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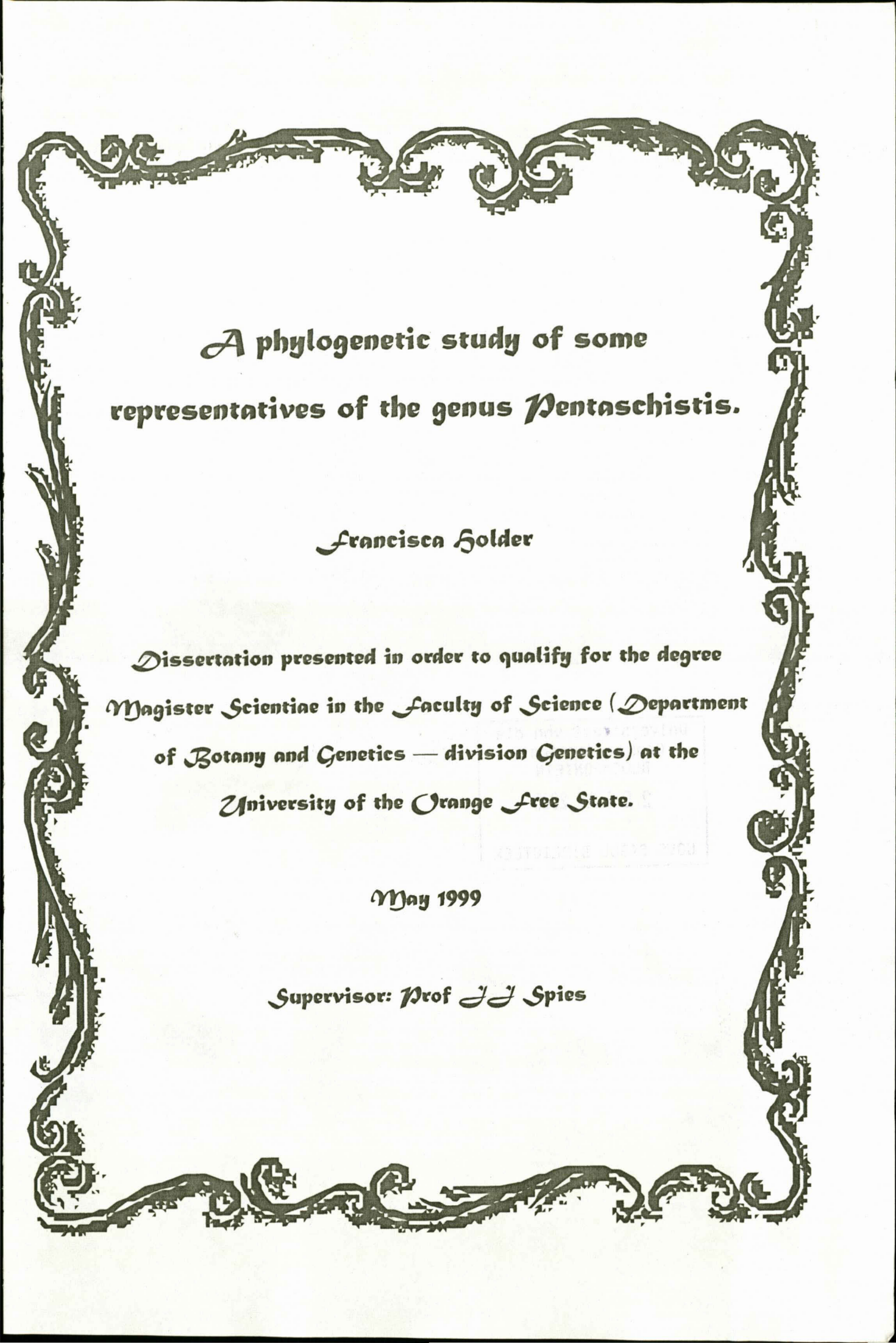
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*A phylogenetic study of some  
representatives of the genus *Pentaschistis*.*

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## LIST OF ABBREVIATIONS

AgNO<sub>3</sub> — Silver Nitrate

*Bgl* I — *Bacillus globigii* I

BLFU — Geo Potts Herbarium, Department of Botany & Genetics, University of the Orange Free State, Bloemfontein.

bp — base pair

CI — Consistency index

CO<sub>2</sub> — Carbon Dioxide

CTAB — Hexadecyltrimethyl ammonium bromide

D — Genetic distance

d H<sub>2</sub>O — distilled water

DAF — DNA amplification fingerprinting

DNA — Deoxyribonucleic acid

dNTP — Deoxynucleotide triphosphate

EDTA — Ethylene diaminetetra acetic acid

ethanol — Ethylalcohol

F — coefficient of similarity

HCl — Hydrochloric acid

HCOH — formaldehyde

HI — homoplacy index

*Hind* III — *Haemophilus influenzae* Rd III

*Hinf* I — *Haemophilus influenzae* RF I

HNO<sub>3</sub> — Nitric acid

i.e. — *it est* (that is)

*ITS* — Internal transcribed spacer

K<sub>2</sub>Cr<sub>2</sub>O<sub>3</sub> — potassiumdichromate

km — kilometers

M — molar

m/v — mass per volume

MgCl<sub>2</sub> — Magnesium chloride

ml — milliliter

mM — millimolar

NaCl — sodium chloride

Na<sub>2</sub>CO<sub>3</sub> — sodium carbonate

Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> — sodium thiosulfate

OPA — Operon primer kit A

OPB — Operon primer kit B

OPC — Operon primer kit C  
PAUP — phylogenetic analysis using parsimony  
PCR — polymerase chain reaction  
pmol — picomoles  
RAPD — Random amplified polymorphic DNA  
RC — rescaled consistency index  
RFLP — restriction fragment length polymorphisms  
RI — retention index  
RNA — Ribonucleic acid  
*rpoC<sub>2</sub>* — RNA polymerase II gene  
SNL — signal to noise  
subsp. — subspecies  
TAE — Tris-acetic acid EDTA  
*Taq* — *Thermus aquaticus*  
Tris — 2-amino-2-(hydroxymethyl)-1,3-propanediol  
UOFS — University of the Orange Free State  
UV — ultraviolet light  
V — Volt  
v/v — volume per volume  
% — percentage  
°C — degrees Celsius  
μg — micrograms  
μl — microlitre  
μM — micromoles



## CHAPTER 1

### INTRODUCTION



#### 1.1 The tribe Arundineae (Arundinoideae, Poaceae).

The world wide family Poaceae consist of 770 genera and 9700 species of grasses (Watson & Dallwitz 1989). There are 194 genera and 967 species and intraspecific taxa in southern Africa, with 847 indigenous species, including 329 endemic species (Gibbs Russell *et al.* 1990). The family Poaceae is divided into six subfamilies, namely Arundinoideae Tateoka, Pooideae Macfarlane & Watson, Panicoideae A. Br., Chloridoideae Rouy, Bambusoideae Asch & Graeb and the smaller Centothecoideae Soderstrom (Clayton & Renvoize 1986). Of interest in this study is the subfamily Arundinoideae.

The subfamily Arundinoideae is poorly defined, lacking reliable diagnostic features and is the least specialised of all the grass subfamilies (Conert 1986, Ellis 1986). Arundinoideae was first described by Tateoka (1957). Many features that are taxonomically discriminating in the other subfamilies vary in this group and, consequently, there is no clearly defined central core group. Phylogenetic relationships of the whole family Poaceae inferred from the sequence data of rDNA (ITS) recently supported the hypothesis that the Arundinoids are monophyletic (Hsiao *et al.* 1998) and not polyphyletic as previously proposed (Ellis 1986, Kellog & Campbell 1987, Barker *et al.* 1995). The tribes recognised have been reduced by transferring some to other subfamilies (Barker 1995). Five major tribal classification systems are recognised (Table 1.1) and the tribes included vary from 17 (Tateoka 1957) to as few as three or four (Watson *et al.* 1985, Clayton & Renvoize 1986, Conert 1986, Watson 1990, Verboom *et al.* 1994). The tribe Arundineae Dumortier is defined by embryo features, non-kranz leaf anatomy and a generally simple spikelet structure. To date all attempts to clarify the relationships of, and within the subfamily have been based on extensive surveys of morphology and anatomy, and to a lesser extent on micromolecular characters. The relationships among the African Arundineae (Clayton & Renvoize 1986) are unclear and the tribe has no solid foundation (Renvoize 1981, Barker 1995). *Pentaschistis* (Nees) Spach is one of the genera included in the tribe Arundineae. *Pentaschistis* was

