies 6291</i>	<i>Spies 6181</i>	<i>Spies 6249</i>	<i>Spies 5976</i>	<i>Spies 6237</i>	<i>Spies 6047</i>	<i>Spies 6286</i>	<i>Spies 6061</i>	<i>Spies 6254</i>	<i>Spies 6101</i>	<i>Spies 6213</i>	
<i>Spies 6084</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 5967</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6106</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6240</i>	—	—	—	—	0.52	—	—	—	0.42	—	0.46	0.38	0.50	0.00	—	—	0.30	0.34	0.41	0.67	
<i>Spies 6025</i>	—	—	—	0.66	—	—	—	—	0.54	—	0.69	0.37	0.52	0.32	—	—	0.47	0.63	0.63	0.61	
<i>Spies 6096</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6203</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6085</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6201</i>	—	—	—	0.86	0.62	—	—	—	—	0.45	0.47	0.47	0.27	—	—	—	0.54	0.60	0.65	0.64	
<i>Spies 6291</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6181</i>	—	—	—	0.77	0.87	—	—	—	0.80	—	—	0.62	0.38	0.22	—	—	0.62	0.58	0.58	0.63	
<i>Spies 6249</i>	—	—	—	1.10	0.99	—	—	—	0.75	—	0.49	—	0.58	0.13	—	—	0.44	0.48	0.48	0.33	
<i>Spies 5976</i>	—	—	—	0.69	0.06	—	—	—	0.75	—	0.96	0.54	—	0.25	—	—	0.30	0.48	0.34	0.33	
<i>Spies 6237</i>	—	—	—	#	1.15	—	—	—	1.32	—	1.50	2.08	1.39	—	—	—	0.21	0.19	0.38	0.18	
<i>Spies 6047</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6286</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6061</i>	—	—	—	1.22	0.76	—	—	—	0.62	—	0.48	0.81	1.22	1.56	—	—	—	0.81	0.69	0.55	
<i>Spies 6254</i>	—	—	—	1.06	0.47	—	—	—	0.60	—	0.54	0.73	0.73	1.66	—	—	0.21	—	0.71	0.51	
<i>Spies 6101</i>	—	—	—	0.88	0.47	—	—	—	0.43	—	0.54	0.73	1.06	0.97	—	—	0.37	0.35	—	0.63	
<i>Spies 6213</i>	—	—	—	0.41	0.50	—	—	—	0.45	—	0.47	1.10	1.10	1.70	—	—	0.61	0.66	0.46	—	
<i>Spies 6252</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6256</i>	—	—	—	1.45	2.30	—	—	—	2.05	—	1.56	1.04	1.04	#	—	—	1.20	1.30	2.40	2.44	
<i>Spies 6072</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6154</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6225</i>	—	—	—	0.74	1.10	—	—	—	1.25	—	1.06	1.25	1.66	#	—	—	1.10	1.18	1.18	0.99	
<i>Spies 6160</i>	—	—	—	1.25	0.54	—	—	—	1.07	—	0.50	0.97	1.25	1.87	—	—	1.10	0.96	1.18	0.99	
<i>Spies 6166</i>	—	—	—	1.04	0.52	—	—	—	0.47	—	0.49	0.89	0.89	1.47	—	—	0.52	0.49	0.57	0.51	
<i>Spies 6235</i>	—	—	—	1.45	0.92	—	—	—	1.35	—	0.86	1.04	1.04	1.50	—	—	0.92	1.01	1.30	0.83	
<i>Spies 6316</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6140</i>	—	—	—	1.50	0.56	—	—	—	1.39	—	0.92	1.10	1.10	#	—	—	0.97	1.06	1.06	0.88	
<i>Spies 6285</i>	—	—	—	0.92	0.69	—	—	—	0.67	—	0.41	0.92	1.14	1.04	—	—	0.44	0.63	0.41	0.44	
<i>Spies 6227</i>	—	—	—	1.50	1.25	—	—	—	1.39	—	1.20	1.10	1.50	1.61	—	—	0.97	1.34	0.83	1.10	
<i>Spies 6145</i>	—	—	—	2.08	2.25	—	—	—	1.61	—	2.20	1.39	0.98	0.69	—	—	2.25	1.66	1.66	2.40	
<i>Spies 6288</i>	—	—	—	0.79	0.64	—	—	—	0.34	—	0.61	0.79	0.79	1.06	—	—	0.53	0.59	0.25	0.43	
<i>Spies 6257</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6241</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6244</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6575</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6574</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>Spies 6573</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	

Appendix N (continued). Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 4. Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies</i> 6252	<i>Spies</i> 6256	<i>Spies</i> 6072	<i>Spies</i> 6154	<i>Spies</i> 6225	<i>Spies</i> 6160	<i>Spies</i> 6166	<i>Spies</i> 6235	<i>Spies</i> 6316	<i>Spies</i> 6140	<i>Spies</i> 6285	<i>Spies</i> 6227	<i>Spies</i> 6145	<i>Spies</i> 6288	<i>Spies</i> 6257	<i>Spies</i> 6241	<i>Spies</i> 6244	<i>Spies</i> 6575	<i>Spies</i> 6574	<i>Spies</i> 6573
<i>Spies</i> 6084	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 5967	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6106	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6240	—	0.24	—	—	0.40	0.29	0.35	0.24	—	0.22	0.40	0.22	0.13	0.45	—	—	—	—	—	—
<i>Spies</i> 6025	—	0.10	—	—	0.33	0.58	0.59	0.40	—	0.57	0.50	0.29	0.11	0.53	—	—	—	—	—	—
<i>Spies</i> 6096	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6203	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6085	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6201	—	0.13	—	—	0.29	0.34	0.63	0.26	—	0.25	0.51	0.25	0.20	0.71	—	—	—	—	—	—
<i>Spies</i> 6291	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6181	—	0.21	—	—	0.35	0.61	0.61	0.42	—	0.40	0.67	0.30	0.11	0.55	—	—	—	—	—	—
<i>Spies</i> 6249	—	0.35	—	—	0.29	0.38	0.41	0.35	—	0.33	0.40	0.33	0.25	0.45	—	—	—	—	—	—
<i>Spies</i> 5976	—	0.35	—	—	0.19	0.29	0.41	0.35	—	0.33	0.32	0.22	0.38	0.45	—	—	—	—	—	—
<i>Spies</i> 6237	—	0.00	—	—	0.00	0.15	0.23	0.22	—	0.00	0.35	0.20	0.50	0.35	—	—	—	—	—	—
<i>Spies</i> 6047	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6286	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6061	—	0.30	—	—	0.33	0.33	0.59	0.40	—	0.38	0.64	0.38	0.11	0.59	—	—	—	—	—	—
<i>Spies</i> 6254	—	0.27	—	—	0.31	0.38	0.62	0.36	—	0.35	0.53	0.26	0.19	0.56	—	—	—	—	—	—
<i>Spies</i> 6101	—	0.09	—	—	0.31	0.31	0.56	0.27	—	0.35	0.67	0.43	0.19	0.78	—	—	—	—	—	—
<i>Spies</i> 6213	—	0.09	—	—	0.37	0.37	0.60	0.43	—	0.42	0.65	0.33	0.09	0.65	—	—	—	—	—	—
<i>Spies</i> 6252	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6256	—	—	—	—	0.14	0.14	0.37	0.20	—	0.00	0.22	0.00	0.22	0.17	—	—	—	—	—	—
<i>Spies</i> 6072	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6154	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6225	—	1.95	—	—	—	0.44	0.32	0.29	—	0.27	0.18	0.40	0.15	0.29	—	—	—	—	—	—
<i>Spies</i> 6160	—	1.95	—	—	0.81	—	0.45	0.29	—	0.27	0.27	0.40	0.00	0.29	—	—	—	—	—	—
<i>Spies</i> 6166	—	0.99	—	—	1.31	0.79	—	0.37	—	0.36	0.57	0.21	0.15	0.54	—	—	—	—	—	—
<i>Spies</i> 6235	—	1.61	—	—	1.25	1.25	0.99	—	—	0.55	0.33	0.18	0.22	0.33	—	—	—	—	—	—
<i>Spies</i> 6316	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6140	—	#	—	—	1.32	1.32	1.03	0.61	—	—	0.32	0.33	0.00	0.24	—	—	—	—	—	—
<i>Spies</i> 6285	—	1.50	—	—	1.70	1.30	0.56	1.10	—	1.15	—	0.21	0.24	0.69	—	—	—	—	—	—
<i>Spies</i> 6227	—	#	—	—	0.92	0.92	1.54	1.70	—	1.10	1.56	—	0.40	0.40	—	—	—	—	—	—
<i>Spies</i> 6145	—	1.50	—	—	1.87	#	1.87	1.50	—	3	1.45	0.92	—	0.35	—	—	—	—	—	—
<i>Spies</i> 6288	—	1.79	—	—	1.25	1.25	0.62	1.10	—	1.43	0.37	0.92	1.06	—	—	—	—	—	—	—
<i>Spies</i> 6257	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6241	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6244	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6575	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6574	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6573	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Appendix O. Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 5.

Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies 6084</i>	<i>Spies 5967</i>	<i>Spies 6106</i>	<i>Spies 6240</i>	<i>Spies 6025</i>	<i>Spies 6096</i>	<i>Spies 6203</i>	<i>Spies 6085</i>	<i>Spies 6201</i>	<i>Spies 6291</i>	<i>Spies 6181</i>	<i>Spies 6249</i>	<i>Spies 5976</i>	<i>Spies 6237</i>	<i>Spies 6047</i>	<i>Spies 6286</i>	<i>Spies 6061</i>	<i>Spies 6254</i>	<i>Spies 6101</i>	<i>Spies 6213</i>
<i>Spies 6084</i>		0.38	0.57	0.53	0.42	0.20	0.33	—	—	0.35	0.31	0.36	0.37	0.42	0.21	0.30	0.40	0.32	0.19	0.31
<i>Spies 5967</i>	0.98		0.43	0.32	0.42	0.30	0.22	—	—	0.43	0.15	0.24	0.15	0.25	0.21	0.10	0.32	0.32	0.48	0.23
<i>Spies 6106</i>	0.56	0.85		0.35	0.55	0.44	0.38	—	—	0.29	0.25	0.32	0.32	0.27	0.24	0.22	0.26	0.24	0.21	0.25
<i>Spies 6240</i>	0.64	1.15	1.04		0.44	0.09	0.19	—	—	0.31	0.48	0.44	0.40	0.37	0.27	0.35	0.36	0.27	0.33	0.28
<i>Spies 6025</i>	0.88	0.88	0.61	0.81		0.36	0.38	—	—	0.32	0.59	0.49	0.40	0.44	0.44	0.43	0.42	0.30	0.48	0.41
<i>Spies 6096</i>	1.61	1.20	0.81	2.44	1.03		0.64	—	—	0.37	0.40	0.32	0.52	0.36	0.43	0.42	0.34	0.52	0.48	0.27
<i>Spies 6203</i>	1.10	1.50	0.98	1.66	0.96	0.45		—	—	0.32	0.36	0.29	0.34	0.31	0.38	0.36	0.37	0.48	0.43	0.36
<i>Spies 6085</i>	—	—	—	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6201</i>	—	—	—	—	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6291</i>	1.06	0.83	1.25	1.18	1.13	0.99	1.14	—	—		0.36	0.35	0.41	0.52	0.38	0.30	0.62	0.54	0.50	0.42
<i>Spies 6181</i>	1.18	1.87	1.39	0.73	0.53	0.92	1.03	—	—	1.01		0.47	0.59	0.59	0.48	0.60	0.51	0.34	0.31	0.44
<i>Spies 6249</i>	1.01	1.42	1.13	0.81	0.72	1.13	1.25	—	—	1.05	0.77		0.50	0.59	0.17	0.32	0.38	0.17	0.21	0.51
<i>Spies 5976</i>	0.99	1.91	1.14	0.92	0.92	0.66	1.06	—	—	0.89	0.52	0.69		0.74	0.27	0.39	0.39	0.40	0.38	0.43
<i>Spies 6237</i>	0.88	1.39	1.30	0.99	0.83	1.03	1.18	—	—	0.66	0.53	0.54	0.30		0.22	0.36	0.36	0.37	0.41	0.35
<i>Spies 6047</i>	1.56	1.56	1.45	1.30	0.81	0.83	0.97	—	—	0.96	0.73	1.79	1.32	1.50		0.78	0.50	0.73	0.50	0.34
<i>Spies 6286</i>	1.20	2.30	1.50	1.06	0.85	0.88	1.01	—	—	1.22	0.51	1.13	0.95	1.03	0.25		0.55	0.52	0.32	0.27
<i>Spies 6061</i>	0.92	1.14	1.34	1.03	0.86	1.06	0.99	—	—	0.47	0.66	0.97	0.94	1.01	0.69	0.59		0.57	0.40	0.51
<i>Spies 6254</i>	1.15	1.15	1.45	1.30	1.22	0.65	0.74	—	—	0.62	1.06	1.79	0.92	0.99	0.32	0.65	0.56		0.67	0.41
<i>Spies 6101</i>	1.66	0.74	1.56	1.10	0.73	0.73	0.83	—	—	0.69	1.13	1.56	0.98	0.88	0.69	1.14	0.92	0.41		0.19
<i>Spies 6213</i>	1.18	1.47	1.39	1.29	0.89	1.32	1.03	—	—	0.86	0.81	0.67	0.84	1.04	1.06	1.32	0.66	0.88	1.64	
<i>Spies 6252</i>	2.25	1.56	#	1.30	0.81	1.06	1.25	—	—	1.87	0.59	1.28	1.61	1.50	0.79	0.83	1.54	1.01	1.10	1.29
<i>Spies 6256</i>	1.22	1.91	1.14	1.32	0.66	0.79	0.73	—	—	0.89	0.52	1.01	1.00	0.92	1.10	0.79	0.81	1.10	1.16	0.72
<i>Spies 6072</i>	1.50	1.91	1.43	1.61	1.07	0.79	0.59	—	—	0.89	0.62	0.61	0.86	0.78	1.10	0.79	0.94	1.10	1.16	0.97
<i>Spies 6154</i>	1.95	1.25	#	1.13	0.69	1.39	1.10	—	—	0.78	0.75	0.81	0.09	0.81	1.13	1.16	0.72	0.79	0.72	0.75
<i>Spies 6225</i>	1.29	1.29	1.50	1.39	1.13	0.86	1.13	—	—	0.41	0.57	1.06	0.60	0.72	0.98	0.86	0.46	0.58	0.89	0.67
<i>Spies 6160</i>	2.64	1.54	2.56	1.35	0.81	0.83	1.10	—	—	0.92	0.64	0.81	0.77	0.81	0.95	1.16	0.84	0.79	0.86	1.00
<i>Spies 6166</i>	0.88	1.29	1.22	1.39	0.72	0.72	0.79	—	—	0.49	0.89	0.65	0.80	0.72	0.83	1.01	0.64	0.58	0.64	0.89
<i>Spies 6235</i>	0.81	1.50	0.98	0.74	0.77	1.01	1.20	—	—	1.14	0.85	1.48	1.06	1.18	0.56	0.61	0.99	0.56	0.83	1.25
<i>Spies 6316</i>	1.15	2.25	1.45	0.79	0.99	1.06	1.25	—	—	1.87	0.59	1.50	1.10	1.50	0.79	0.65	1.03	0.79	0.88	1.29
<i>Spies 6140</i>	0.69	1.79	0.79	0.99	0.69	0.69	1.18	—	—	0.79	0.75	0.72	0.56	0.58	1.22	1.03	1.01	1.22	1.29	1.45
<i>Spies 6285</i>	1.43	1.43	1.75	1.54	1.42	0.59	1.22	—	—	1.16	1.07	0.97	0.81	1.01	1.03	0.88	0.64	0.85	1.10	1.49
<i>Spies 6227</i>	0.77	1.87	1.39	0.88	0.75	0.92	1.03	—	—	0.61	0.49	0.67	0.43	0.44	0.88	0.76	0.78	0.88	1.35	0.59
<i>Spies 6145</i>	0.92	1.32	1.25	0.88	0.46	0.75	0.98	—	—	0.72	0.29	0.52	0.72	0.64	0.61	0.75	0.57	0.72	0.92	0.60
<i>Spies 6288</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6257</i>	1.34	1.34	1.25	1.13	0.95	0.52	0.73	—	—	0.92	0.86	0.80	0.53	0.79	0.96	0.99	1.39	0.96	1.03	1.01
<i>Spies 6241</i>	1.18	0.96	1.39	1.06	0.89	0.51	0.56	—	—	0.86	0.61	0.87	0.84	1.04	0.73	0.76	0.78	0.73	0.66	0.69
<i>Spies 6244</i>	0.85	1.54	1.18	1.13	0.49	0.98	0.92	—	—	1.07	0.64	0.63	0.77	0.81	0.95	0.98	1.13	1.13	1.01	0.86
<i>Spies 6575</i>	1.06	1.75	1.66	0.77	0.79	1.22	0.92	—	—	1.61	0.72	0.69	1.04	0.95	0.62	0.52	0.98	0.98	1.25	1.19
<i>Spies 6574</i>	0.88	1.29	1.50	0.98	0.43	1.19	1.13	—	—	0.94	0.57	0.43	0.69	0.52	1.16	0.72	0.86	1.39	1.04	0.89
<i>Spies 6573</i>	0.88	1.29	1.22	0.98	0.62	1.01	0.95	—	—	0.81	0.57	0.43	0.92	0.62	1.16	0.72	0.64	0.96	0.89	0.89