**TABLE 1.1** The tribal classification systems described in the literature of the subfamily Arundinoideae.

Classification systems	Tribes
Tateoka 1957	<b>Arundineae</b> <b>Ehrharteae</b> <b>Aristideae</b> <b>Thysanolaeneae</b> <b>Lygeae</b> <b>Nardeae</b> <b>Centosteceae</b> <b>Micraireae</b> <b>Stipeae</b> <b>Steyermarkochloae</b> <b>Danthonieae</b> <b>Sportochloae</b> <b>Cyperchloae</b> <b>Eriachneae</b> <b>Cortaderieae</b> <b>Brachyelytreae</b>
Watson <i>et al.</i> 1985	<b>Aristideae</b> <b>Stipeae</b> <b>Arundinoids</b> <b>Danthonoids</b>
Clayton & Renvoize 1986	<b>Arundineae</b> <b>Aristideae</b> <b>Thysanolaneae</b> <b>Micraireae</b>
Conert 1986	<b>Arundineae</b> <b>Danthonieae</b> <b>Cortaderieae</b>
Watson 1990	<b>Aristideae</b> <b>Arundineae</b> <b>Cyperochloae</b> <b>Danthonieae</b> <b>Eriachneae</b> <b>Lygeaea</b> <b>Nardeae</b> <b>Micraireae</b> <b>Spartochloae</b> <b>Steyermarkochloae</b> <b>Stipeae</b>

previously placed in the tribe Danthoneae Zotov, which was originally placed in the Aveneae and Festuceae (Hubbard 1934), but due to superficial similarities in spikelet morphology, they were moved near the Arundineae on the basis of anatomical and cytological characters (DeWet 1954, 1956, 1960). However, Renvoize (1981) and Clayton and Renvoize (1986) included the Danthoneae grasses in the Arundineae (Verboom *et al.* 1994).

The genus *Pentaschistis* mostly forms part of the Cape Flora, but can also be found in the Drakensberg, with a few species in Cameroon, Sudan, Tanzania and Madagascar. Bond & Goldblatt (1984) listed almost 200 species of Poaceae for the Cape Floristic Region. Of these virtually all the endemic species belong to the Arundinoideae in the tribes Arundineae and Ehrharteae. These fynbos grasses are restricted to mountain heaths (Clayton & Renvoize 1986). The Poaceae, although generally ranking high in most floras, is only the 13<sup>th</sup> largest family in the Cape Floristic Region (Bond & Goldblatt 1984). The levels of endemism are high, both at species (68%) and at generic (20%) levels.

The Cape Floristic region is essentially a Mediterranean region, with winter rain and dry summers. The mountain ranges, which run more or less parallel to the coast, greatly influence the amount and distribution of precipitation. The rainfall along the coastal slopes is about four times higher than inland. The soils are generally deficient in nutrients and the combinations of dry summers and low soil nutrient status results in slow growth rates (Linder & Ellis 1990b).

The vegetation is a shrubland or heathland, from 0.5 to 3m high, with very small herbaceous components and virtually no annual component (Taylor 1978, Kruger 1979, Campbell 1985, Linder & Ellis 1990b). The vegetation is dense in the coastal ranges with, a high basal cover and no bare ground, whereas the shrubs are well scattered inland. Although the fynbos vegetation is pyrophytic, fires are spaced well apart, with at least four years between fires (Linder 1989, Linder & Ellis 1990b). The species of the genus *Pentaschistis* are mostly mesophytic and a few even pyrophytic (Linder & Ellis 1990b). Linder (in Linder & Ellis 1990b) has reviewed the phytogeographical patterns inherent in the grasses of the Cape Floristic Region, showing that several taxa may help elucidate the origins and evolution of the Cape Flora.

The Cape Poaceae show a remarkable diversity of habitats from the classical caespitose tussock grass, to a complex growth form, somewhat similar to a divaricate herbaceous plant. These plants appear to be adapted to a range of habitats, both spatial and temporal in the Cape Floristic Region. They may be highly informative on the ecology and

selective restraints operative in the area, but they have not received much attention from researchers.

## **1.2 The genus *Pentaschistis*.**

The genus *Pentaschistis* consists of 68 species and is one of the largest genera in the tribe Arundineae. It is endemic to Africa, with the greatest number of species, 57, indigenous to South Africa, with at least 40 of them being endemic. The generic limits and delimitations of species in *Pentaschistis* are considered as being difficult (Chippindall 1955, Clayton & Renvoize 1986, Linder & Ellis 1990b, Phillips 1994). The genus is considered a homogenous genus (Clayton & Renvoize 1986) and contains all the Arundinoid species that have two, or rarely one, floret per spikelet and lack a terminal tuft of bristles on the ovary. The species included in the genus do not have glumes with three strong veins. However, little confidence in the correct delimitation of the genus *Pentaschistis* exists. Linder & Ellis (1990b) classified *Pentaschistis* into six groupings according to either their morphology or to their leaf anatomy (Table 1.2). A core group of species is common to both groupings, illustrating the complementary aspects of these two data sets and so enhancing the applicability of these groupings (Ellis & Linder 1990b). The groupings are purely for classification and do not have any phylogenetic significance (Linder & Ellis 1990b).

### **1.2.1 Morphology.**

*Pentaschistis* species range from annual to weakly perennial to perennial. Most perennial grasses have herbaceous culms that annually die back to the base and are replaced by shoots arising from axillary basal buds (Gould 1968). Various structures can develop the perennial grass resulting in several to many lateral shoots, initiated at the base, by varying the length and thickness of the internodes in the basal portion of the culm. One of these is the bulbous base which is modified into a distinct storage organ and occurs in *P. viscidula*, *P. argentea*, *P. aristidoides* and *P. velutina*. Another form is the knotty tillering base which has no clearly differentiated storage organ and is capable of coppicing from the base after fire (*P. pallescens*, *P. pyrophila* and *P. eriostoma*) (Linder & Ellis 1990a).

Plants that develop from weak bases are loosely tufted and short lived perennials with stooling (Gould 1968), which gives a "cushion" type of growth (*P. pallida*, *P. densifolia*, *P.*

*rosea* subsp. *rosea*, *P. alticola*, *P. aspera* and *P. acinosa*). The annual grasses are mostly found in the mountains in the most nutrient poor soils (Kruger 1979), but are better diversified in the arid margins of the Cape Floristic Region (*P. airoides* subsp. *airoides*, *P. aristifolia* and *P. capillaris*).

The lateral shoots, mentioned earlier, develop from innovation buds, which are enclosed and protected by prophyll. Two basic variants can be recognised. First the basal innovation shoots that develop from the buds at the base of the plant. This type of lateral shoot innovation is well adapted to the regular annual or biennial fires, characteristic of the subtropical savannas and grasslands. (e.g. *P. glandulosa* and *P. pyrophila*). The other type is the cauline innovation shoots which are produced from nodes higher up on the culms. This is found in perennial grasses. Three different patterns occur in the cauline innovation species (Linder & Ellis 1990a).

In one group the culms are more or less erect and the branches well spread (*P. aspera*, *P. acinosa* and *P. scandens*). In the second group the culms are more or less decumbent and they develop into cushions, often low on the ground (*P. densifolia*, *P. rosea* subsp. *purpurascens* and *P. alticola*). In the third group the plant is initially caespitose, with basal innovation shoots (*P. colorata*) (Linder & Ellis 1990a).

These morphological features are also reflected in anatomical features such as glands. During this study the morphological and anatomical features will be compared with cytogenetic and molecular features.

### **1.2.2 Glands in *Pentaschistis*.**

The leaves of the genus *Pentaschistis* can be either hard, rolled and sclerenchymatous or soft and expanded. The soft leaves are usually associated with glands, whereas the hard rolled leaves usually lack glands. In *Pentaschistis* it appears as if glands arise directly from epidermal tissues (Linder *et al.* 1990). The co-occurrence of the glands with all the other normal emergences, such as bicellular hairs, macro hairs and prickles indicates that the glands cannot be homologous with one of these structures, because homologous structures cannot occur in the same organism (Patterson 1988).

Although glands may occur on the leaf sheaths, pedicels or glumes of various species of *Pentaschistis*, the glands within any one species appear to be almost identical, irrespective of where they occur. Thus, glume glands differ from leaf glands only by their position, not by

**TABLE 1.2** The six groupings in the genus *Pentaschistis* according to their morphology (Linder & Ellis 1990a) and leaf anatomy (Ellis & Linder 1990). A questionmark indicates that the leaf anatomy has not been studied.

Species	Morphological group	Anatomical group
<i>P. acinosa</i>	4	6
<i>P. airoides</i> subsp. <i>airoides</i>	1	1
<i>P. airoides</i> subsp. <i>jugorum</i>	1	2
<i>P. alticola</i>	3	5
<i>P. ampla</i>	1	2
<i>P. andringitrensis</i>	5	?
<i>P. argentea</i>	2	4
<i>P. aristidoides</i>	2	3
<i>P. aristifolia</i>	1	1
<i>P. aspera</i>	1	2
<i>P. aurea</i> subsp. <i>area</i>	2	2
<i>P. aurea</i> subsp. <i>pilosogluma</i>	2	2
<i>P. barbata</i> subsp. <i>barbata</i>	1	1
<i>P. barbata</i> subsp. <i>orientalis</i>	1	2
<i>P. basutorum</i>	3	3
<i>P. borussica</i>	1	2
<i>P. calsicola</i> var. <i>calsicola</i>	3	5
<i>P. calsicola</i> var. <i>hirsuta</i>	3	5
<i>P. capensis</i>	5	?
<i>P. capillaris</i>	1	1
<i>P. caulescens</i>	4	6
<i>P. chippindalliae</i>	3	3
<i>P. chrysurus</i>	6	?
<i>P. cirrhulosa</i>	1	3, 5
<i>P. colorata</i>	3	5
<i>P. curvifolia</i>	4	6
<i>P. densifolia</i>	1	1
<i>P. ecklonii</i>	1	2
<i>P. elegans</i>	3	5
<i>P. eriostoma</i>	6	6
<i>P. exerta</i>	3	3
<i>P. galpinii</i>	1	2
<i>P. glandulosa</i>	1	2
<i>P. gracilis</i>	1	?
<i>P. heptamera</i>	6	2
<i>P. holciformis</i>	3	5
<i>P. humbertii</i>	5	?
<i>P. imantongensis</i>	1	?
<i>P. lima</i>	1	3

<i>P. longipes</i>	1	2
<i>P. malouinensis</i>	3	5
<i>P. manni</i>	1	?
<i>P. microphyla</i>	1	2
<i>P. minor</i>	1	?
<i>P. montana</i>	3	3
<i>P. natalensis</i>	1	2
<i>P. oreodoxa</i>	1	2
<i>P. pallescens</i>	2	2
<i>P. pallida</i>	1	1, 2
<i>P. papillosa</i>	1	2
<i>P. patula</i>	1	1
<i>P. pictigluma</i>	1	?
<i>P. praecox</i>	3	5
<i>P. pseudopallescens</i>	2	2
<i>P. pungens</i>	4	6
<i>P. pusilla</i>	3	5
<i>P. pyrophila</i>	3	6
<i>P. reflexa</i>	1	2
<i>P. rigidissima</i>	3	3
<i>P. rosea</i> subsp. <i>purpurascens</i>	2	2
<i>P. rosea</i> subsp. <i>rosea</i>	2	2
<i>P. rupestris</i>	1	1
<i>P. scandens</i>	4	6
<i>P. setifolia</i>	1	2
<i>P. tomentella</i>	1	1
<i>P. tortuosa</i>	3	5
<i>P. triseta</i>	2	2
<i>P. trisetoides</i>	1	?
<i>P. tysonii</i>	3	5
<i>P. velutina</i>	2	3
<i>P. veneta</i>	1	1
<i>P. viscudula</i>	2	4

their anatomy or structure. The most complex glands are those found in *P. airoides* subsp. *jugorum*. The glands in *Pentastichis* may be analogous to glands recorded from other genera. The phylogenetic implications of these data are that those species of *Pentastichis* possessing glands form a monophyletic group, and that similarly, the possession of clavate and sunken glands, defines a secondary group, whereas a third group is defined by having sunken glands (Linder *et al.* 1990). Consequently, the shared possession of linear glands does not necessarily define a monophyletic group, as this is the primitive state common to all glandular species. Both Stapf (1899) and Ellis & Linder (1990a) used glands as species

delimiters. The leaf anatomy confirms that most species delimitations are based on morphology.

### 1.2.3 Cytogenetics.

De Wet (1954b) described a single somatic chromosome number of  $2n=52$  for *Pentaschistis thunbergii* (Kunth) Stapf, but later studies revealed that this count was erroneous (Du Plessis & Spies 1992). Most literature shows that cytogenetic investigations indicated basic chromosome numbers of  $x = 7$  &  $13$  (Hedberg & Hedberg 1977, Davidse, Hoshino & Simon 1986, Du Plessis & Spies 1988, Spies & Du Plessis 1988, Du Plessis & Spies 1992, Spies *et al.* 1994, Klopper *et al.* 1998). The most common basic chromosome number being  $x = 7$  (26 species). The second basic chromosome number of  $x = 13$  was observed in only four species. Du Plessis & Spies (1992) suggested that  $x = 13$  could have developed in two different ways: as the result of either aneuploidy after polyploidization of  $x = 7$  ( $n = 2x - 1 = 13$ ) or hybridisation with some *Merxmuellera* species ( $x = 6$ ) resulting in a dibasic chromosome number of  $x = 6 + 7$ . Further studies are needed to assess the validity of these hypotheses.

### 1.3 Molecular systematics.

The analysis of genetic diversity and relatedness between or within different species, populations and individuals is a central task for many disciplines of biological science (Weising *et al.* 1995). During the past decade, classical strategies for evaluating genetic variability, such as comparative anatomy, morphology, etceteras have been increasingly complemented by molecular techniques. These include the analysis of chemical constituents. The development of so-called molecular markers, which are based on polymorphisms found in proteins or DNA, has greatly facilitated research in a variety of disciplines, such as taxonomy, phylogeny, ecology, genetics and plant breeding.

The molecular methods used in assisting taxonomical studies include restriction fragment length polymorphisms in the nuclear and chloroplast genomes (Wang *et al.* 1992), random amplified polymorphic DNA fragment patterns (RAPD) (Williams *et al.* 1990), DNA amplification fingerprinting (Caetano-Anollés *et al.* 1991a, b) and sequencing of various genes or DNA segments.

RAPDs, DAFs and sequencing will be used in this study.



### 1.3.1 Random amplified polymorphic DNA (RAPD).

Molecular markers have become fundamental tools for plant biologists; they are useful for fingerprinting, phylogenetic studies, tagging genes and mapping of plant genomes. Several methods for comparing plants at molecular level have been developed, since inception of the polymerase chain reaction (PCR) (Mullis 1987). PCR result in amplification of specific portions of template DNA that occur between sequences that bind DNA synthesis primers (Yu *et al.* 1993).

The advent of automated PCR technology supplied a new set of markers available to scientists interested in comparing organisms at molecular level. In particular, the use of arbitrary primers to obtain random amplified polymorphic DNA (RAPD) markers. RAPD markers are obtained by PCR amplification of random DNA segments from single arbitrary primers. Williams *et al.* (1990) were the first scientists to use this procedure on plant samples and suggested it be named RAPD. The arbitrary primers used for the procedure are usually 9 to 10 bp in size, they have a CG content of 50 to 80% and do not contain palindromic sequences. The number of DNA fragments that are amplified is dependent on the primer and the genomic DNA used. A single nucleotide substitution in a primer can result in a complete change in the RAPD pattern, this is an indication of the sensitivity of the system. However, the method is not 100% stringent, because much larger numbers of fragments are generally observed, when bacterial genomes are used as templates, than would be predicted. Only DNA fragments within the size range of 100 to 3000bp, occurring in DNA sequences, and that are complementary to the primer sequence, are amplified. Polymorphisms for RAPDs may be due to single base pair changes, deletions of primer sites, insertions that increase the separation of primer sites over the 3000bp limits and small insertions/deletions that result in changes in the size of the PCR product. The advantages of using RAPDs are:

- 1) an universal set of primers can be used for all species,
- 2) no probe libraries or primer sequence information are required,
- 3) only the primer sequence information is needed for information transfer and
- 4) the process can be automated.

The limitation in the use of RAPDs is that they are dominant. This can be overcome by using more than one closely related marker. Efficient use of RAPD markers requires quick DNA extraction, optimum amplification conditions and appropriate data analysis.

- This same technique was used by Welsh *et al.* (1990), but under a different name (arbitrarily primed polymerase chain reaction) to fingerprint human, rice and virus genomes. With this technology, inbred maize lines could be distinguished from each other (Welsh *et al.* 1991).
- Kerr *et al.* (1995) described RAPDs as a simple and effective method to determine the relatedness of *Pseudomonas aeruginosa* isolates, and typing results are available within a single working day, thus dramatically increasing its clinical relevance over existing molecular methods.
- Stiller *et al.* (1995) successfully used RAPD analysis to determine the genetic history and current structure of *Spartina alterniflora* (Poaceae) clones in Wallapa Bay.
- RAPDs was successfully used:
  - ❖ to develop molecular markers linked to a gene controlling fruit acidity in citrus (Fang *et al.* 1997),
  - ❖ in the analysis of tetraploid *Elymus* species (Sun *et al.* 1997) and
  - ❖ for the identification of markers for percentage hull in oat (Ronald *et al.* 1997).