Appendix O (continued). Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 5. Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies 6252</i>	<i>Spies 6256</i>	<i>Spies 6072</i>	<i>Spies 6154</i>	<i>Spies 6225</i>	<i>Spies 6160</i>	<i>Spies 6166</i>	<i>Spies 6235</i>	<i>Spies 6316</i>	<i>Spies 6140</i>	<i>Spies 6285</i>	<i>Spies 6227</i>	<i>Spies 6145</i>	<i>Spies 6288</i>	<i>Spies 6257</i>	<i>Spies 6241</i>	<i>Spies 6244</i>	<i>Spies 6575</i>	<i>Spies 6574</i>	<i>Spies 6573</i>
<i>Spies 6084</i>	0.11	0.30	0.22	0.14	0.28	0.07	0.41	0.44	0.32	0.50	0.24	0.46	0.40	—	0.26	0.31	0.43	0.35	0.41	0.41
<i>Spies 5967</i>	0.21	0.15	0.15	0.29	0.28	0.21	0.28	0.22	0.11	0.17	0.24	0.15	0.27	—	0.26	0.38	0.21	0.17	0.28	0.28
<i>Spies 6106</i>	0.00	0.32	0.24	0.00	0.22	0.08	0.30	0.38	0.24	0.45	0.17	0.25	0.29	—	0.29	0.25	0.31	0.19	0.22	0.30
<i>Spies 6240</i>	0.27	0.27	0.20	0.32	0.25	0.26	0.25	0.48	0.45	0.37	0.21	0.41	0.42	—	0.31	0.34	0.32	0.46	0.38	0.38
<i>Spies 6025</i>	0.44	0.51	0.34	0.50	0.32	0.44	0.49	0.46	0.37	0.50	0.24	0.47	0.63	—	0.39	0.41	0.61	0.45	0.65	0.54
<i>Spies 6096</i>	0.35	0.45	0.45	0.25	0.42	0.44	0.48	0.36	0.35	0.50	0.55	0.40	0.47	—	0.59	0.60	0.38	0.30	0.30	0.36
<i>Spies 6203</i>	0.29	0.48	0.55	0.33	0.32	0.33	0.45	0.30	0.29	0.31	0.30	0.36	0.38	—	0.48	0.57	0.40	0.40	0.32	0.39
<i>Spies 6085</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6201</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6291</i>	0.15	0.41	0.41	0.46	0.67	0.40	0.61	0.32	0.15	0.45	0.31	0.55	0.49	—	0.40	0.42	0.34	0.20	0.39	0.44
<i>Spies 6181</i>	0.55	0.59	0.54	0.47	0.56	0.53	0.41	0.43	0.55	0.47	0.34	0.61	0.75	—	0.42	0.44	0.53	0.48	0.56	0.56
<i>Spies 6249</i>	0.28	0.36	0.55	0.44	0.35	0.44	0.52	0.23	0.22	0.49	0.38	0.51	0.60	—	0.45	0.42	0.53	0.50	0.65	0.65
<i>Spies 5976</i>	0.20	0.37	0.42	0.41	0.55	0.46	0.45	0.34	0.33	0.59	0.44	0.65	0.49	—	0.59	0.43	0.46	0.35	0.50	0.40
<i>Spies 6237</i>	0.22	0.40	0.46	0.44	0.49	0.44	0.49	0.31	0.22	0.56	0.36	0.65	0.53	—	0.45	0.35	0.44	0.39	0.59	0.54
<i>Spies 6047</i>	0.45	0.33	0.33	0.32	0.38	0.39	0.44	0.57	0.45	0.30	0.36	0.41	0.55	—	0.38	0.48	0.39	0.54	0.31	0.31
<i>Spies 6286</i>	0.43	0.45	0.45	0.31	0.42	0.31	0.36	0.55	0.52	0.36	0.41	0.47	0.47	—	0.37	0.47	0.38	0.59	0.46	0.48
<i>Spies 6061</i>	0.21	0.44	0.39	0.49	0.63	0.43	0.53	0.37	0.36	0.36	0.53	0.46	0.56	—	0.25	0.46	0.32	0.38	0.42	0.53
<i>Spies 6254</i>	0.36	0.33	0.33	0.45	0.56	0.45	0.56	0.57	0.45	0.30	0.43	0.41	0.48	—	0.38	0.48	0.32	0.38	0.25	0.38
<i>Spies 6101</i>	0.33	0.31	0.31	0.48	0.41	0.42	0.53	0.43	0.52	0.28	0.33	0.26	0.40	—	0.36	0.52	0.36	0.29	0.35	0.41
<i>Spies 6213</i>	0.28	0.49	0.38	0.47	0.51	0.37	0.41	0.29	0.28	0.24	0.23	0.56	0.55	—	0.36	0.50	0.42	0.30	0.41	0.41
<i>Spies 6252</i>	—	0.53	0.40	0.45	0.31	0.45	0.44	0.48	0.55	0.30	0.36	0.34	0.55	—	0.21	0.41	0.45	0.62	0.50	0.38
<i>Spies 6256</i>	0.63	—	0.58	0.46	0.50	0.41	0.50	0.34	0.47	0.46	0.44	0.49	0.49	—	0.24	0.38	0.46	0.47	0.50	0.65
<i>Spies 6072</i>	0.92	0.55	—	0.46	0.60	0.41	0.55	0.21	0.40	0.34	0.39	0.43	0.49	—	0.41	0.43	0.46	0.41	0.45	0.50
<i>Spies 6154</i>	0.79	0.77	0.77	—	0.49	0.70	0.54	0.13	0.19	0.33	0.43	0.47	0.57	—	0.51	0.47	0.50	0.40	0.68	0.44
<i>Spies 6225</i>	1.16	0.69	0.51	0.72	—	0.54	0.57	0.39	0.50	0.32	0.37	0.51	0.47	—	0.39	0.46	0.44	0.39	0.43	0.57
<i>Spies 6160</i>	0.79	0.89	0.89	0.36	0.62	—	0.54	0.27	0.32	0.39	0.43	0.42	0.62	—	0.57	0.42	0.45	0.46	0.54	0.54
<i>Spies 6166</i>	0.83	0.69	0.60	0.62	0.56	0.62	—	0.45	0.31	0.49	0.47	0.41	0.65	—	0.50	0.56	0.59	0.44	0.62	0.62
<i>Spies 6235</i>	0.74	1.06	1.58	2.01	0.95	1.32	0.79	—	0.67	0.38	0.37	0.43	0.44	—	0.32	0.50	0.47	0.64	0.39	0.39
<i>Spies 6316</i>	0.61	0.76	0.92	1.64	0.69	1.13	1.16	0.41	—	0.30	0.29	0.28	0.36	—	0.23	0.41	0.45	0.62	0.31	0.50
<i>Spies 6140</i>	1.22	0.78	1.07	1.10	1.13	0.94	0.72	0.98	1.22	—	0.61	0.65	0.58	—	0.45	0.41	0.56	0.39	0.54	0.49
<i>Spies 6285</i>	1.03	0.81	0.94	0.84	1.00	0.84	0.75	0.99	1.25	0.50	—	0.34	0.46	—	0.31	0.46	0.38	0.38	0.42	0.37
<i>Spies 6227</i>	1.06	0.72	0.94	0.75	0.67	0.86	0.89	0.85	1.29	0.44	1.07	—	0.70	—	0.55	0.50	0.53	0.42	0.62	0.56
<i>Spies 6145</i>	0.61	0.72	0.72	0.56	0.77	0.48	0.43	0.83	1.01	0.55	0.77	0.36	—	—	0.59	0.60	0.67	0.59	0.65	0.56
<i>Spies 6288</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6257</i>	1.18	1.45	0.89	0.66	0.94	0.56	0.69	1.14	1.47	0.79	1.16	0.61	0.52	—	—	0.73	0.63	0.73	0.61	0.39
<i>Spies 6241</i>	0.88	0.97	0.84	0.75	0.77	0.86	0.57	0.69	0.88	0.89	0.78	0.69	0.51	—	0.32	—	0.63	0.61	0.62	0.46
<i>Spies 6244</i>	0.79	0.77	0.77	0.69	0.92	0.80	0.54	0.76	0.79	0.59	0.97	0.64	0.41	—	0.46	0.46	—	0.63	0.73	0.59
<i>Spies 6575</i>	0.49	0.75	0.89	0.92	0.94	0.78	0.61	0.45	0.49	0.95	0.98	0.86	0.52	—	0.31	0.50	0.46	—	0.61	0.50
<i>Spies 6574</i>	0.68	0.69	0.80	0.38	0.85	0.62	0.48	0.95	1.16	0.62	0.86	0.49	0.43	—	0.49	0.49	0.31	0.49	—	0.67
<i>Spies 6573</i>	0.98	0.43	0.69	0.82	0.56	0.62	0.48	0.95	0.69	0.72	1.00	0.57	0.58	—	0.94	0.77	0.64	0.69	0.41	—

Appendix P. Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 6.

Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies 6084</i>	<i>Spies 5967</i>	<i>Spies 6106</i>	<i>Spies 6240</i>	<i>Spies 6025</i>	<i>Spies 6096</i>	<i>Spies 6203</i>	<i>Spies 6085</i>	<i>Spies 6201</i>	<i>Spies 6291</i>	<i>Spies 6181</i>	<i>Spies 6249</i>	<i>Spies 5976</i>	<i>Spies 6237</i>	<i>Spies 6047</i>	<i>Spies 6286</i>	<i>Spies 6061</i>	<i>Spies 6254</i>	<i>Spies 6101</i>	<i>Spies 6213</i>
<i>Spies 6084</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 5967</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6106</i>	—	—	—	0.83	0.43	0.35	—	0.52	0.48	0.73	0.77	0.62	0.57	0.40	0.24	0.24	0.32	0.48	0.64	0.45
<i>Spies 6240</i>	—	—	0.19	—	0.33	0.33	—	0.50	0.52	0.71	0.74	0.59	0.64	0.38	0.11	0.11	0.30	0.54	0.46	0.43
<i>Spies 6025</i>	—	—	0.83	1.10	—	0.33	—	0.44	0.57	0.48	0.48	0.38	0.13	0.00	0.17	0.17	0.29	0.30	0.30	0.35
<i>Spies 6096</i>	—	—	1.06	1.10	1.10	—	—	0.33	0.19	0.32	0.29	0.48	0.38	0.13	0.00	0.17	0.29	0.29	0.50	0.30
<i>Spies 6203</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6085</i>	—	—	0.65	0.69	0.81	1.10	—	—	0.29	0.48	0.57	0.78	0.39	0.27	0.00	0.17	0.29	0.40	0.50	0.00
<i>Spies 6201</i>	—	—	0.77	0.66	0.56	1.66	—	1.25	—	0.57	0.33	0.17	0.32	0.22	0.13	0.13	0.24	0.43	0.17	0.50
<i>Spies 6291</i>	—	—	0.31	0.34	0.73	1.14	—	0.73	0.56	—	0.57	0.64	0.43	0.36	0.21	0.11	0.38	0.59	0.44	0.42
<i>Spies 6181</i>	—	—	0.26	0.30	0.74	1.25	—	0.56	1.10	0.56	—	0.58	0.42	0.33	0.13	0.13	0.35	0.43	0.61	0.50
<i>Spies 6249</i>	—	—	0.49	0.52	0.97	0.74	—	0.27	1.79	0.44	0.54	—	0.42	0.22	0.13	0.27	0.35	0.43	0.61	0.10
<i>Spies 5976</i>	—	—	0.56	0.45	2.08	0.98	—	0.98	1.15	0.83	0.86	0.86	—	0.46	0.20	0.20	0.33	0.56	0.56	0.27
<i>Spies 6237</i>	—	—	0.92	0.97	#	2.01	—	1.32	1.50	1.01	1.10	1.50	0.77	—	0.00	0.00	0.18	0.47	0.47	0.14
<i>Spies 6047</i>	—	—	1.45	2.20	1.79	#	—	#	2.01	1.56	2.01	2.01	1.61	#	—	0.67	0.25	0.14	0.29	0.55
<i>Spies 6286</i>	—	—	1.45	2.20	1.79	1.79	—	1.79	2.01	2.25	2.01	1.32	1.61	#	0.41	—	0.50	0.29	0.29	0.36
<i>Spies 6061</i>	—	—	1.15	1.20	1.25	1.25	—	1.25	1.45	0.97	1.04	1.04	1.10	1.70	1.39	0.69	—	0.63	0.25	0.31
<i>Spies 6254</i>	—	—	0.73	0.62	1.20	0.69	—	0.92	0.83	0.52	0.83	0.83	0.59	0.75	1.95	1.25	0.47	—	0.36	0.32
<i>Spies 6101</i>	—	—	0.45	0.77	1.20	1.20	—	0.69	1.75	0.81	0.50	0.50	0.59	0.75	1.25	1.25	1.39	1.01	—	0.32
<i>Spies 6213</i>	—	—	0.79	0.83	1.04	#	—	#	0.69	0.88	0.69	2.30	1.32	1.95	0.61	1.01	1.18	1.15	1.15	—
<i>Spies 6252</i>	—	—	0.92	1.25	#	1.32	—	1.32	1.50	1.01	1.10	1.10	1.18	1.10	1.50	1.50	1.01	1.45	0.75	1.95
<i>Spies 6256</i>	—	—	0.52	0.69	1.01	1.01	—	0.45	1.14	0.59	0.73	0.45	0.92	0.86	1.38	0.98	0.81	0.69	0.54	1.66
<i>Spies 6072</i>	—	—	0.45	0.65	1.04	2.14	—	1.04	0.92	0.69	0.89	0.92	0.63	0.85	1.01	1.01	0.49	0.86	0.46	0.69
<i>Spies 6154</i>	—	—	0.45	0.26	0.92	1.20	—	0.92	0.50	0.41	0.50	0.83	0.81	1.05	1.95	1.95	0.96	0.61	1.30	0.86
<i>Spies 6225</i>	—	—	1.06	0.92	0.88	0.88	—	0.88	0.99	0.66	1.22	0.81	1.01	1.26	2.20	1.50	0.92	0.62	0.77	1.34
<i>Spies 6160</i>	—	—	2.14	2.20	#	#	—	#	2.01	2.25	2.01	#	1.61	0.81	#	#	1.39	1.95	1.95	1.70
<i>Spies 6166</i>	—	—	1.10	0.86	1.87	#	—	1.18	1.39	1.20	0.98	1.39	1.01	0.92	#	#	1.50	2.01	0.92	1.10
<i>Spies 6235</i>	—	—	0.50	0.69	1.50	2.20	—	0.81	1.66	0.92	0.74	0.74	0.69	0.63	1.10	1.10	1.95	0.92	0.36	1.04
<i>Spies 6316</i>	—	—	1.39	0.92	1.15	2.25	—	1.56	1.01	1.18	1.30	1.30	1.04	0.98	1.87	1.87	1.32	1.25	0.74	1.10
<i>Spies 6140</i>	—	—	1.20	0.97	2.01	2.01	—	0.92	2.20	1.30	1.50	1.10	1.18	1.10	#	#	1.70	1.45	0.75	#
<i>Spies 6285</i>	—	—	0.74	0.61	#	2.08	—	1.39	1.56	0.83	0.86	1.15	0.85	0.49	1.61	1.61	1.79	0.81	1.10	1.32
<i>Spies 6227</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6145</i>	—	—	0.79	1.06	1.04	1.45	—	2.14	0.92	0.88	1.20	2.30	1.32	1.25	1.01	1.70	1.18	0.86	1.15	0.69
<i>Spies 6288</i>	—	—	0.66	0.85	1.30	1.01	—	1.30	0.73	0.48	0.92	1.14	1.61	0.86	2.08	#	1.50	1.10	0.88	1.25
<i>Spies 6257</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6241</i>	—	—	0.63	0.54	1.14	0.92	—	0.92	1.54	0.47	0.69	0.56	1.06	1.01	1.56	1.56	0.97	0.52	0.66	0.88
<i>Spies 6244</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6575</i>	—	—	0.59	0.51	1.10	1.10	—	1.10	0.52	0.66	0.81	0.99	1.01	0.97	#	#	1.20	0.77	0.96	1.34
<i>Spies 6574</i>	—	—	0.37	0.41	0.69	1.39	—	0.88	0.81	0.44	0.41	0.66	1.01	0.74	1.50	1.50	0.92	0.49	0.62	0.65
<i>Spies 6573</i>	—	—	0.48	0.31	0.69	1.39	—	0.88	0.99	0.34	0.52	0.52	1.30	1.66	1.50	2.20	1.20	0.96	0.77	0.83

Appendix P (continued). Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 6. Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies 6252</i>	<i>Spies 6256</i>	<i>Spies 6072</i>	<i>Spies 6154</i>	<i>Spies 6225</i>	<i>Spies 6160</i>	<i>Spies 6166</i>	<i>Spies 6235</i>	<i>Spies 6316</i>	<i>Spies 6140</i>	<i>Spies 6285</i>	<i>Spies 6227</i>	<i>Spies 6145</i>	<i>Spies 6288</i>	<i>Spies 6257</i>	<i>Spies 6241</i>	<i>Spies 6244</i>	<i>Spies 6575</i>	<i>Spies 6574</i>	<i>Spies 6573</i>
<i>Spies 6084</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 5967</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6106</i>	0.40	0.59	0.64	0.64	0.34	0.12	0.33	0.61	0.25	0.30	0.48	—	0.45	0.52	—	0.53	—	0.56	0.69	0.62
<i>Spies 6240</i>	0.29	0.50	0.52	0.77	0.40	0.11	0.42	0.50	0.40	0.38	0.55	—	0.35	0.43	—	0.58	—	0.60	0.67	0.73
<i>Spies 6025</i>	0.00	0.36	0.35	0.40	0.42	0.00	0.15	0.22	0.32	0.13	0.00	—	0.35	0.27	—	0.32	—	0.33	0.50	0.50
<i>Spies 6096</i>	0.27	0.36	0.12	0.30	0.42	0.00	0.00	0.11	0.11	0.13	0.13	—	0.24	0.36	—	0.40	—	0.33	0.25	0.25
<i>Spies 6203</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6085</i>	0.27	0.64	0.35	0.40	0.42	0.00	0.31	0.44	0.21	0.40	0.25	—	0.12	0.27	—	0.40	—	0.33	0.42	0.42
<i>Spies 6201</i>	0.22	0.32	0.40	0.61	0.37	0.13	0.25	0.19	0.36	0.11	0.21	—	0.40	0.48	—	0.21	—	0.59	0.44	0.37
<i>Spies 6291</i>	0.36	0.55	0.50	0.67	0.52	0.11	0.30	0.40	0.31	0.27	0.43	—	0.42	0.62	—	0.63	—	0.52	0.65	0.71
<i>Spies 6181</i>	0.33	0.48	0.50	0.61	0.30	0.13	0.39	0.48	0.27	0.22	0.42	—	0.30	0.40	—	0.50	—	0.44	0.67	0.59
<i>Spies 6249</i>	0.33	0.64	0.40	0.43	0.44	0.00	0.25	0.48	0.27	0.33	0.32	—	0.10	0.32	—	0.57	—	0.37	0.52	0.59
<i>Spies 5976</i>	0.31	0.40	0.53	0.44	0.36	0.20	0.36	0.50	0.35	0.31	0.43	—	0.27	0.20	—	0.35	—	0.36	0.36	0.27
<i>Spies 6237</i>	0.33	0.42	0.43	0.35	0.29	0.44	0.40	0.53	0.39	0.33	0.62	—	0.29	0.42	—	0.36	—	0.38	0.48	0.19
<i>Spies 6047</i>	0.22	0.25	0.36	0.14	0.11	0.00	0.00	0.33	0.15	0.00	0.20	—	0.36	0.13	—	0.21	—	0.00	0.22	0.22
<i>Spies 6286</i>	0.22	0.38	0.36	0.14	0.20	0.00	0.00	0.33	0.15	0.00	0.20	—	0.18	0.00	—	0.21	—	0.00	0.22	0.11
<i>Spies 6061</i>	0.36	0.44	0.62	0.39	0.40	0.25	0.22	0.14	0.27	0.18	0.17	—	0.31	0.22	—	0.38	—	0.30	0.40	0.30
<i>Spies 6254</i>	0.24	0.50	0.42	0.55	0.54	0.14	0.13	0.40	0.29	0.24	0.44	—	0.42	0.33	—	0.59	—	0.46	0.62	0.38
<i>Spies 6101</i>	0.47	0.58	0.63	0.27	0.46	0.14	0.40	0.70	0.48	0.47	0.33	—	0.32	0.42	—	0.52	—	0.38	0.54	0.46
<i>Spies 6213</i>	0.14	0.19	0.50	0.42	0.26	0.18	0.33	0.35	0.33	0.00	0.27	—	0.50	0.29	—	0.42	—	0.26	0.52	0.43
<i>Spies 6252</i>	—	0.53	0.57	0.35	0.38	0.22	0.40	0.40	0.36	0.33	0.31	—	0.00	0.53	—	0.18	—	0.38	0.38	0.29
<i>Spies 6256</i>	0.64	—	0.67	0.50	0.64	0.25	0.24	0.73	0.43	0.53	0.40	—	0.29	0.46	—	0.48	—	0.36	0.71	0.50
<i>Spies 6072</i>	0.56	0.41	—	0.42	0.43	0.36	0.67	0.59	0.44	0.43	0.27	—	0.38	0.38	—	0.42	—	0.43	0.61	0.43
<i>Spies 6154</i>	1.04	0.69	0.86	—	0.38	0.14	0.27	0.40	0.40	0.24	0.56	—	0.32	0.50	—	0.30	—	0.46	0.54	0.62
<i>Spies 6225</i>	0.97	0.44	0.83	0.96	—	0.33	0.21	0.50	0.72	0.48	0.27	—	0.17	0.36	—	0.65	—	0.33	0.53	0.47
<i>Spies 6160</i>	1.50	1.39	1.01	1.95	1.10	—	0.29	0.33	0.46	0.22	0.00	—	0.00	0.13	—	0.21	—	0.11	0.22	0.00
<i>Spies 6166</i>	0.92	1.45	0.41	1.32	1.56	1.25	—	0.46	0.29	0.40	0.18	—	0.17	0.35	—	0.30	—	0.32	0.32	0.32
<i>Spies 6235</i>	0.92	0.32	0.53	0.92	0.96	1.10	0.77	—	0.53	0.53	0.50	—	0.24	0.27	—	0.48	—	0.25	0.58	0.42
<i>Spies 6316</i>	0.98	0.83	0.81	0.74	0.33	0.77	1.25	0.64	—	0.50	0.35	—	0.11	0.35	—	0.46	—	0.40	0.40	0.48
<i>Spies 6140</i>	1.10	0.64	0.85	1.45	0.74	1.50	0.92	0.63	0.69	—	0.46	—	0.29	0.32	—	0.45	—	0.38	0.29	0.48
<i>Spies 6285</i>	1.19	0.92	1.32	0.59	1.30	#	1.70	0.69	1.04	0.77	—	—	0.40	0.40	—	0.43	—	0.36	0.36	0.45
<i>Spies 6227</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6145</i>	#	1.25	0.98	1.15	1.75	#	1.79	1.45	2.20	1.25	0.92	—	—	0.57	—	0.42	—	0.52	0.35	0.52
<i>Spies 6288</i>	0.64	0.77	0.97	0.69	1.03	2.08	1.04	1.30	1.06	1.15	0.92	—	0.56	—	—	0.41	—	0.64	0.43	0.64
<i>Spies 6257</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6241</i>	1.70	0.73	0.88	1.22	0.44	1.56	1.20	0.73	0.77	0.79	0.83	—	0.88	0.88	—	—	—	0.39	0.58	0.65
<i>Spies 6244</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6575</i>	0.97	1.03	0.83	0.77	1.10	2.20	1.15	1.39	0.92	0.97	1.01	—	0.85	0.44	—	0.95	—	—	0.47	0.60
<i>Spies 6574</i>	0.97	0.34	0.50	0.62	0.63	1.50	1.15	0.54	0.92	1.25	1.01	—	1.06	0.85	—	0.54	—	0.76	—	0.60
<i>Spies 6573</i>	1.25	0.69	0.83	0.49	0.76	#	1.15	0.88	0.73	0.74	0.79	—	0.65	0.44	—	0.44	—	0.51	0.51	—

Appendix Q. Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 9.

Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies 6084</i>	<i>Spies 5967</i>	<i>Spies 6106</i>	<i>Spies 6240</i>	<i>Spies 6025</i>	<i>Spies 6096</i>	<i>Spies 6203</i>	<i>Spies 6085</i>	<i>Spies 6201</i>	<i>Spies 6291</i>	<i>Spies 6181</i>	<i>Spies 6249</i>	<i>Spies 5976</i>	<i>Spies 6237</i>	<i>Spies 6047</i>	<i>Spies 6286</i>	<i>Spies 6061</i>	<i>Spies 6254</i>	<i>Spies 6101</i>	<i>Spies 6213</i>
<i>Spies 6084</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 5967</i>	—	—	0.63	0.56	0.59	—	—	0.41	0.58	0.38	0.53	—	—	0.54	0.63	0.55	0.64	0.65	0.54	0.39
<i>Spies 6106</i>	—	0.46	—	0.41	0.70	—	—	0.47	0.60	0.43	0.60	—	—	0.57	0.54	0.71	0.78	0.79	0.62	0.49
<i>Spies 6240</i>	—	0.57	0.89	—	0.55	—	—	0.50	0.79	0.63	0.59	—	—	0.65	0.51	0.56	0.51	0.48	0.50	0.56
<i>Spies 6025</i>	—	0.54	0.36	0.61	—	—	—	0.47	0.68	0.38	0.56	—	—	0.57	0.59	0.71	0.73	0.71	0.57	0.59
<i>Spies 6096</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6203</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6085</i>	—	0.88	0.75	0.69	0.75	—	—	—	0.42	0.56	0.45	—	—	0.60	0.40	0.55	0.54	0.50	0.33	0.41
<i>Spies 6201</i>	—	0.55	0.51	0.23	0.39	—	—	0.86	—	0.54	0.64	—	—	0.65	0.59	0.73	0.72	0.69	0.61	0.71
<i>Spies 6291</i>	—	0.98	0.84	0.46	0.97	—	—	0.58	0.62	—	0.59	—	—	0.48	0.36	0.61	0.50	0.46	0.30	0.50
<i>Spies 6181</i>	—	0.64	0.50	0.54	0.58	—	—	0.79	0.45	0.53	—	—	—	0.62	0.47	0.67	0.61	0.62	0.62	0.58
<i>Spies 6249</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 5976</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6237</i>	—	0.62	0.56	0.43	0.56	—	—	0.51	0.43	0.72	0.49	—	—	—	0.55	0.59	0.67	0.64	0.53	0.65
<i>Spies 6047</i>	—	0.47	0.62	0.66	0.52	—	—	0.92	0.54	1.03	0.75	—	—	0.61	—	0.67	0.60	0.62	0.61	0.44
<i>Spies 6286</i>	—	0.60	0.34	0.58	0.34	—	—	0.61	0.31	0.48	0.41	—	—	0.54	0.41	—	0.83	0.81	0.59	0.60
<i>Spies 6061</i>	—	0.45	0.25	0.67	0.31	—	—	0.62	0.33	0.69	0.50	—	—	0.41	0.51	0.19	—	0.98	0.62	0.55
<i>Spies 6254</i>	—	0.43	0.23	0.74	0.34	—	—	0.69	0.37	0.77	0.47	—	—	0.45	0.49	0.21	0.02	—	0.64	0.56
<i>Spies 6101</i>	—	0.62	0.48	0.69	0.56	—	—	1.10	0.50	1.19	0.49	—	—	0.64	0.50	0.54	0.47	0.45	—	0.32
<i>Spies 6213</i>	—	0.94	0.72	0.57	0.54	—	—	0.88	0.34	0.69	0.55	—	—	0.43	0.83	0.51	0.61	0.58	1.13	—
<i>Spies 6252</i>	—	0.82	0.50	0.45	0.43	—	—	0.64	0.39	0.72	0.43	—	—	0.41	0.72	0.47	0.37	0.41	0.48	0.46
<i>Spies 6256</i>	—	0.56	0.67	0.28	0.52	—	—	0.78	0.35	0.64	0.53	—	—	0.67	1.00	0.57	0.51	0.56	0.77	0.65
<i>Spies 6072</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6154</i>	—	0.72	0.85	0.29	0.48	—	—	0.76	0.36	1.01	0.41	—	—	0.46	0.61	0.62	0.55	0.53	0.55	0.52
<i>Spies 6225</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6160</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6166</i>	—	0.50	0.64	0.81	1.15	—	—	0.77	0.65	1.06	0.78	—	—	0.64	0.59	0.84	0.54	0.60	0.89	0.86
<i>Spies 6235</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6316</i>	—	0.66	0.69	0.89	0.59	—	—	1.10	0.80	1.22	1.42	—	—	0.83	0.52	0.92	0.77	0.75	0.69	1.13
<i>Spies 6140</i>	—	0.76	0.66	1.01	0.56	—	—	1.34	0.88	1.47	1.16	—	—	0.79	0.62	1.04	0.86	0.84	0.66	1.32
<i>Spies 6285</i>	—	0.83	0.72	1.25	0.52	—	—	1.14	0.94	1.25	1.04	—	—	1.01	0.69	0.81	0.80	0.77	0.72	1.16
<i>Spies 6227</i>	—	1.00	0.50	1.07	0.50	—	—	0.95	0.85	0.89	0.69	—	—	0.89	0.75	0.48	0.43	0.41	0.57	1.00
<i>Spies 6145</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6288</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6257</i>	—	0.55	0.58	0.46	0.67	—	—	1.13	0.52	0.89	0.69	—	—	0.57	0.44	0.56	0.50	0.47	0.33	0.64
<i>Spies 6241</i>	—	0.38	0.31	0.51	0.55	—	—	0.80	0.39	0.77	0.48	—	—	0.54	0.50	0.47	0.37	0.35	0.47	0.59
<i>Spies 6244</i>	—	0.80	0.55	0.67	0.63	—	—	0.86	0.56	0.94	0.97	—	—	0.46	0.81	0.89	0.61	0.67	0.94	0.69
<i>Spies 6575</i>	—	0.94	0.72	0.33	0.72	—	—	0.88	0.34	0.69	0.75	—	—	0.52	0.69	0.80	0.79	0.67	0.72	0.81
<i>Spies 6574</i>	—	0.62	0.57	0.45	0.43	—	—	0.64	0.39	0.52	0.50	—	—	0.41	0.52	0.41	0.37	0.41	0.65	0.62
<i>Spies 6573</i>	—	0.56	0.51	0.05	0.69	—	—	0.85	0.61	0.66	0.43	—	—	1.10	0.95	0.41	0.43	0.41	0.81	1.07

Appendix Q (continued). Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 9. Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies 6252</i>	<i>Spies 6256</i>	<i>Spies 6072</i>	<i>Spies 6154</i>	<i>Spies 6225</i>	<i>Spies 6160</i>	<i>Spies 6166</i>	<i>Spies 6235</i>	<i>Spies 6316</i>	<i>Spies 6140</i>	<i>Spies 6285</i>	<i>Spies 6227</i>	<i>Spies 6145</i>	<i>Spies 6288</i>	<i>Spies 6257</i>	<i>Spies 6241</i>	<i>Spies 6244</i>	<i>Spies 6575</i>	<i>Spies 6574</i>	<i>Spies 6573</i>
<i>Spies 6084</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 5967</i>	0.44	0.57	—	0.49	—	—	0.61	—	0.52	0.47	0.44	0.37	—	—	0.58	0.68	0.45	0.39	0.54	0.57
<i>Spies 6106</i>	0.61	0.51	—	0.43	—	—	0.53	—	0.50	0.51	0.49	0.60	—	—	0.56	0.73	0.58	0.49	0.57	0.60
<i>Spies 6240</i>	0.64	0.76	—	0.75	—	—	0.44	—	0.41	0.36	0.29	0.34	—	—	0.63	0.60	0.51	0.72	0.64	0.95
<i>Spies 6025</i>	0.65	0.60	—	0.62	—	—	0.32	—	0.56	0.57	0.59	0.60	—	—	0.51	0.58	0.53	0.49	0.85	0.50
<i>Spies 6096</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6203</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6085</i>	0.53	0.46	—	0.47	—	—	0.46	—	0.33	0.26	0.32	0.39	—	—	0.32	0.45	0.42	0.41	0.53	0.49
<i>Spies 6201</i>	0.68	0.71	—	0.70	—	—	0.52	—	0.45	0.41	0.39	0.49	—	—	0.60	0.68	0.57	0.71	0.68	0.55
<i>Spies 6291</i>	0.49	0.53	—	0.36	—	—	0.34	—	0.30	0.23	0.29	0.41	—	—	0.41	0.47	0.39	0.50	0.58	0.52
<i>Spies 6181</i>	0.65	0.59	—	0.67	—	—	0.48	—	0.24	0.31	0.35	0.50	—	—	0.50	0.65	0.38	0.47	0.60	0.65
<i>Spies 6249</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 5976</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6237</i>	0.67	0.51	—	0.63	—	—	0.53	—	0.44	0.45	0.36	0.41	—	—	0.56	0.58	0.63	0.39	0.67	0.33
<i>Spies 6047</i>	0.49	0.37	—	0.55	—	—	0.55	—	0.59	0.54	0.50	0.47	—	—	0.65	0.60	0.44	0.50	0.59	0.39
<i>Spies 6286</i>	0.62	0.57	—	0.54	—	—	0.43	—	0.40	0.35	0.44	0.62	—	—	0.57	0.63	0.41	0.45	0.67	0.67
<i>Spies 6061</i>	0.69	0.60	—	0.58	—	—	0.59	—	0.46	0.43	0.45	0.65	—	—	0.61	0.69	0.54	0.45	0.69	0.65
<i>Spies 6254</i>	0.67	0.57	—	0.59	—	—	0.55	—	0.47	0.42	0.46	0.67	—	—	0.62	0.70	0.51	0.42	0.67	0.67
<i>Spies 6101</i>	0.62	0.47	—	0.58	—	—	0.41	—	0.50	0.52	0.48	0.56	—	—	0.72	0.63	0.39	0.49	0.52	0.44
<i>Spies 6213</i>	0.63	0.52	—	0.59	—	—	0.42	—	0.32	0.27	0.31	0.37	—	—	0.53	0.55	0.50	0.44	0.54	0.34
<i>Spies 6252</i>	—	0.68	—	0.62	—	—	0.47	—	0.28	0.23	0.32	0.58	—	—	0.70	0.62	0.58	0.54	0.65	0.50
<i>Spies 6256</i>	0.38	—	—	0.60	—	—	0.41	—	0.43	0.33	0.37	0.55	—	—	0.59	0.72	0.57	0.52	0.47	0.49
<i>Spies 6072</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6154</i>	0.48	0.50	—	—	—	—	0.47	—	0.44	0.39	0.36	0.41	—	—	0.56	0.58	0.44	0.59	0.57	0.39
<i>Spies 6225</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6160</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6166</i>	0.75	0.89	—	0.75	—	—	—	—	0.36	0.30	0.41	0.40	—	—	0.57	0.68	0.54	0.442	0.53	0.56
<i>Spies 6235</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6316</i>	1.28	0.94	—	0.83	—	—	1.03	—	—	0.88	0.74	0.55	—	—	0.55	0.57	0.51	0.52	0.50	0.20
<i>Spies 6140</i>	1.48	1.10	—	0.95	—	—	1.22	—	0.13	—	0.77	0.50	—	—	0.44	0.49	0.53	0.53	0.51	0.21
<i>Spies 6285</i>	1.13	1.00	—	1.01	—	—	0.88	—	0.30	0.26	—	0.65	—	—	0.47	0.56	0.56	0.50	0.49	0.32
<i>Spies 6227</i>	0.58	0.61	—	0.89	—	—	0.92	—	0.61	0.69	0.44	—	—	—	0.65	0.69	0.57	0.37	0.60	0.54
<i>Spies 6145</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6288</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6257</i>	0.36	0.53	—	0.57	—	—	0.56	—	0.61	0.83	0.75	0.43	—	—	—	0.73	0.52	0.53	0.60	0.43
<i>Spies 6241</i>	0.49	0.33	—	0.54	—	—	0.38	—	0.56	0.72	0.58	0.37	—	—	0.31	—	0.71	0.55	0.58	0.57
<i>Spies 6244</i>	0.55	0.57	—	0.82	—	—	0.62	—	0.66	0.64	0.59	0.56	—	—	0.65	0.35	—	0.65	0.62	0.36
<i>Spies 6575</i>	0.62	0.65	—	0.52	—	—	0.86	—	0.66	0.63	0.69	1.00	—	—	0.64	0.59	0.43	—	0.63	0.34
<i>Spies 6574</i>	0.43	0.76	—	0.56	—	—	0.64	—	0.69	0.66	0.72	0.50	—	—	0.50	0.55	0.47	0.46	—	—
<i>Spies 6573</i>	0.69	0.72	—	0.94	—	—	0.58	—	1.61	1.58	1.13	0.62	—	—	0.94	0.57	1.02	1.07	0.43	—

Appendix R. Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 11.

Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies</i> 6084	<i>Spies</i> 5967	<i>Spies</i> 6106	<i>Spies</i> 6240	<i>Spies</i> 6025	<i>Spies</i> 6096	<i>Spies</i> 6203	<i>Spies</i> 6085	<i>Spies</i> 6201	<i>Spies</i> 6291	<i>Spies</i> 6181	<i>Spies</i> 6249	<i>Spies</i> 5976	<i>Spies</i> 6237	<i>Spies</i> 6047	<i>Spies</i> 6286	<i>Spies</i> 6061	<i>Spies</i> 6254	<i>Spies</i> 6101	<i>Spies</i> 6213		
<i>Spies</i> 6084	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
<i>Spies</i> 5967	—	—	0.71	—	0.40	—	0.40	—	0.57	—	0.53	0.33	0.00	0.44	0.27	0.67	0.40	0.17	0.15	0.53		
<i>Spies</i> 6106	—	0.85	—	—	0.36	—	0.18	—	0.45	—	0.50	0.31	0.00	0.74	0.50	0.50	0.50	0.15	0.29	0.50		
<i>Spies</i> 6240	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
<i>Spies</i> 6025	—	0.92	1.01	—	—	—	—	—	0.50	—	0.13	—	0.31	0.00	0.00	0.33	0.22	0.44	0.44	0.33	0.00	0.22
<i>Spies</i> 6096	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6203	—	0.92	1.70	—	0.69	—	—	—	0.13	—	0.31	0.83	0.00	0.17	0.22	0.22	0.22	0.00	0.00	0.00	0.00	0.00
<i>Spies</i> 6085	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6201	—	0.56	0.79	—	2.01	—	2.01	—	—	—	0.50	0.35	0.27	0.52	0.30	0.50	0.40	0.47	0.33	0.50	—	—
<i>Spies</i> 6291	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6181	—	0.64	0.69	—	1.18	—	1.18	—	0.69	—	—	0.40	0.31	0.57	0.44	0.56	0.67	0.40	0.38	0.33	—	—
<i>Spies</i> 6249	—	1.10	1.18	—	#	—	1.10	—	1.04	—	0.92	—	0.00	0.29	0.18	0.18	0.18	0.00	0.22	0.00	—	—
<i>Spies</i> 5976	—	#	#	—	#	—	#	—	1.32	—	1.18	#	—	0.17	0.22	0.22	0.22	0.67	0.29	0.22	—	—
<i>Spies</i> 6237	—	0.81	0.31	—	1.10	—	1.79	—	0.65	—	0.56	1.25	1.79	—	0.59	0.59	0.59	0.43	0.53	0.47	—	—
<i>Spies</i> 6047	—	1.32	0.69	—	1.50	—	1.50	—	1.20	—	0.81	1.70	1.50	0.53	—	0.29	0.29	0.36	0.00	0.14	—	—
<i>Spies</i> 6286	—	0.41	0.69	—	0.81	—	1.50	—	0.69	—	0.59	1.70	1.50	0.53	1.25	—	0.57	0.36	0.33	0.57	—	—
<i>Spies</i> 6061	—	0.92	0.69	—	0.81	—	1.50	—	0.92	—	0.41	1.70	1.50	0.53	1.25	0.56	—	0.36	0.33	0.29	—	—
<i>Spies</i> 6254	—	1.79	1.87	—	1.10	—	#	—	0.75	—	0.92	#	0.41	0.85	1.01	1.01	1.01	—	0.22	0.36	—	—
<i>Spies</i> 6101	—	1.87	1.25	—	#	—	#	—	1.10	—	0.98	1.50	1.25	0.63	#	1.10	1.10	1.50	—	0.33	—	—
<i>Spies</i> 6213	—	0.63	0.69	—	1.50	—	#	—	0.69	—	1.10	#	1.50	0.75	1.95	0.56	1.25	1.01	1.10	—	—	—
<i>Spies</i> 6252	—	1.70	#	—	#	—	#	—	1.39	—	0.85	#	0.92	1.87	#	0.92	0.92	1.25	1.39	0.92	—	—
<i>Spies</i> 6256	—	0.98	0.75	—	0.92	—	1.61	—	0.74	—	0.64	#	1.61	0.4	1.32	0.63	0.63	1.10	0.77	0.63	—	—
<i>Spies</i> 6072	—	0.85	#	—	#	—	1.39	—	0.86	—	0.53	1.81	0.69	2.08	1.87	1.18	1.97	0.92	1.01	1.18	—	—
<i>Spies</i> 6154	—	0.41	0.45	—	1.32	—	1.32	—	0.37	—	0.41	1.45	1.32	0.65	1.20	0.69	0.69	1.04	0.81	0.69	—	—
<i>Spies</i> 6225	—	0.65	0.36	—	1.18	—	1.87	—	0.88	—	1.01	#	#	0.56	1.10	1.10	1.10	2.01	0.98	0.81	—	—
<i>Spies</i> 6160	—	0.56	0.92	—	0.69	—	1.39	—	0.86	—	1.04	#	1.39	0.69	1.18	0.49	0.77	0.92	1.70	0.77	—	—
<i>Spies</i> 6166	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6235	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6316	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6140	—	0.56	1.32	—	#	—	#	—	1.15	—	0.75	1.61	1.39	1.39	#	1.18	1.18	1.61	0.61	1.18	—	—
<i>Spies</i> 6285	—	0.68	0.51	—	#	—	#	—	1.39	—	#	#	#	0.77	0.92	1.61	#	1.25	#	1.61	—	—
<i>Spies</i> 6227	—	#	#	—	#	—	#	—	1.95	—	#	#	#	#	#	1.39	#	#	#	#	—	—
<i>Spies</i> 6145	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6288	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6257	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6241	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6244	—	0.92	0.96	—	0.81	—	1.50	—	1.20	—	0.41	#	1.50	0.75	1.25	0.34	0.56	1.01	1.10	0.85	—	—
<i>Spies</i> 6575	—	0.53	0.41	—	1.01	—	0.45	—	0.45	—	0.92	1.87	1.70	0.46	1.39	0.29	0.69	1.18	0.85	0.47	—	—
<i>Spies</i> 6574	—	0.32	0.36	—	1.39	—	0.52	—	0.52	—	0.58	1.50	2.08	0.41	1.25	0.41	0.97	1.50	0.64	0.56	—	—
<i>Spies</i> 6573	—	0.50	0.69	—	1.45	—	0.69	—	0.69	—	0.37	1.56	2.14	0.58	1.30	0.79	0.81	1.56	0.69	0.79	—	—

Appendix R (continued). Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 11. Missing— values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies</i> 6252	<i>Spies</i> 6256	<i>Spies</i> 6072	<i>Spies</i> 6154	<i>Spies</i> 6225	<i>Spies</i> 6160	<i>Spies</i> 6166	<i>Spies</i> 6235	<i>Spies</i> 6316	<i>Spies</i> 6140	<i>Spies</i> 6285	<i>Spies</i> 6227	<i>Spies</i> 6145	<i>Spies</i> 6288	<i>Spies</i> 6257	<i>Spies</i> 6241	<i>Spies</i> 6244	<i>Spies</i> 6575	<i>Spies</i> 6574	<i>Spies</i> 6573
<i>Spies</i> 6084	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 5967	<i>0.18</i>	<i>0.38</i>	<i>0.43</i>	<i>0.67</i>	<i>0.53</i>	<i>0.57</i>	—	—	—	<i>0.57</i>	<i>0.20</i>	<i>0.00</i>	—	—	—	—	<i>0.40</i>	<i>0.59</i>	<i>0.73</i>	<i>0.61</i>
<i>Spies</i> 6106	<i>0.00</i>	<i>0.47</i>	<i>0.00</i>	<i>0.64</i>	<i>0.70</i>	<i>0.40</i>	—	—	—	<i>0.27</i>	<i>1.67</i>	<i>0.00</i>	—	—	—	—	<i>0.38</i>	<i>0.67</i>	<i>0.70</i>	<i>0.50</i>
<i>Spies</i> 6240	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6025	<i>0.00</i>	<i>0.40</i>	<i>0.00</i>	<i>0.27</i>	<i>0.31</i>	<i>0.50</i>	—	—	—	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>	—	—	—	—	<i>0.44</i>	<i>0.36</i>	<i>0.25</i>	<i>0.24</i>
<i>Spies</i> 6096	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6203	<i>0.00</i>	<i>0.20</i>	<i>0.25</i>	<i>0.27</i>	<i>0.51</i>	<i>0.25</i>	—	—	—	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>	—	—	—	—	<i>0.22</i>	<i>0.18</i>	<i>0.13</i>	<i>0.24</i>
<i>Spies</i> 6085	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6201	<i>0.25</i>	<i>0.48</i>	<i>0.42</i>	<i>0.69</i>	<i>0.42</i>	<i>0.42</i>	—	—	—	<i>0.32</i>	<i>0.25</i>	<i>0.14</i>	—	—	—	—	<i>0.30</i>	<i>0.64</i>	<i>0.59</i>	<i>0.50</i>
<i>Spies</i> 6291	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6181	<i>0.43</i>	<i>0.53</i>	<i>0.59</i>	<i>0.67</i>	<i>0.36</i>	<i>0.35</i>	—	—	—	<i>0.47</i>	<i>0.00</i>	<i>0.00</i>	—	—	—	—	<i>0.67</i>	<i>0.40</i>	<i>0.56</i>	<i>0.69</i>
<i>Spies</i> 6249	<i>0.00</i>	<i>0.00</i>	<i>0.20</i>	<i>0.24</i>	<i>0.00</i>	<i>0.00</i>	—	—	—	<i>0.20</i>	<i>0.00</i>	<i>0.00</i>	—	—	—	—	<i>0.00</i>	<i>0.15</i>	<i>0.22</i>	<i>0.21</i>
<i>Spies</i> 5976	<i>0.40</i>	<i>0.20</i>	<i>0.50</i>	<i>0.27</i>	<i>0.00</i>	<i>0.25</i>	—	—	—	<i>0.25</i>	<i>0.00</i>	<i>0.00</i>	—	—	—	—	<i>0.22</i>	<i>0.18</i>	<i>0.13</i>	<i>0.12</i>
<i>Spies</i> 6237	<i>0.15</i>	<i>0.67</i>	<i>0.13</i>	<i>0.52</i>	<i>0.57</i>	<i>0.50</i>	—	—	—	<i>0.25</i>	<i>0.46</i>	<i>0.00</i>	—	—	—	—	<i>0.47</i>	<i>0.63</i>	<i>0.67</i>	<i>0.56</i>
<i>Spies</i> 6047	<i>0.00</i>	<i>0.27</i>	<i>0.15</i>	<i>0.30</i>	<i>0.33</i>	<i>0.31</i>	—	—	—	<i>0.00</i>	<i>0.40</i>	<i>0.00</i>	—	—	—	—	<i>0.29</i>	<i>0.25</i>	<i>0.29</i>	<i>0.27</i>
<i>Spies</i> 6286	<i>0.40</i>	<i>0.53</i>	<i>0.31</i>	<i>0.50</i>	<i>0.33</i>	<i>0.62</i>	—	—	—	<i>0.31</i>	<i>0.20</i>	<i>0.25</i>	—	—	—	—	<i>0.71</i>	<i>0.75</i>	<i>0.67</i>	<i>0.45</i>
<i>Spies</i> 6061	<i>0.40</i>	<i>0.53</i>	<i>0.15</i>	<i>0.50</i>	<i>0.33</i>	<i>0.46</i>	—	—	—	<i>0.31</i>	<i>0.00</i>	<i>0.00</i>	—	—	—	—	<i>0.57</i>	<i>0.50</i>	<i>0.38</i>	<i>0.55</i>
<i>Spies</i> 6254	<i>0.29</i>	<i>0.33</i>	<i>0.40</i>	<i>0.35</i>	<i>0.13</i>	<i>0.40</i>	—	—	—	<i>0.20</i>	<i>0.29</i>	<i>0.00</i>	—	—	—	—	<i>0.36</i>	<i>0.31</i>	<i>0.22</i>	<i>0.21</i>
<i>Spies</i> 6101	<i>0.25</i>	<i>0.46</i>	<i>0.36</i>	<i>0.44</i>	<i>0.38</i>	<i>0.18</i>	—	—	—	<i>0.55</i>	<i>0.00</i>	<i>0.00</i>	—	—	—	—	<i>0.33</i>	<i>0.43</i>	<i>0.53</i>	<i>0.50</i>
<i>Spies</i> 6213	<i>0.40</i>	<i>0.53</i>	<i>0.31</i>	<i>0.50</i>	<i>0.44</i>	<i>0.46</i>	—	—	—	<i>0.31</i>	<i>0.20</i>	<i>0.00</i>	—	—	—	—	<i>0.43</i>	<i>0.63</i>	<i>0.57</i>	<i>0.45</i>
<i>Spies</i> 6252	—	<i>0.36</i>	<i>0.44</i>	<i>0.25</i>	<i>0.00</i>	<i>0.22</i>	—	—	—	<i>0.44</i>	<i>0.00</i>	<i>0.00</i>	—	—	—	—	<i>0.40</i>	<i>0.17</i>	<i>0.24</i>	<i>0.33</i>
<i>Spies</i> 6256	<i>1.01</i>	—	<i>0.29</i>	<i>0.67</i>	<i>0.63</i>	<i>0.57</i>	—	—	—	<i>0.29</i>	<i>0.36</i>	<i>0.00</i>	—	—	—	—	<i>0.53</i>	<i>0.59</i>	<i>0.64</i>	<i>0.61</i>
<i>Spies</i> 6072	<i>0.81</i>	<i>1.25</i>	—	<i>0.42</i>	<i>0.24</i>	<i>0.33</i>	—	—	—	<i>0.67</i>	<i>0.00</i>	<i>0.00</i>	—	—	—	—	<i>0.46</i>	<i>0.13</i>	<i>0.40</i>	<i>0.48</i>
<i>Spies</i> 6154	<i>1.39</i>	<i>0.41</i>	<i>0.86</i>	—	<i>0.58</i>	<i>0.53</i>	—	—	—	<i>0.42</i>	<i>0.13</i>	<i>0.00</i>	—	—	—	—	<i>0.40</i>	<i>0.64</i>	<i>0.67</i>	<i>0.64</i>
<i>Spies</i> 6225	#	<i>0.46</i>	<i>1.45</i>	<i>0.54</i>	—	<i>0.59</i>	—	—	—	<i>0.24</i>	<i>0.29</i>	<i>0.00</i>	—	—	—	—	<i>0.44</i>	<i>0.50</i>	<i>0.64</i>	<i>0.54</i>
<i>Spies</i> 6160	<i>1.50</i>	<i>0.56</i>	<i>1.10</i>	<i>0.64</i>	<i>0.53</i>	—	—	—	—	<i>0.33</i>	<i>0.22</i>	<i>0.00</i>	—	—	—	—	<i>0.46</i>	<i>0.53</i>	<i>0.50</i>	<i>0.38</i>
<i>Spies</i> 6166	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6235	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6316	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6140	<i>0.81</i>	<i>1.25</i>	<i>0.41</i>	<i>0.86</i>	<i>1.45</i>	<i>1.10</i>	—	—	—	—	<i>0.00</i>	<i>0.00</i>	—	—	—	—	<i>0.46</i>	<i>0.27</i>	<i>0.60</i>	<i>0.57</i>
<i>Spies</i> 6285	#	<i>1.01</i>	#	<i>2.08</i>	<i>1.25</i>	<i>1.50</i>	—	—	—	#	—	<i>0.00</i>	—	—	—	—	<i>0.00</i>	<i>0.17</i>	<i>0.24</i>	<i>0.11</i>
<i>Spies</i> 6227	#	#	#	#	#	#	—	—	—	#	#	—	—	—	—	—	<i>0.25</i>	<i>0.20</i>	<i>0.13</i>	<i>0.00</i>
<i>Spies</i> 6145	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6288	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6257	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6241	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies</i> 6244	<i>0.92</i>	<i>0.63</i>	<i>0.77</i>	<i>0.92</i>	<i>0.81</i>	<i>0.77</i>	—	—	—	<i>0.77</i>	#	<i>1.39</i>	—	—	—	—	—	<i>0.50</i>	<i>0.57</i>	<i>0.55</i>
<i>Spies</i> 6575	<i>1.79</i>	<i>0.53</i>	<i>2.01</i>	<i>0.45</i>	<i>0.69</i>	<i>0.63</i>	—	—	—	<i>1.32</i>	<i>1.79</i>	<i>1.61</i>	—	—	—	—	<i>0.69</i>	—	<i>0.70</i>	<i>0.42</i>
<i>Spies</i> 6574	<i>1.45</i>	<i>0.45</i>	<i>0.92</i>	<i>0.41</i>	<i>0.45</i>	<i>0.69</i>	—	—	—	<i>0.51</i>	<i>1.45</i>	<i>2.01</i>	—	—	—	—	<i>0.56</i>	<i>0.36</i>	—	<i>0.69</i>
<i>Spies</i> 6573	<i>1.10</i>	<i>0.50</i>	<i>0.74</i>	<i>0.44</i>	<i>0.62</i>	<i>0.97</i>	—	—	—	<i>0.56</i>	<i>2.20</i>	#	—	—	—	—	<i>0.61</i>	<i>0.88</i>	<i>0.37</i>	—