Landry *et al.* (1997) tried to clarify some questions related to the application of RAPDs for phylogenetic reconstruction purposes. They found that by using more primers, stability increased. They also indicated that at least 12 primers should be used to obtain a stable phylogeny. Their results also indicated that RAPDs should not be used to study phylogenetic relationships at higher taxonomic levels. In 1996 Klopper did a preliminary study on the genus *Pentaschistis* and indicated that RAPDs could have some potential in determining the phylogenetic relationships in the genus.

In this study the RAPD technique is used to determine the genetic variation in and between the species and to determine the phylogenetic relationships between 18 different *Pentaschistis* species and the six species groups described by Linder & Ellis (1990b).

### **1.3.2 DNA amplified fingerprinting (DAF).**

The amplification of anonymous polymorphic DNA fragments is based on standard PCR methodology, the distinction being that usually only one primer, with an arbitrary nucleotide sequence, is used. However, the simultaneous use of two different primers is also possible and often yields additional information (Caetano-Annollès *et al.* 1991a, b). Several

people have described different names for the use of arbitrary primers in PCR, each having a different protocol (reviewed by Caetano-Anollès *et al.* 1993). One of them being DAF (DNA amplified fingerprinting).

DAF was introduced by Caetano-Anollès *et al.* (1991a, b). Short primers are used, often only five to eight nucleotides long, with either low or high stringency annealing steps and a three-temperature, cycling programme. Resulting fragments are separated on polyacrylamide gels and visualised by silver staining. Reaction conditions could be tailored to obtain the desired level of pattern complexity. The differences between RAPD and DAF protocols are:

1. higher primer concentrations in DAF,
2. wider range of primer lengths (e.g. very short primers are often used) in DAF,
3. lower DNA concentrations in DAF and
4. a higher amount of polymorphisms in DAF.

This technique often results in highly complex banding patterns.

- Caetano-Anollès *et al.* (1995, 1996) successfully studied the origin of Bermudagrass (*Cynodon*) off-types by DNA amplification fingerprinting and laid the ground work for the identification of mistakes in plantings, mislabelled plant materials and contamination or substitutions of sod fields.
- Caetano-Anollès (1996) used DAFs with mini hairpins, harbouring arbitrary “core” sequences at their 3’ termini to fingerprint a variety of templates, including PCR products and whole genomes, to establish genetic relationships between plant taxa at the interspecific and intraspecific level, and to identify closely related fungal isolations and plant accessions.
- Weaver *et al.* (1995) used DAFs to infer genetic relationships in Centipedegrass.
- Caetano-Anollès *et al.* (1993) successfully used DAFs in the identification of markers tightly linked to the supernodulation locus in soybean.
- In 1993, Calahan *et al.*, used single or multiple primers to enhance understanding of genome divergence, cultivar identity and genetic mapping of relevant adaptive gene loci in Turfgrasses.
- Baum *et al.* (1994) achieved species identification and quantifications of genotypic diversity in root-knot nematodes by DNA amplification fingerprinting.

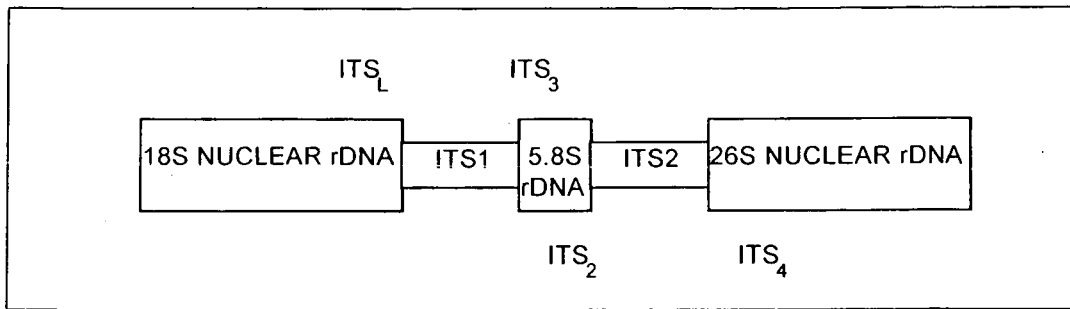
To our knowledge there have never been any studies done on *Pentascistis* by using DAFs. We will use DAFs for the first time, to determine the genetic variation in and between the species and to determine the phylogenetic relationships in the genus *Pentascistis*.

### 1.3.3 Sequencing.

Until a few years ago, most plant systematists reserved DNA sequencing for phylogenetic analyses of taxa with sequences too divergent to be easily interpreted by restriction site mapping. Consequently, only moderately to slowly evolving DNA sequences, have been widely used in plant phylogenetics (Chase *et al.* 1990, Hamby & Zimmer 1988, Hamby & Zimmer 1992). With the advent of polymerase chain reaction (PCR) technology, however, DNA sequencing is now sufficiently inexpensive and easy to use for phylogenetic studies at all taxonomic levels. This sequencing option offers increased precision and resolution by permitting better homology assessment of molecular characters and character states than is possible by restriction site mapping. The primary challenge in using nucleotide characters for lower level phylogenetic studies, is the identification of easily amplified and relatively rapidly evolving DNA regions that can provide sufficient variation with a short sequence segment (Baldwin *et al.* 1994). An example of this is the internal transcribed spacers (*ITS*) region of 18-26S nuclear ribosomal DNA. This region includes three components (Figure 1.1): the 5.8 S subunit, an evolutionary highly conserved sequence and, importantly from the perspective of this study, two spacer regions designated *ITS1* and *ITS2*. The *ITS* regions are part of the transcriptional unit of nuclear ribosomal DNA (nrDNA), but the spacer segments of the transcript are not incorporated into mature ribosomes. Instead *ITS1* and *ITS2* regions of the nrDNA transcript may function in the maturation of nrRNA's.

Several aspects of the *ITS* region promote *ITS* use for phylogenetic analysis. Firstly, along with the other components of the nrDNA multigene family, the *ITS* region is one of the most highly repeated sequences in the plant nuclear genome. The entire nrDNA repeat unit, including the subunits, *ITS1*, *ITS2*, the intergenic spacer (*IGS*) and the external transcribed spacer (*ETS*), is present in up to many thousands of copies, arranged in tandem repeats of a chromosomal locus or at multiple loci (Rodgers & Bendich 1987, Hamby & Zimmer 1992). This high copy number promotes detection, amplification, cloning and sequencing of nrDNA. Secondly, and most importantly from the phylogenetic standpoint, this gene family undergoes rapid concerted evolution (Arnheim *et al.* 1980, Hills *et al.* 1991), via unequal crossing over

and gene conversion, a property that promotes intragenomic uniformity of repeat unit and accurate reconstruction of species relationships from these sequences (Hamby & Zimmer 1992, Sanderson & Doyle 1992). Thirdly, the small size of the *ITS* region and the presence of highly conserved sequences flanking each of the two spacers makes this region easy to amplify.



**Figure 1.1.** Repeat unit of 18-26S nuclear ribosomal DNA, minus the intergenic spacer and the larger part of the 26S subunit.

- Cordesse *et al.* (1993) sequenced the rDNA Intergenic Spacer in Rice. Comparison of the rice sequence with those of maize, wheat and rye, shows that, despite considerable divergence from the ancestral sequence, several regions have been highly conserved, suggesting that they may play an important role in the structure and/or expression of the ribosomal genes.
- Hsiao *et al.* (1995a, b) studied the phylogenetic relationships of 30 diploid species of *Triticeae* and *Pooideae* (Poaceae) representing 19 genomes, from the sequences of the *ITS* region of nuclear ribosomal DNA.
- Susanna *et al.* (1995) came to the conclusion that phylogenetic analysis of *ITS* sequence variation supports the monophyly of *Cardueae* (Asteraceae).
- Kamser *et al.* (1997) and Grebenstein (1998) did the same with *Aveneae* (Poaceae) and other grasses as well as Guinea Yam species as deduced from *ITS1* and *ITS2* rDNA sequences.
- The *ITS* sequences of the following taxa were successfully investigated:
  - ❖ 22 diploid and tetraploid annual *Bromus* species of section *Bromus* (Poaceae) and three species belonging to other *Bromus* sections (Ainouche 1997),

- ❖ perennial and annual *Medicago* species (Diwan *et al.* 1997),
  - ❖ *Eragrostis tef* (Zucc.) Trotter (Pillay 1997),
  - ❖ *Abies* (Vendramin 1997),
  - ❖ of brown trout (*Salmo trutta*) (Castro *et al.* 1997),
  - ❖ the family Fouquieriaceae (Schultheis & Baldwin 1999), and
  - ❖ *Lupinus* (Aïnouche & Bayer 1999).
- Brochmann (1998) analysed fifteen populations of *Saxifraga* by using random amplified polymorphic DNA (RAPD) and nucleotide sequences of the chloroplast gene *matK* and the internal transcribed spacers of nuclear ribosomal DNA (rDNA).