Appendix S. Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 12.

Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies 6084</i>	<i>Spies 5967</i>	<i>Spies 6106</i>	<i>Spies 6240</i>	<i>Spies 6025</i>	<i>Spies 6096</i>	<i>Spies 6203</i>	<i>Spies 6085</i>	<i>Spies 6201</i>	<i>Spies 6291</i>	<i>Spies 6181</i>	<i>Spies 6249</i>	<i>Spies 5976</i>	<i>Spies 6237</i>	<i>Spies 6047</i>	<i>Spies 6286</i>	<i>Spies 6061</i>	<i>Spies 6254</i>	<i>Spies 6101</i>	<i>Spies 6213</i>
<i>Spies 6084</i>		0.54	0.44	0.38	—	0.13	0.29	0.32	0.26	0.32	0.28	0.13	0.36	0.32	0.34	0.48	0.35	0.35	0.44	0.26
<i>Spies 5967</i>	0.62		0.55	0.44	—	0.27	0.46	0.45	0.43	0.40	0.43	0.42	0.50	0.54	0.69	0.58	0.48	0.48	0.73	0.43
<i>Spies 6106</i>	0.81	0.61		0.61	—	0.26	0.56	0.56	0.42	0.38	0.65	0.46	0.46	0.63	0.44	0.44	0.47	0.47	0.59	0.40
<i>Spies 6240</i>	0.96	0.83	0.50		—	0.27	0.57	0.45	0.43	0.40	0.52	0.53	0.36	0.59	0.57	0.39	0.55	0.55	0.55	0.34
<i>Spies 6025</i>	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6096</i>	2.08	1.30	1.34	1.30	—		0.16	0.19	0.15	0.27	0.19	0.29	0.33	0.37	0.32	0.10	0.53	0.53	0.26	0.21
<i>Spies 6203</i>	1.29	0.78	0.59	0.56	—	1.83		0.59	0.45	0.14	0.58	0.49	0.26	0.55	0.37	0.35	0.44	0.44	0.39	0.31
<i>Spies 6085</i>	1.14	0.79	0.58	0.79	—	1.66	0.53		0.56	0.33	0.59	0.43	0.37	0.50	0.47	0.27	0.43	0.43	0.38	0.36
<i>Spies 6201</i>	1.35	0.84	0.86	0.84	—	1.91	0.80	0.59		0.40	0.60	0.51	0.36	0.52	0.65	0.56	0.47	0.47	0.37	0.35
<i>Spies 6291</i>	1.15	0.92	0.96	0.92	—	1.32	1.95	1.10	0.92		0.29	0.26	0.48	0.20	0.50	0.25	0.18	0.18	0.38	0.18
<i>Spies 6181</i>	1.28	0.85	0.43	0.65	—	1.67	0.55	0.54	0.52	1.25		0.79	0.32	0.68	0.58	0.49	0.41	0.41	0.51	0.41
<i>Spies 6249</i>	2.08	0.86	0.77	0.64	—	1.25	0.72	0.84	0.67	1.35	0.23		0.41	0.65	0.63	0.43	0.51	0.51	0.56	0.40
<i>Spies 5976</i>	1.01	0.69	0.73	1.03	—	1.10	1.35	0.99	1.01	0.74	1.15	0.89		0.42	0.52	0.44	0.32	0.32	0.46	0.40
<i>Spies 6237</i>	1.13	0.62	0.46	0.52	—	0.99	0.60	0.69	0.65	1.61	0.38	0.43	0.86		0.50	0.59	0.59	0.59	0.53	0.59
<i>Spies 6047</i>	1.06	0.38	0.91	0.56	—	1.14	1.00	0.75	0.43	0.69	0.55	0.46	0.66	0.69		0.59	0.50	0.50	0.56	0.31
<i>Spies 6286</i>	0.73	0.54	0.83	0.95	—	2.35	1.04	1.32	0.59	1.39	0.72	0.84	0.81	0.69	0.53		0.43	0.43	0.50	0.36
<i>Spies 6061</i>	1.06	0.73	0.76	0.59	—	0.64	0.83	0.85	0.75	1.70	0.89	0.66	1.14	0.53	0.69	0.85		1.00	0.40	0.38
<i>Spies 6254</i>	1.06	0.73	0.76	0.59	—	0.64	0.83	0.85	0.75	1.70	0.89	0.66	1.14	0.53	0.69	0.85	0.00		0.40	0.38
<i>Spies 6101</i>	0.81	0.32	0.53	0.61	—	1.34	0.94	0.98	1.00	0.96	0.67	0.57	0.73	0.64	0.59	0.69	0.92	0.92		0.40
<i>Spies 6213</i>	1.34	1.06	0.92	1.06	—	1.56	1.16	1.03	1.04	1.70	0.89	0.92	0.92	0.53	1.16	1.01	0.96	0.96	0.92	
<i>Spies 6252</i>	0.95	0.43	0.46	0.72	—	1.91	0.92	0.69	0.48	0.63	0.52	0.77	0.86	0.85	0.51	0.69	1.04	1.04	0.81	1.04
<i>Spies 6256</i>	0.92	0.66	1.39	1.35	—	2.35	0.89	0.63	0.49	1.10	0.82	1.13	0.99	1.10	0.64	0.92	1.25	1.25	0.98	0.69
<i>Spies 6072</i>	1.06	0.88	0.76	0.73	—	2.25	0.83	0.85	0.89	1.30	0.89	0.78	0.92	0.75	0.98	1.03	1.18	1.18	0.63	0.77
<i>Spies 6154</i>	1.03	0.44	0.66	0.64	—	1.39	0.84	1.01	0.49	0.99	0.61	0.51	1.10	0.67	0.35	0.50	0.54	0.54	0.56	1.35
<i>Spies 6225</i>	1.10	0.49	0.72	0.69	—	1.47	0.77	1.25	0.54	1.29	0.65	0.56	0.98	0.62	0.41	0.56	0.61	0.61	0.62	1.10
<i>Spies 6160</i>	1.18	0.98	1.19	0.83	—	1.70	0.92	0.95	0.43	1.43	0.65	0.55	1.25	0.75	0.46	0.54	0.48	0.48	1.42	0.73
<i>Spies 6166</i>	0.81	0.72	1.45	1.01	—	2.44	0.94	0.98	0.46	1.87	0.67	0.67	0.88	0.72	0.49	0.47	0.76	0.76	1.22	1.01
<i>Spies 6235</i>	0.88	0.76	1.35	1.10	—	1.61	1.01	1.29	0.78	1.34	0.92	0.94	1.18	0.92	0.72	0.59	0.81	0.81	0.79	0.81
<i>Spies 6316</i>	1.22	0.86	0.44	1.01	—	1.34	0.59	0.69	0.64	1.47	0.30	0.49	1.29	0.46	0.81	0.69	0.76	0.76	0.75	0.63
<i>Spies 6140</i>	0.83	0.46	0.26	0.64	—	1.54	0.62	0.62	0.58	1.13	0.41	0.53	0.53	0.58	0.62	0.62	0.78	0.78	0.41	0.78
<i>Spies 6285</i>	1.10	0.81	0.52	0.69	—	1.18	1.18	0.92	0.72	0.73	0.50	0.56	0.83	0.54	0.57	0.78	0.86	0.86	0.62	0.61
<i>Spies 6227</i>	0.92	0.49	0.52	0.49	—	1.18	0.67	0.78	0.46	0.73	0.43	0.48	0.58	0.46	0.57	0.46	0.72	0.72	0.52	0.86
<i>Spies 6145</i>	1.06	0.73	0.29	1.06	—	#	0.98	0.56	0.75	1.01	0.49	0.78	0.92	0.89	0.69	0.69	1.87	1.87	1.10	1.47
<i>Spies 6288</i>	1.06	0.66	0.49	0.78	—	1.83	0.75	0.44	0.51	1.03	0.28	0.54	0.79	0.43	0.64	0.64	0.69	0.69	0.69	0.83
<i>Spies 6257</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6241</i>	1.03	0.44	0.78	0.89	—	1.39	0.62	0.61	0.67	1.50	0.69	0.69	0.76	0.57	0.62	0.61	0.79	0.79	0.78	1.13
<i>Spies 6244</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6575</i>	1.14	0.79	0.98	0.95	—	1.66	0.75	0.63	0.69	0.69	0.54	0.52	0.99	0.69	0.64	1.10	1.03	1.03	0.98	0.85
<i>Spies 6574</i>	1.06	0.79	0.49	0.56	—	1.14	0.75	1.04	0.80	1.25	0.41	0.38	0.95	0.51	0.45	0.75	0.58	0.58	0.59	0.83
<i>Spies 6573</i>	1.47	0.69	1.01	0.98	—	1.70	0.92	0.95	0.62	1.43	0.97	0.75	1.03	0.72	0.46	0.54	1.06	1.06	0.72	0.88

Appendix S (continued). Genetic distances (D) (lower diagonal) and coefficients of similarity (F) (in italics – upper diagonal) between the studied specimens, using DAF primer 12. Missing values are indicated by —. Values that could not be calculated, due to division by zero, are indicated with #.

	<i>Spies 6252</i>	<i>Spies 6256</i>	<i>Spies 6072</i>	<i>Spies 6154</i>	<i>Spies 6225</i>	<i>Spies 6160</i>	<i>Spies 6166</i>	<i>Spies 6235</i>	<i>Spies 6316</i>	<i>Spies 6140</i>	<i>Spies 6285</i>	<i>Spies 6227</i>	<i>Spies 6145</i>	<i>Spies 6288</i>	<i>Spies 6257</i>	<i>Spies 6241</i>	<i>Spies 6244</i>	<i>Spies 6575</i>	<i>Spies 6574</i>	<i>Spies 6573</i>
<i>Spies 6084</i>	0.39	0.40	0.35	0.36	0.33	0.31	0.44	0.42	0.30	0.44	0.33	0.40	0.35	0.34	—	0.36	—	0.32	0.34	0.23
<i>Spies 5967</i>	0.65	0.52	0.41	0.65	0.61	0.38	0.48	0.47	0.42	0.63	0.44	0.61	0.49	0.51	—	0.65	—	0.45	0.46	0.50
<i>Spies 6106</i>	0.63	0.25	0.47	0.51	0.49	0.30	0.24	0.26	0.65	0.77	0.59	0.59	0.40	0.61	—	0.46	—	0.38	0.61	0.36
<i>Spies 6240</i>	0.49	0.26	0.48	0.53	0.50	0.44	0.36	0.33	0.36	0.53	0.50	0.61	0.34	0.46	—	0.41	—	0.39	0.57	0.38
<i>Spies 6025</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6096</i>	0.15	0.10	0.11	0.25	0.23	0.18	0.09	0.20	0.26	0.21	0.31	0.31	0.00	0.16	—	0.25	—	0.19	0.32	0.18
<i>Spies 6203</i>	0.40	0.41	0.44	0.43	0.46	0.40	0.39	0.36	0.56	0.54	0.31	0.51	0.38	0.47	—	0.54	—	0.47	0.47	0.40
<i>Spies 6085</i>	0.50	0.53	0.43	0.36	0.29	0.39	0.38	0.28	0.50	0.54	0.40	0.46	0.57	0.65	—	0.55	—	0.53	0.35	0.39
<i>Spies 6201</i>	0.62	0.61	0.41	0.62	0.59	0.65	0.63	0.46	0.53	0.56	0.49	0.63	0.47	0.60	—	0.51	—	0.50	0.45	0.54
<i>Spies 6291</i>	0.53	0.33	0.27	0.37	0.28	0.24	0.15	0.26	0.23	0.32	0.48	0.48	0.36	0.36	—	0.22	—	0.50	0.29	0.24
<i>Spies 6181</i>	0.60	0.44	0.41	0.55	0.52	0.52	0.51	0.40	0.74	0.67	0.61	0.65	0.62	0.76	—	0.50	—	0.59	0.67	0.38
<i>Spies 6249</i>	0.47	0.32	0.46	0.60	0.57	0.58	0.51	0.39	0.62	0.59	0.57	0.62	0.46	0.59	—	0.50	—	0.59	0.68	0.47
<i>Spies 5976</i>	0.42	0.37	0.40	0.33	0.38	0.29	0.41	0.31	0.28	0.59	0.44	0.56	0.40	0.45	—	0.47	—	0.37	0.39	0.36
<i>Spies 6237</i>	0.43	0.33	0.47	0.51	0.54	0.49	0.47	0.40	0.63	0.56	0.59	0.63	0.41	0.65	—	0.56	—	0.50	0.60	0.49
<i>Spies 6047</i>	0.60	0.53	0.38	0.70	0.67	0.63	0.61	0.48	0.44	0.54	0.56	0.56	0.50	0.53	—	0.54	—	0.53	0.63	0.63
<i>Spies 6286</i>	0.50	0.40	0.36	0.61	0.57	0.58	0.63	0.55	0.50	0.54	0.46	0.63	0.50	0.53	—	0.55	—	0.33	0.47	0.58
<i>Spies 6061</i>	0.35	0.29	0.31	0.58	0.55	0.62	0.47	0.44	0.47	0.46	0.42	0.48	0.15	0.50	—	0.45	—	0.36	0.56	0.34
<i>Spies 6254</i>	0.35	0.29	0.31	0.58	0.55	0.62	0.47	0.44	0.47	0.46	0.42	0.49	0.15	0.50	—	0.45	—	0.36	0.56	0.34
<i>Spies 6101</i>	0.74	0.38	0.53	0.57	0.54	0.24	0.29	0.45	0.47	0.67	0.54	0.59	0.33	0.50	—	0.46	—	0.38	0.56	0.48
<i>Spies 6213</i>	0.35	0.50	0.46	0.26	0.36	0.48	0.33	0.44	0.53	0.46	0.55	0.42	0.23	0.44	—	0.32	—	0.43	0.44	0.41
<i>Spies 6252</i>	—	0.67	0.47	0.67	0.63	0.43	0.37	0.46	0.53	0.70	0.63	0.63	0.47	0.65	—	0.51	—	0.50	0.50	0.38
<i>Spies 6256</i>	0.41	—	0.36	0.36	0.40	0.52	0.44	0.41	0.44	0.43	0.40	0.51	0.43	0.53	—	0.36	—	0.53	0.35	0.26
<i>Spies 6072</i>	0.75	1.03	—	0.39	0.36	0.34	0.40	0.22	0.47	0.51	0.48	0.55	0.54	0.38	—	0.39	—	0.36	0.44	0.34
<i>Spies 6154</i>	0.41	1.01	0.95	—	0.95	0.71	0.63	0.69	0.51	0.60	0.53	0.58	0.32	0.54	—	0.61	—	0.42	0.65	0.53
<i>Spies 6225</i>	0.46	0.92	1.01	0.05	—	0.87	0.65	0.71	0.49	0.62	0.55	0.60	0.30	0.51	—	0.58	—	0.40	0.62	0.56
<i>Spies 6160</i>	0.84	0.66	1.06	0.35	0.41	—	0.67	0.60	0.42	0.42	0.39	0.50	0.34	0.57	—	0.47	—	0.58	0.51	0.56
<i>Spies 6166</i>	1.00	0.83	0.92	0.46	0.43	0.41	—	0.58	0.47	0.56	0.38	0.54	0.53	0.56	—	0.63	—	0.38	0.44	0.48
<i>Spies 6235</i>	0.78	0.88	1.50	0.37	0.35	0.51	0.54	—	0.32	0.39	0.41	0.41	0.15	0.42	—	0.44	—	0.34	0.42	0.47
<i>Spies 6316</i>	0.64	0.83	0.76	0.66	0.72	0.86	0.75	1.13	—	0.72	0.65	0.65	0.53	0.61	—	0.51	—	0.56	0.67	0.36
<i>Spies 6140</i>	0.36	0.84	0.66	0.51	0.48	0.86	0.57	0.94	0.33	—	0.62	0.67	0.51	0.68	—	0.60	—	0.49	0.59	0.47
<i>Spies 6285</i>	0.46	0.92	0.72	0.64	0.60	0.94	0.97	0.89	0.43	0.48	—	0.75	0.48	0.56	—	0.37	—	0.51	0.62	0.39
<i>Spies 6227</i>	0.46	0.66	0.61	0.55	0.51	0.69	0.62	0.89	0.43	0.41	0.29	—	0.67	0.67	—	0.47	—	0.57	0.67	0.39
<i>Spies 6145</i>	0.75	0.85	0.62	1.13	1.19	1.06	0.63	1.91	0.63	0.66	0.7	0.41	—	0.63	—	0.45	—	0.57	0.44	0.34
<i>Spies 6288</i>	0.43	0.64	0.98	0.62	0.67	0.56	0.59	0.86	0.49	0.38	0.57	0.41	0.47	—	—	0.54	—	0.59	0.42	0.29
<i>Spies 6257</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6241</i>	0.67	1.01	0.95	0.49	0.55	0.75	0.46	0.83	0.66	0.51	1.00	0.75	0.79	0.62	—	—	—	0.42	0.49	0.59
<i>Spies 6244</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Spies 6575</i>	0.69	0.63	1.03	0.86	0.92	0.54	0.98	1.06	0.58	0.72	0.66	0.56	0.56	0.53	—	0.86	—	—	0.47	0.39
<i>Spies 6574</i>	0.69	1.04	0.83	0.43	0.49	0.66	0.81	0.86	0.41	0.54	0.49	0.41	0.83	0.86	—	0.72	—	0.75	—	0.40
<i>Spies 6573</i>	0.97	1.35	1.06	0.64	0.58	0.59	0.72	0.76	1.01	0.75	0.94	0.94	1.06	1.25	—	0.53	—	0.95	0.92	—

Appendix T. Aligned sequences of the studied specimens, using CLUSTALW. Voucher

numbers refer to specimens collected by Spies. * indicate sequences obtained from Genbank.

	1	60
<i>P. dentatum</i> 6286	TCGTGACCCGAAACCAAAA-CCGACCGCGAA-CGGGTCA-CCCTGCCTGGTCGCGCGC--	
<i>K. tenella</i> 6290	TCGTGACCCGAAACCAAAA-CCGACCGCGAA-CGCGTCA-CCCTGCCCGGTGCGCGC--	
<i>T. pusillum</i> 6256	TCGTGACCCGAAACCAAAA-CCGACCGCGAA-CGCGTCA-CCCTGTGAGGCGCGCGC--	
<i>T. echinatum</i> 6255	TCGTGACCCGAAACCAAAA-CCGACCGCGAA-CG-GTCA-CC-TGCCGTGGTCGCGCGC--	
<i>T. utriculosum</i> 5892	TCGTGACCCGAAACCAAAA-CCGACCGCGAA-CGTGTCA-CCCTGCCTGGTCGCGCGC--	
<i>T. pusillum</i> 6296	TCGTGACCCGAAACCAAAA-CTGACCGCGAA-CGCGTCA-CCCTGTGAGGCGCGCGC--	
<i>T. obtusifolium</i> 6245	TCGTGACCCGAAACCAAAA-CCGACCGCGAA-CGCGTCA-CCCTGCCCGGTGCGCGAC--	
<i>T. brachystachyum</i> 6249	TCGTGACCCGAAACCAAAA-CCGACCGCGAA-CGTGTCA-CCCTGCCTGGCCGCGCGC--	
<i>K. purpurea</i> *	TCGTGACCCGAAACCAAAA-CCGACCGCGAA-CGCGTCA-CCCTGCCCGGTGCGCGC--	
<i>A. donax</i> *	CCGTACCCTGA-CCAAAA-CAGACCGCGCA-CGCGTCA-TCCA-TGCCCGCGGGTGC--	
<i>P. australis</i> *	TCGTGACCTGA-CCAAAA-CAGACCGCGAA-CGCGTCA-TCCA-CGCCGGCGGGCGC--	
<i>D. pumila</i> *	TCGTGACCTGA-CCGAAA-CAGACCGCGCA-CGCGTCA-TCCA-TGCCCGCGGGCGT--	
<i>M. rangei</i> *	TCGTGACCTGA-CCAAAA-CAGACCGCGAA-CGTGTCA-TCCG-TGCCCGCGGACGC--	
<i>C. glauca</i> *	TCGTGACCTGA-CCAAAA-CAGACCGCGAA-CGCGTCA-CCCA-TGCCCGCGGGCGG--	
<i>M. macowanii</i> *	TCGTGACCTGA-CCAAAA-CTGACCGCGAA-CGCGTCA-TCCC-TGCCCGCGGGCGT--	
<i>M. arundinacea</i> 4322	TCGGGACCCGAAACCAAAA-CTGACCGCGAA-CGCGTCA-CCTTGTCTGGCCGCGGG--	
<i>K. tenella</i> 4350	TCGTGACCCGAAACCAAAA--CTGACC-CGAA-CGCGTCA-CCTTGTGTTGGCCGCGCG--	
<i>K. purpurea</i> 4748	TCGAGAACCAGAAACCAAAA--GGACAGTGAA--GGGTAA-CCTGATCGGGCCGGAGGC--	
<i>M. setacea</i> *	TCGTGACCCGAAACCAAAA-CTGACCGCGAA-CGCGTCA--CCTGTCTGGCCGCGCG--	
<i>M. stricta</i> *	TCGTGACCCGAAACCAAAA-CCGACCGCGAA-CGCGTCA-CCCTGTCCGGCCGCGCT--	
<i>C. selloana</i> *	TCGTGACCCGAAACCAAAA-CCGACCGCGAA-CGCGTCA-CCCTGTCCGGCCACGCGG--	
<i>P. aristifolia</i> 6295	TCGTGACC-GAAACCAATG-CAGACCGTGCAACAAGTCA-CATTGTCCGGCCACACGA--	
<i>P. rupestris</i> 6308	TCGTGAGTCAGTAGG--TG-CTGACCATGAACCAAGTCAATGTTGCCCGACGCG--	
<i>P. aspera</i> *	TCGTGACCCGAAACCAAAA-CTGACCGCGAA-CAAGTCA-CCTTGTCCGGCCACGCG--	
<i>P. ecklonii</i> *	TCGTGACCCGAAACCAAAA-CTGACCGCGAA-CAAGTCA-CCTTGTCCGGTCCGACG--	
<i>P. macrocalycina</i> *	TCGTGACCCGAAACCAAAA-CTGACCGCGAA-CGCGTCA-CCTTGTCCGGCCGACGG--	
<i>M. dura</i> 5307	TCGTGACCCGAAACCAAAAACCGACCGCGAA-TGTGTCA-CCCTGTCCGGCCGCGCG--	
<i>M. dura</i> 6285	TCGTGACCCGAAACCAAAAACCGACCGCGAA-TGCGTCA-CCCTGTCCGGCCGCGCG--	
<i>M. dura</i> *	TCGTGACCCGAAACCAAAAACCGACCGCGAA-TGCGTCA-CCCTGTCCGGCCGCGCG--	
<i>S. barbatus</i> *	TCGTGACCCGAAACCAAAA-CCGACCGCGAA-CGCGTCA-CCCTGTCCGGCTGCGCGT--	
<i>S. scaberrimus</i> 4660	TCGTGACCCGAAACCAAAA-CTGACCGTGAA-CGTGTCA-CCCTGTCCGGCTGCGCGT--	
<i>S. barbatus</i> 6353	TCGTGACCCGAAACCAAAA-CCGACCGCGAA-CGCGTCA-CCCTGTCCGGCTGCGCGT--	
<i>E. capensis</i> 6095	TCGTCA-----CCGTC-CCGGCCACGCGTGC	
<i>E. villosa</i> 6095	TCGTGAC-----CCGGCCACGCG--C	