The *rpoC2* gene encodes the *B* subunit of the RNA polymerase II. A portion of the *rpoC2* gene of two *Pentaschistis* species (*P. aspera* & *P. curvifolia*) has been sequenced by Barker (1995). He determined that the two species were monophyletic in their origin. In 1998 Hsiao *et al.* sequenced the *ITS* region of *Pentaschistis aspera*. These are the only sequences available on the genus *Pentaschistis*. In this study we will sequence the internal transcribed spacer region of ribosomal DNA in *Pentaschistis*.

#### 1.4 Aim.

Almost a third of the world's representatives of the tribe Arundineae are indigenous to South Africa, and thus we have an ideal opportunity to study the phylogenetic relationships within the tribe, as well as within the genus *Pentaschistis*. Therefore, the aim of this study is to determine the phylogenetic relationships between some representatives of the genus *Pentaschistis*. This will be done by a cytogenetic investigation as well as by using random amplified polymorphic DNA, DNA amplified fingerprinting and sequencing of the *ITS* region of nrDNA. The data will also be combined to give us clearer indications of the phylogenetic relationships.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 Materials.

The plants used in this study were collected in the veld. Voucher herbarium specimens (Table 2.1) are housed in the Geo Potts Herbarium (BLFU).

In this study Taq polymerase (Advanced Biotechnology's, Surrey, UK) and DNA Molecular marker VI (pBR328 DNA cleaved with a mixture of *Bgl* I and *Hinf* I) (Boehringer Mannheim Cat. no. 1062590) were used. Different primers were used for RAPDs (Operon Technologies, California), DAFs (Boehringer Mannheim) and *ITS* sequencing (DNAgency Cat. No. GK 071101 and GK 071102). The Thermo Sequence™ dye terminator cycle sequencing pre-mix kit (Amersham Life Science, US 79765 and US 79865) was used to sequence the purified nrDNA. All the other chemicals used were of analytical grade.

#### 2.2 Methods.

##### 2.2.1 Cytogenetics.

Young inflorescence were collected and fixed in Carnoy's (1886) fixative. The fixative was replaced with 70% (v/v) ethylalcohol 24-48 hours after fixation. The anthers were squashed in 2% (m/v) aceto-carmin (Darlington & La Cour 1976) on a microscope slide. Improved staining of the chromosomes was achieved by adding iron acetate (Thomas 1940) and heating the slide over a spirit flame. Liquid CO<sub>2</sub> (Bowen 1956) was used to separate the cover slip from the microscope slide for mounting the slides permanently. Removal of the cover slip was followed by dehydration in ethylalcohol and mounting in Euparal. A Nikon microphot-FXa photomicroscope and Ilford Pan-F films were used for the photomicrographs.

At least twenty cells per specimen were studied for each meiotic stage, except where otherwise indicated.

**TABLE 2.1** List of specimens studied, their voucher numbers and localities according to the degree reference system (Edwards & Leistner 1971).

*P. airoides* (Nees) Stapf subsp. *airoides*

WESTERN CAPE.—3219 (Wuppertal): In Uitkyk Pass at stream (–AC), *Spies* 6311<sup>◊</sup>.  
3323 (Willowmore): 13 km from Oudshoorn to Uniondale in Potjiesberg Pass (–CA),  
*Spies* 6152<sup>◊</sup>. 3420 (Bredasdorp): 500 m north of De Hoop nature reserve (–AB), *Spies*  
6205<sup>\*◊◊</sup>.

*P. aristifolia* Schweick.

WESTERN CAPE.—3119 (Calvinia): In Vanrhyns Pass (–AC), *Spies* 6295<sup>\*</sup>.

*P. barbata* (Nees) Linder subsp. *barbata*

WESTERN CAPE.—3119 (Calvinia): 6 km from Algeria to Citrusdal (–AC), *Spies*  
6086<sup>\*</sup>. 3218 (Clanwilliam): Leipolt's grave in Pakhuis Pass (–BB), *Spies* 6267<sup>\*◊◊</sup>.  
3219 (Wuppertal): on top of Nieuwoudt Pass (–AC), *Spies* 6321<sup>◊</sup>.

*P. calsicola* Linder var. *calsicola*

WESTERN CAPE.—3420 (Bredasdorp): 1 km North from de Hoop Nature Reserve  
(–AB), *Spies* 6209<sup>^</sup>, 6210<sup>\*</sup>.

*P. capensis* (Nees) Stapf

WESTERN CAPE.—3322 (Oudtshoorn): Swartberg Pass (–BC), *Spies* 6169<sup>\*◊</sup>.

*P. colorata* (Steud.) Stapf

WESTERN CAPE.—3219 (Wuppertal): on top of Uitkyk Pass (–AC), *Spies* 6313<sup>\*◊</sup>.

*P. curvifolia* (Schrad.) Stapf

WESTERN CAPE.—3218 (Clanwilliam): 21 km from Clanwilliam to Nieuwoudtville  
(Pakhuis Pass) (–BB), *Spies* 6024<sup>\*</sup>, 6270<sup>◊◊</sup>. 3219 (Wuppertal): in Uitkyk Pass at  
Bosbouhuis turn (–AA), *Spies* 6074<sup>\*</sup>; on top of Uitkyk Pass (–AC), *Spies* 6315<sup>\*</sup>. 3419  
(Caledon): at MTN tower on scenic route at Hermanus (–AC), *Spies* 6215<sup>\*◊◊</sup>, 6221<sup>◊</sup>;  
Shaw's Pass (–AD), *Spies* 6231<sup>◊</sup>; Galgeberg (–BA), *Spies* 6236<sup>\*</sup>.

*P. densifolia* (Nees) Stapf

WESTERN CAPE.—3219 (Wuppertal): On top of Nieuwoudt Pass (–AC), *Spies*  
6328<sup>\*◊</sup>.

*P. eriostoma* (Nees) Stapf

NORTHERN CAPE.—2917 (Springbok): 24 km from Springbok to Hondeklipbaai (–  
DB), *Spies* 5919<sup>\*</sup>. 3018 (Kamiesberg): 8 km from Kamieskroon to Leliehoek, on top



of Kamiesberg Pass (-AC), *Spies 6000*<sup>\*</sup>.

WESTERN CAPE.—3118 (Vanrhynsdorp): on top of the Gifberg Pass (-DA), *Spies 6055*<sup>\*</sup>. 3218 (Clanwilliam): 21 km from Clanwilliam to Nieuwoudtville (-BB), *Spies 6041*<sup>\*</sup>., *6269*<sup>\*<sup>o</sup></sup>. 3219 (Wuppertal): On top of Nieuwoudt Pass (-AC), *Spies 6329*<sup>\*</sup>. 3322 (Oudtshoorn): In Swartberg Pass (-AC), *Spies 6173*<sup>\*\*<sup>o</sup><sup>o</sup></sup>; 9 km from Uniondale to Oudtshoorn (-CA), *Spies 6144*<sup>o</sup>

*P. lima* (Nees) Stapf

NORTHERN CAPE.—3018 (Kamiesberg): 8 km from Kamieskroon to Leliehoek, on top of Kamiesberg Pass (-AC), *Spies 5961*<sup>\*</sup>.

*P. pallida* (Thunb.) Linder

NORTHERN CAPE.—2917 (Springbok): 24 km from Springbok to Hondeklipbaai (-DB), *Spies 5922*<sup>\*</sup>, *5924*<sup>\*</sup>.

WESTERN CAPE.—3218 (Clanwilliam): 39 km from Clanwilliam to Nieuwoudtville (-BB), *Spies 6276*<sup>o</sup>. 3419 (Caledon): At MTN tower on scenic route at Hermanus (-AC), *Spies 6281*<sup>\*\*<sup>o</sup><sup>o</sup></sup>. 3420 (Bredasdorp): 4 km North from Waenhuiskrans (-CA), *Spies 6189*<sup>o</sup>; 1 km North from de Hoop Nature Reserve (-AB), *Spies 6208*<sup>\*\*<sup>o</sup><sup>o</sup></sup>.

*P. papillosa* (Steud.) Linder

WESTERN CAPE.—3419 (Caledon): At MTN tower on scenic route at Hermanus (-AC), *Spies 6226*<sup>o<sup>o</sup></sup>.

*P. patula* (Nees) Stapf

WESTERN CAPE.—3218 (Clanwilliam): 32 km from Clanwilliam to Nieuwoudtville at Klawer turnoff (-BB), *Spies 6036*<sup>\*</sup>. 3219 (Wuppertal): Klawer-Elizabethfontein turn-off, in Pakhuis Pass (-AA) *Spies 6272*<sup>o</sup>; in Uitkyk Pass (-AC), *Spies 6069*<sup>\*</sup>.

*P. rigidissima* Pilg. ex Linder

WESTERN CAPE.—3319 (Worcester): FM tower on Matroosberg (-BC), *Spies 6243*<sup>\*\*<sup>o</sup><sup>o</sup></sup>; on top of Du Toitskloof Pass (-CA), *Spies 6109*<sup>\*\*<sup>o</sup></sup>.

*P. rupestris* (Nees) Stapf

NORTHERN CAPE.—2917 (Springbok): 24 km from Springbok to Hondeklipbaai (-DB), *Spies 5927*<sup>\*</sup>.

WESTERN CAPE.— 3219 (Wuppertal): In Uitkyk Pass (-AC), *Spies 6309*<sup>\*\*<sup>o</sup><sup>o</sup></sup>, *6308*<sup>\*\*<sup>o</sup><sup>o</sup></sup>, *6310*<sup>\*\*<sup>o</sup><sup>o</sup></sup>; on top of Nieuwoudt Pass (-AC), *Spies 6330*<sup>\*\*<sup>o</sup><sup>o</sup></sup>.

*P. tomentella* Stapf

NORTHERN CAPE.— 2916 (Port Nolloth): 3 km from Steinkopf to Port Nolloth (-

BD), *Spies* 6356<sup>\*\*^\*</sup>.2917 (Springbok): 9 km from Steinkopf to Port Nolloth on top of the Aninaus Pass (-BC), *Spies* 5898<sup>\*</sup>. 3018 (Kamiesberg): 7 km from Kamieskroon to Leliefontein (-AC), *Spies* 6342<sup>\*\*^\*</sup>, 6343<sup>\*</sup>, 6344<sup>\*\*^\*</sup>; 4 km from Kamieskroon to Leliefontein (-AC), *Spies* 6346<sup>\*</sup>; 8 km from Kamieskroon to Leliehoek on top of Kamiesberg Pass (-AC), *Spies* 5960<sup>\*</sup>; 4 km from Kamieskroon to Leliefontein (-AC), *Spies* 6345<sup>\*\*^\*</sup>; 22 km from Garies to Kamieskroon (-CA), *Spies* 6337<sup>\*\*^\*</sup>.

WESTERN CAPE.—3218 (Clanwilliam): 39 km from Clanwilliam to Nieuwoudtville (-BB), *Spies* 6277<sup>\*\*^\*</sup>, 6280<sup>\*</sup>.

*P. tortuosa* (Trin.) Stapf

WESTERN CAPE.—3420 (Bredasdorp): 5 km from Ouplaas to Malgas (-AB), *Spies* 6214<sup>\*\*^\*</sup>. 3319 (Worcester): On top of Du Toitskloof Pass (-CA), *Spies* 6102<sup>\*</sup>.3322 (Oudtshoorn): Robinson Pass (-CC), *Spies* 6179<sup>\*\*^\*</sup>.

*P. veneta* Linder

WESTERN CAPE.—3119 (Calvinia): 14 km from Nieuwoudtville to Clanwilliam (-AC), *Spies* 6001<sup>\*\*^\*</sup>.3219 (Wuppertal): on top of Nieuwoudt Pass (-AC), *Spies*6327<sup>\*\*^\*</sup>.

*P. viscidula* (Nees) Stapf

WESTERN CAPE.—3322 (Oudtshoorn): Robinson Pass (-CC), *Spies* 6178<sup>\*\*^\*</sup>.

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\* Specimens cytogenetically studied.

\*\* Specimens used for the RAPD study.

^ Specimens used for the DAF study.

^ Specimens used for sequencing.

### 2.2.2 DNA extraction.

The leaves of the different specimens (Table 2.1) were collected and stored in a saturated sodium chloride (NaCl) and hexadecyltrimethylammonium bromide (CTAB) solution (Rogstad 1992). The leaves were washed with distilled water and cut into pieces. One gram of the material was ground to a fine powder in liquid nitrogen. The tissue was immediately resuspended in warm (65°C) 1% CTAB (500µl) extraction buffer [1% (m/v) CTAB, 50mM Tris-HCl (2-amino-2(hydroxymethyl)-1,3-propanediol) (pH 8,0), 10mM

ethylene diaminetetra-acetic acid (EDTA), 0.7M NaCl (Hills & Moritz 1990), to which 1% (v/v)  $\beta$ -mercapto-ethylalcohol had been added prior to use]. This mixture was incubated for half an hour at 65°C. After incubation the same volume of chloroform:iso-amylalcohol (24:1) was added to the mixture and thoroughly mixed. This solution was centrifuged at 3 000g for five minutes. The supernatant (which contained the DNA) was transferred to a clean eppendorf tube. Ethylalcohol (two times the volume of the supernatant), containing 3M sodium acetate (25:1), was added to the supernatant to precipitate the DNA at -20°C for half an hour. This eppendorf tube was centrifuged at 10 000g for 10 minutes. The supernatant was discarded and the pellet washed with 70% (v/v) ethylalcohol, containing 10mM ammonium acetate. This pellet was air dried and dissolved in sterile water (Rogstad 1992). The DNA was visualised by intercalating it with Ethidium Bromide and studying it on an ultraviolet (UV) light illuminator.

### **2.2.3 Gel electrophoresis.**

An agarose gel [1.2% for RAPD or 0.8% for genomic DNA] was prepared by melting the desired amount of electrophoresis grade agarose in 1x TAE buffer [Tris, acetic acid and EDTA (pH 8.0)]. This mixture was cooled down to 55°C and Ethidium Bromide was added to a final concentration of 0.05  $\mu$ g/ml. Electrophoresis was done in 1x TAE running buffer.

The DNA samples (6 $\mu$ l) were mixed with 2  $\mu$ l 6x loading buffer [0.25% (m/v) bromophenol blue, 0.25% (m/v) xylene cyanol and 40% (m/v) glycerol in water] and loaded into the slots of the submerged gel. The DNA fragments were separated at 80V for 30 minutes and examined under ultraviolet (UV) light.

### **2.2.4 Photographs.**

All gels were photographed using a KALIMAR K-90 35 mm SLR camera. The image of each gel was also electronically stored with the Molecular Analyst Software Image Analysis System version 1.4 (Anonymous 1995). A thermal printer was used for some gels.

### **2.2.5 Taguchi optimisation.**

The optimisations of the PCR based RAPD and DAF analyses were done according to

the Taguchi method (Cobb & Clarkson 1994). According to this method, four reaction component optima can be assessed by using nine reactions. Each reaction component are varied in an orthogonal array by three different concentrations of each variable (Table 2.2). The primer, dNTP, MgCl<sub>2</sub> and DNA concentrations were the variable components and DNA polymerase and buffer concentrations were kept constant.

**TABLE 2.2** The orthogonal arrangement of the components to be optimised by the Taguchi method (Cobb & Clarkson 1994). Three different volumes of the four components, which are to be optimised, are varied in an orthogonal array.

Reactions	[Primer] 3.5pmol/ μl	[dNTP] 2mM/ μl	[MgCl <sub>2</sub> ] 25mM/ μl	[DNA] 1:40/ μl
1	1	2	3	2
2	2	3	3	2.5
3	3	4	3	3
4	1	3	4	3
5	2	4	4	2
6	3	2	4	2.5
7	1	4	5	2.5
8	2	2	5	3
9	3	3	5	2

### 2.2.5 RAPD.

The optimized RAPD reaction volume was 25μl and contained 2.5μl of a 10x reaction buffer with 4μl of 25mM MgCl<sub>2</sub>, 2μl of 3.5pmol primer, 4μl of 2mM dNTP mixture, 0.8 units of Taq polymerase and 2μl of a 1:40 dilution of DNA (~100ng) (diluted with sterile water). Seven primers were used. OPA<sub>7</sub> — 5'-GAA ACG GGT G-3', OPA<sub>11</sub> — 5'-CAA TCG CCG T-3', OPA<sub>16</sub> — 5'-AGC CAG CGA A-3', OPA<sub>20</sub> — 5'-GTT GCG ATC C-3' OPB<sub>3</sub> — 5'-CAT CCC CCT G-3', OPB<sub>6</sub> — 5'-TGC TCT GCC C-3' and OPC<sub>4</sub> — 5'-CCG CAT CTA C-3'. Standard amplifications were carried out through an initial denaturation step of 94°C for 60

seconds and 40 amplification cycles of 94°C for 10 seconds, 37°C for 15 seconds and 72°C for 75 seconds. Reactions were cooled down to 4°C and stored at this temperature. They were heated to 65°C for 5 minutes prior to electrophoreses.