	61	120
<i>P. dentatum</i> 6286	-CGGGGATCCGTCCTCCCGTCCGCGTGACCA-AGGCCGCGGACCTCC--GTCAGGA-GGGGAG	
<i>K. tenella</i> 6290	-CGGGGATCCGTCCTTCGTGCGGTGGCCATAGGCCCGGACCTCC--GTCAGGA-GGGGAG	
<i>T. pusillum</i> 6256	-CGGGGATCCGTCCTCCCGTCCGCGGACCA-AGGCCGCGGACCTCC--GTCAGGA-GGGGAG	
<i>T. echinatum</i> 6255	-CGGGGATCCGTCCTCCCGTCCGCGTGACCA-AGGCCGCGGACCTCC--GTCAGGA-GGGGAG	
<i>T. utriculosum</i> 5892	-CGGGGATCCGTTCCCGTCCGCGTGCCCA-AGGCCGCGGACCTCC--GTCAGGA-GGGGAG	
<i>T. pusillum</i> 6296	-CGGGGATCCGTCCTCCCGTCCGCGGACCA-AGGCCGCGGACCTCC--GTCAGGA-GGGGAG	
<i>T. obtusifolium</i> 6245	-CGGGGATCCGTCCTCCCGT-GCGTGACCA-AGGCCGCGGACCTCC--GTCAGGA-GGGGAG	
<i>T. brachystachyum</i> 6249	-CGAGGATCCGTCCTCCGTCGCGCGGCCA-AGGCCGCGGACCTCC--GTCAGGA-GGGGAG	
<i>K. purpurea</i> *	-TGGGGATCCGTCCTTCGTGCGGTGGCCATAGGCCCGGACCTCC--GTCAGGA-GGGGAG	
<i>A. donax</i> *	-GGGGGCT--TGCCCGACCGTGGGCT-CAGGCCCGGACCTCC-GTCAGGA-A-GGGGAG	
<i>P. australis</i> *	-GGGGGCG-CTCCCCCGTCCGCTCGGCC-CCGGCCCGGACCTCC-GTCAGGA-GGGGAG	
<i>D. pumila</i> *	-CGGGGCT--CGCCTTGGCCCGCGCA-CAGGCCCGGACCTCC-T-TCCGGA-GGGGAC	
<i>M. rangei</i> *	-CGGGGCT-CGCCCCCGTCCCGCGCA-CAGGCCCGGACCTCCGTCCTCCGG-G-GGGGAG	
<i>C. glauca</i> *	-CGGGGCT-CGCCCCCGCGCGCGCA-CAGGCCCGGACCTCC--GTCAGGA-GGGGAG	
<i>M. macowanii</i> *	-CGGGGCTTCGCCCCCGCGCACGGCC-CAGGCCCGGACCTCC--GTCAGGA-GGGGAG	
<i>M. arundinacea</i> 4322	-ATGGGGCAAGTCCCGCGCGCGGCC-CAGGCCCGGACCTCC--GTCAGGA-GGG-AG	
<i>K. tenella</i> 4350	-ATGGGGTATGTCCTCCCGCGCGCGGCC-CAGGCCCGGACCTCC--GTCAGGA-GGGGAG	
<i>K. purpurea</i> 4748	-AAGGGGATGTTCCCGCGCGGGGCC-CAGGCCCGGACCTCC--GTTAAGG-GGGGGG	
<i>M. setacea</i> *	-ATGGGGCTCGTCCCGCGCGCGGCC-CAGGCCCGGACCTCC--GTCAGGA-GGGGAG	
<i>M. stricta</i> *	-CGGGGCT-TGTCCCTGCGCGACGGCC-TAGGCCCGGACCTCC--GTCAGGA-GGGGAG	
<i>C. selloana</i> *	-CGGGGCC-TGTCCCGCGCGCGGCCCTAGGATGCGGACCTCC--GCCAGGA-GGGGAG	
<i>P. aristifolia</i> 6295	-----GCTCAGCCGCGGTGGCC-TAGGCCCGGACCTCC--GCAAGGACGGGGAG	
<i>P. rupestris</i> 6308	-----GCTCAGCCGCGCGGCC-TAGGCCCGGACCTCC--GCAAGGAGGGGGAG	
<i>P. aspera</i> *	-----GCTCAGCCGCGCGGCC-TAGGCCCGGACCTCC--GTCAGGA-GGGGAG	
<i>P. ecklonii</i> *	-----GCTCAGCCGCGCGGCC-TAGGCCCGGACCTCC--GCAAGGACGGGGAG	
<i>P. macrocalycina</i> *	-CGTGCT-CGCTCAGCCCGCGGTGGCC-TAGGCCCGGACCTCC--GCCAGGATGGGGAG	
<i>M. dura</i> 5307	-CGGGGCT-TGTCCCTGTCGCGTGCCCAAGGCCCGCAACCTCC--GTAGGG-GGGCAG	
<i>M. dura</i> 6285	-CGGGGCT-TGTCCCTGTCGCGTGCCCAAGGCCCGCAACCTCC--GTAGGG-GGGCAG	
<i>M. dura</i> *	-CGGGGCT-TGTCCCTGTCGCGTGCCCAAGGCCCGGACCTCC--GTAGGG-GGGCAG	
<i>S. barbatus</i> *	-CGGGGCT-TGTCCCTGACGTGTGGCCTAAGGCCCGGACCTCC--GTCAGGA-GGAGAG	
<i>S. scaberrimus</i> 4660	-CGGGGCT-TGTCCCTGACGTGTGGCCTAAGGCCCGGACCTCC--GTCAGGA-GGAGAG	
<i>S. barbatus</i> 6353	-CGGGGCT-TGTCCCTGACGTGTGGCCTAAGGCCCGGACCTCC--GTCAGGA-GGAGAG	
<i>E. capensis</i> 6095	TAAGGCTCAGCCCCACCGTGTGGCCACCGT-GCAAGCCCTCC--TCGG--AGGGCC	
<i>E. villosa</i> 6095	TAAGGCTCAGCCCCACCGTGTGGCCACCGT-TGAAGCCCTCC--TCGG--AGGGCC	

Appendix U. Pairwise distance analysis between species studied using DNA sequencing of the *ITS* region. 1 = *Prionanthium dentatum* (Spies 6286), 2 = *Karroochloa tenella* (Spies 6290), 3 = *Tribolium pusillum* (Spies 6256), 4 = *T. echinatum* (Spies 6255), 5 = *T. utriculosum* (Spies 5892), 6 = *T. pusillum* (Spies 6296), 7 = *T. obtusifolium* (Spies 6245), 8 = *T. brachystachyum* (Spies 6249), 9 = *Karroochloa purpurea*, 10 = *Arundo donax*, 11 = *Phragmites australis*, 12 = *Dregeochloa pumila*, 13 = *Merxmuellera rangei*, 14 = *Centropodia glauca*, 15 = *Merxmuellera macowanii*, 16 = *M. arundinacea* (Spies 4322), 17 = *Karroochloa tenella* (Spies 4350), 18 = *K. purpurea* (Spies 4748), 19 = *Merxmuellera setacea*, 20 = *M. stricta*, 21 = *Cortaderia selloana*, 22 = *Pentaschistis aristifolia* (Spies 6295), 23 = *P. rupestris* (Spies 6308), 24 = *P. aspera*, 25 = *Prionanthium ecklonii*, 26 = *Pentameris macrocalycina*, 27 = *Merxmuellera dura* (Spies 5307), 28 = *M. dura* (Spies 6285), 29 = *M. dura*, 30 = *Schismus barbatus*, 31 = *S. scaberrimus* (Spies 4660), 32 = *S. barbatus* (Spies 6353), 33 = *Ehrharia capensis* (Spies 6095), 34 = *E. villosa* (Spies 6299).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
1	-	.150	.150	.145	.126	.169	.174	.205	.235	.488	.453	.500	.457	.426	.444	.442	.484	.550	.394	.364	.346	.439	.426	.385	.377	.326	.386	.400	.362	.385	.385	.412	.555	.565
2		-	.180	.183	.189	.237	.220	.242	.218	.508	.465	.480	.462	.454	.432	.454	.473	.569	.422	.392	.374	.452	.439	.398	.407	.346	.376	.382	.366	.366	.374	.379	.523	.534
3			-	.183	.162	.119	.174	.205	.258	.456	.461	.444	.395	.395	.411	.388	.414	.512	.346	.318	.315	.415	.385	.344	.377	.318	.386	.385	.338	.369	.385	.397	.588	.548
4				-	.128	.147	.115	.169	.200	.472	.476	.452	.417	.417	.393	.425	.429	.543	.344	.307	.297	.380	.367	.325	.350	.291	.354	.367	.297	.328	.328	.364	.479	.460
5					-	.108	.072	.108	.108	.415	.404	.406	.349	.349	.317	.389	.346	.495	.271	.266	.227	.330	.333	.265	.265	.239	.255	.282	.227	.264	.255	.309	.505	.505
6						-	.093	.127	.153	.398	.400	.389	.336	.302	.315	.374	.333	.500	.246	.233	.214	.336	.312	.248	.275	.233	.274	.282	.222	.256	.256	.282	.519	.500
7							-	.106	.083	.384	.406	.381	.341	.310	.306	.403	.398	.473	.260	.233	.208	.309	.295	.254	.262	.202	.290	.300	.231	.246	.254	.328	.517	.491
8								-	.106	.392	.406	.405	.302	.310	.315	.380	.383	.465	.244	.256	.231	.325	.295	.254	.246	.209	.275	.300	.223	.246	.246	.305	.517	.491
9									-	.413	.395	.378	.346	.315	.304	.408	.380	.469	.250	.269	.229	.282	.285	.228	.220	.200	.273	.282	.214	.237	.244	.303	.504	.478
10										-	.222	.232	.236	.317	.250	.468	.463	.544	.374	.397	.389	.451	.430	.413	.397	.389	.484	.492	.421	.456	.464	.508	.518	.491
11											-	.299	.269	.349	.268	.496	.468	.570	.397	.457	.426	.496	.467	.434	.426	.426	.496	.500	.438	.484	.477	.519	.496	.486
12												-	.258	.315	.246	.432	.395	.532	.323	.362	.370	.443	.430	.397	.405	.362	.441	.437	.389	.405	.413	.457	.500	.464
13													-	.215	.185	.477	.449	.496	.331	.346	.338	.395	.382	.350	.341	.323	.408	.419	.349	.395	.388	.469	.517	.491
14														-	.194	.469	.449	.512	.346	.354	.323	.403	.390	.358	.350	.315	.392	.403	.357	.364	.423	.543	.509	
15															-	.439	.402	.452	.279	.290	.274	.373	.333	.299	.333	.298	.371	.374	.309	.341	.358	.419	.416	.385
16																-	.125	.383	.173	.375	.344	.459	.426	.385	.402	.367	.364	.352	.414	.422	.414	.380	.569	.549
17																	-	.402	.165	.362	.339	.413	.397	.355	.372	.362	.359	.331	.402	.409	.402	.328	.603	.558
18																		-	.354	.481	.481	.472	.475	.459	.467	.457	.496	.523	.469	.523	.516	.581	.632	.593
19																			-	.268	.213	.306	.289	.231	.248	.228	.346	.365	.286	.310	.302	.386	.496	.455
20																				-	.185	.306	.276	.244	.260	.200	.308	.302	.233	.279	.287	.354	.543	.518
21																					-	.266	.252	.195	.228	.192	.290	.285	.223	.246	.269	.321	.479	.425
22																						-	.073	.089	.121	.160	.387	.398	.309	.333	.317	.427	.509	.472
23																							-	.065	.129	.153	.390	.402	.311	.352	.328	.447	.514	.481
24																								-	.081	.113	.333	.344	.254	.295	.287	.390	.505	.472
25																									-	.105	.358	.369	.279	.320	.303	.415	.550	.519
26																										-	.315	.318	.233	.256	.256	.346	.526	.491
27																											-	.053	.122	.260	.260	.250	.605	.591
28																												-	.122	.262	.277	.229	.615	.602
29																													-	.223	.238	.298	.547	.513
30																														-	.061	.115	.564	.549
31																															-	.176	.581	.558
32																																-	.610	.596
33																																	-	.069
34																																		-