Each reaction was duplicated in order to determine the repeatability of reactions. The RAPD products were visualised by separating the fragments on a 1.2% agarose gel at 100V for 1 hour. The gel was photographed and analysed.

### **2.2.6 DNA amplification fingerprinting.**

Amplification of the DAF reactions were similar to RAPDs (paragraph 2.2.5.), except for the annealing temperature, which was 30°C. Taguchi optimisation was also done for DAFs. In this study 12 DAF primers were used. DAF<sub>1</sub> —5'-AAC GGG TG-3', DAF<sub>2</sub> —5'-GTA ACG CC-3', DAF<sub>3</sub> —5'-GAG GGT GG-3', DAF<sub>4</sub> —5'-CCT CGT GG-3', DAF<sub>5</sub> —5'-GAA ACG CC-3', DAF<sub>6</sub> —5'-GTT ACG CC-3' DAF<sub>7</sub> —5'-CTG GAC TA-3', DAF<sub>8</sub> —5'-GTA ACG CC-3', DAF<sub>9</sub> —5'-GTA CTG CC-3', DAF<sub>10</sub> —5'-GTA AGG CC-3', DAF<sub>11</sub> —5'-CCT GCT GG-3', and DAF<sub>12</sub> —5'-CAG CTC GG-3'. Optimized reaction mixtures (25µl reactions) consisted of 10mM of dNTPs, 150mM primer, 75mM MgCl<sub>2</sub> and 10x Buffer and a 1:5 diluted sample (of the 1:40 sample used for the RAPD reactions) (~20ng) of the DNA. The DNA amplified fingerprinting products were separated on vertical poly-acrylamide slab gels (Caetano-Anollés & Bassam 1993). A 5% poly-acrylamide gel was prepared by mixing 10M poly-acrylamide (20:1 acryl-bisacrylamide), 10M urea, 10x TAE, 10% (m/v) APS, 4µl/10µl TEMED (N, N, N',N'- Tetramethylethylene-diamine) and sterile water. The amplified DNA samples were diluted (1:5), mixed with 5µl 10M urea and 2µl loading buffer and then loaded into the slots of the gel. The DNA fragments were separated for 50 minutes at 150V and visualised with silver staining (Bassam 1993, Caetano-Anollés & Gresshof 1994).

#### **2.2.6.1 Silver staining.**

The silver staining of the poly-acrylamide gel was done in eight steps: firstly the gels were fixed in 10% (v/v) acetic acid for 20 minutes and then washed with 10% (v/v) ethylalcohol for 5 minutes. Thirdly the gels were put into a potassiumdichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>3</sub>) (1g/l) and HNO<sub>3</sub> (2ml/l) solution for 5 minutes and then rinsed 3 times in deionised water for 2 minutes each. Fifthly these gels were impregnated for 30 minutes in silver nitrate (AgNO<sub>3</sub>)

(1g/l) and 1.5 ml 37% (v/v) formaldehyde (HCOH/l) (this must be done in the dark) and rinsed with sterile water for 20 seconds. The image was developed for 2-5 minutes in sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) (30g/l), 1.5 ml 37% HCOH/l and sodium thiosulfate (Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.5H<sub>2</sub>O) (2mg/l). The image development was stopped in 10% acetic acid for 5 minutes and the gels photographed.

### **2.2.7 PCR Sequencing.**

The *ITS1* and *ITS2* regions were amplified by using the polymerase chain reaction (PCR), with 40 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 90 seconds. Amplification of *ITS1* (Figure 1.1) was obtained by using the primers ITS<sub>L</sub> and ITS<sub>2</sub> {ITS<sub>L</sub>—5'-TCG TAA CAA GGT TTC CGT AGG TG-3' and ITS<sub>2</sub>—5'-GCT GCG TTC TTC ATC GAT GC-3'} (White *et al.* 1990). The *ITS2* region was amplified using the ITS<sub>3</sub> and ITS<sub>4</sub> primers {ITS<sub>3</sub>—5'-GCA TCG ATG AAG AAC GCA GC-3' and ITS<sub>4</sub>—5'-TCC TCC GCT TAT TGC TAT GC-3'} (White *et al.* 1990). The amplified DNA template was diluted with sterile water (1:60). A total volume of 11µl of this DNA template was mixed with 1 µl of one of the primers and 8 µl of the sequence reagent pre-mix. This total reaction volume of 20µl was amplified in a Perkin Elmer thermal cycler. This reaction consists of 94°C for 60 seconds and then 25 cycles of 94°C for 30 seconds, 45°C for 15 seconds and 60°C for 4 minutes. Single stranded DNA were subsequently produced using the double stranded DNA templates and only one primer. Single stranded DNA fragments were purified according to the following protocol of the Thermo Sequence™ dye terminator cycle sequencing pre-mix kit: a volume of 68µl of concentrated ethylalcohol (-20°C) was added to each reaction and mixed using a vortex and placed on ice for 15-20 minutes to precipitate the DNA. This solution of DNA and ethylalcohol was centrifuged for 15 minutes at 10 000 g and the supernatant discarded. The pellet was washed with 250-500 µl of 70% (v/v) ethylalcohol (-20°C) and briefly centrifuged. The supernatant was discarded and the pellets were vacuum dried for 2-5 minutes. The pellets were resuspended in 4µl of formamide loading dye. Each sample was heated to 70°C for 2-5 minutes and then placed on ice. The entire sample was loaded onto the sequencing gel and run on the ABI 377 automated sequencer for four to six hours.

## **2.3 Statistical analysis.**

### **2.3.1 Analysis of Taguchi products.**

Using the Taguchi method, the product yield for each reaction was used to estimate the effects of individual components on amplification. This is done by using the quadratic loss function, which Taguchi refers to as signal-to-noise ratios:

$$\text{SNL} = -10 \log [1/n \sum 1/y_i^2],$$

where SNL is the signal-to-noise ratio, n is the number of levels and y is the yield. The yield was calculated according to the equation  $P = (r \times s) + 1$ , where P is the product yield, r is the number of products and s is the size range. For each component the optimal conditions are those that give the largest SNL. The reactions are further refined by using the polynomial regression from SNL values for each component to obtain curves, the maximum of which represent the reaction optima.

### **2.3.2 Consistency test.**

The consistency for each primer, the degree of similarity in and between groups, was calculated by using the coefficient of similarity (Nei & Li 1979).

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

where  $X_{1,2}$  is the number of fragments shared,  $X_1$  and  $X_2$  are the number of fragments in each of the individual reactions and F is the coefficient of similarity (Nei 1987; Parker *et al.* 1991). An F value of one illustrates total similarity.

The Nei formula (Nei 1987) was used to calculate the genetic distance.

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

where D is the pairwise genetic distance among the samples.

### 2.3.3 Computer analysis.

The fragments on the schematic representation, given by the Molecular Analyst fingerprinting plus software version 1.4 (Anonymous 1995) computer program, with the three steps of conversion, normalisation and analysis, were scored (1 = fragment present, 0 = fragment absent). Compensation was made for the alignment difficulties of the gel images during scoring. For phylogenetic analyses the computer program Phylogenetic Analysis using Parsimony (PAUP) version 3.1.1 (Swofford 1993), which connect taxa, one at a time, until all the taxa have been added, were used. The heuristic search option with branch swapping was used, with tree bisection-reconnection (TBR) branch swapping and MULTIPARS selected. If more than one equally parsimonious cladogram was obtained, the strict consensus cladogram option was used. A bootstrap was done from 200 replications, using the heuristic option (Felsenstein, 1985). Jackknife (Lanyon 1985, Siddall 1995) was performed where the taxa were sequentially deleted. This program is available through Random Cladistics (Siddall 1994). Relative levels of support for clades present in the most parsimonious trees were assessed by calculating decay values (Bremer 1988), but in most cases the computer resulted in insufficient memory and therefore no decay values were obtained. A search for multiple islands of parsimony was also conducted in cases where the cladogram yielded a RI value of less than 0.67 (Maddison 1991). Another phylogenetic tree was obtained by using the computer program Hennig86 (Farris 1988), which considers the information provided by each character one at a time. The *mhennig*, *branch braking*, *ie* options were performed as well as *nelsen* for the consensus tree.

Some characters were temporarily removed, because their CI values (indicating homoplasy) are higher than their RI values (representing the amount of synapomorphies, retained on the cladogram) (Lipscomb 1998). Because the identification of synapomorphic characters is the first step in a phylogenetic reconstruction, high RI values are preferable for the phylogenetic cladogram. A character with a high homoplasy can thus still be informative if it has a high RI value. Only characters with a CI value of less than 0.2 and a CI:RI ratio of less than 2 were excluded.

The possibility of hybrids present was also tested. Taxon jackknifing, which can identify species, whose removal markedly reduces the number of equally parsimonious trees recovered and increases resolution of the clades, was used. This impact on tree topology could be explained by a hybrid origin of that excluded species. (McDade 1992, Campbell 1997,



Alice & Campbell 1999).

Most sequence analyses were carried out using programs designed for, or adapted to, the Apple Macintosh. Basic analysis was carried out using the Sequence Navigator DNA and Protein Sequence Comparison Software (Applied Biosystems 1994). The sequence of *ITS1* and *ITS2* regions were aligned using the computer program Clustal W version 1.6 (Thompson *et al.* 1994). For further alignment the computer program MALIGN (Wheeler & Gladstein 1991), which uses parsimony as an optimality criterion to select among possible alignments, was used. This constructs alignment topologies via different sequence additions and improve them through branch swapping. Final alignment was visually examined and manually optimised for phylogenetic analysis, using Clustal X (Thompson *et al.* 1997).

## CHAPTER 3

### CYTOGENETICS

#### 3.1 Introduction.

Gregor Mendel (1865), with his artificial hybridisation experiments laid the foundation for the science genetics with the garden pea (*Pisum sativum*). Unfortunately Mendel's paper was not widely known or appreciated until 1900, when, as a result of their own investigations, de Vries, Correns and Von Tschermak, independently rediscovered Mendel's laws of inheritance. From his experiments Mendel formulated three basic principles of heredity: 1) the law of uniformity in F<sub>1</sub>, 2) the law of segregation and 3) the law of independent assortment.

Sutton and Boveri (1903) independently reported that the hereditary factors (genes) are located on the chromosomes and thus cytogenetics was born (Peters 1959). Once it was established that chromosomes are the vehicles of genes, cytogenetics flourished. Cytogenetic studies quickly led to numerous extensions of genetic information and interpretation (Singh 1993).

Cytogenetics is a science that combines cytology and genetics. Such a study includes chromosome staining techniques, mitosis, meiosis and karyotype analysis. Cytogenetic studies have made landmark contributions to biological knowledge (Singh 1993). This combined discipline has been especially powerful in explaining hereditary phenomena. Cytogenetics extends beyond just an understanding of the transmission and continuity of genes and chromosomes. It is also concerned with the molecular structure of the chromosome and attempts to relay this in terms of genetic function (Swanson *et al.* 1967).

From the first experiments of Mendel on *Pisum sativum*, chromosome studies have been used in a wide variety of plants and animals: from cytogenetic experiments on *Drosophila*, to cytogenetic studies on *Zea mays* and other species of the family Poaceae. Because grasses cover a third of the lands surface, Poaceae (grasses) is one of the most important families (Gibbs Russel *et al.* 1990). Therefore, it is important to understand the hereditary factors involved in such a family and many studies have been done on these

species. The Zimbabwean grasses (Poaceae) (Moffet & Hurcombe 1949, Davidse *et al.* 1986), the genus *Danthonia* (= *Merxmuellera*) (De Wet *et al.* 1954a, Pienaar 1955), as well as the chromosome numbers of various other South African grasses (De Wet 1954b, De Wet *et al.* 1956, De Wet 1960, Voster & Liebenberg 1977, Spies & Du Plessis 1986a, b, Liebenberg 1986, De Wet 1987, Du Plessis & Spies 1987a, b, Spies & Jonker 1987, Du Plessis & Spies 1988, Spies & Du Plessis 1988, Spies & Gibbs Russel 1988, Spies & Voges 1988, Spies *et al.* 1989, Spies *et al.* 1990, Spies *et al.* 1991, Spies & Du Plessis 1992, Liebenberg 1993, Spies *et al.* 1994a, b, Strydom & Spies 1994, Visser & Spies 1994a, b, c, d, e, Spies & van Wyk 1995, Spies *et al.* 1996a, b, Spies *et al.* 1997, Klopper *et al.* 1998, Visser *et al.* 1998a, b, c). These are just a few of the studies that have already been done and many more are still in progress.

Hedberg (1957), De Wet (1960), Tateoka (1965a & b), Hedberg & Hedberg 1977, Du Plessis & Spies (1988), Spies & Du Plessis (1988), Du Plessis & Spies (1992), Spies *et al.* (1994) and Klopper *et al.* (1998), have previously studied the genus *Pentaschistis* cytogenetically. These studies indicate that the genus has basic chromosome numbers of  $x = 7$  &  $13$  (Table 3.1). There are thus two basic chromosome numbers present in *Pentaschistis*. The most common being  $x = 7$  (26 species), the second basic chromosome number  $x = 13$  is only observed in six species (Table 1.3). Klopper (1996) compared the groupings of Linder & Ellis (1990b) and Ellis & Linder (1990) with cytogenetic data, but could find no correlation with these groupings. However, she concluded that *P. eriostoma*, the only  $x = 13$  species studied by her, is not closely related to the other species of the genus *Pentaschistis*.

The aim of this study is to determine the polyploid levels and type of ploidy present in the different specimens in order to interpret the molecular studies and the phylogenetic relationships among the species.

### **3.2 Results and Discussion.**

Haploid chromosome numbers determined during this study varied from  $n = 7$  to  $n = 49$ . These numbers confirm the basic chromosome numbers of  $x = 7$ , and multiples thereof, as well as  $x = 13$ , and multiples thereof. The basic chromosome number of 13 was observed in *P. eriostoma* only. This could be due to the fact that we have only worked with southern African species. The basic chromosome number of  $x = 7$  predominates in south-

**TABLE 3.1** List of the polyploid levels in *Pentaschistis* from all existing chromosome data. \* indicates plants used in this study.

SPECIES	n	VOUCHER NUMBER	REFERENCE
<i>P. airoides</i> subsp. <i>airoides</i>	7	Davidse 33330, 33356, 33379, 33399, 33785, Spies 3113, 3130, 3166, 3368, 4586, 5789.	Spies & Du Plessis 1988, Spies <i>et al.</i> 1994, Klopper <i>et al.</i> 1998
	7+0-2B	Davidse 33787, Spies 3036, 3102	Spies <i>et al.</i> 1994
	14	Davidse 33307, Spies 3133, 3145, 3146, 3151, 4296, 4317, 4394, 4573.	Spies & Du Plessis 1988, Spies <i>et al.</i> 1994
	21	Spies 4388.	Klopper <i>et al.</i> 1998
	28	Spies 3088.	Du Plessis & Spies 1988
subsp. <i>jugorum</i>	14	Spies 3390, 4734.	Klopper <i>et al.</i> 1998
<i>P. argentea</i>	21	Ellis 5494, Spies 3258.	Du Plessis & Spies 1992
<i>P. aristidoides</i>	14	Spies 3119, 3132.	Spies & Du Plessis 1988, Du Plessis & Spies 1988
<i>P. aristifolia</i>	14	Spies 3119, 3132.	Spies & Du Plessis 1988, Du Plessis & Spies 1988
<i>P. aurea</i> subsp. <i>aurea</i>	7	Ellis 5539, 5570.	Du Plessis & Spies 1992
<i>P. barbata</i> subsp. <i>barbata</i>	14	Spies 3165.	Du Plessis & Spies 1988
	42	Spies 3184.	Du Plessis & Spies 1988
	91/2	Spies 3178.	Du Plessis & Spies 1988
<i>P. borussica</i>	13	Bie 6612:1,2,5, Hedberg 1176, Tateoka 3379.	Hedberg 1957, Tateoka 1965
	2n = 39	Bie 66124:4.	
<i>P. capensis</i>	7+0-2B	Spies 6169.	*
<i>P. capillaris</i>	7	Davidse 34006, Spies 3388, 3389, 3810, 5804.	Du Plessis & Spies 1992 Du Plessis & Spies 1992

	7+2B	<i>Spies 3413.</i>	Klopper <i>et al.</i> 1998 Klopper <i>et al.</i> 1998 Du Plessis & Spies 1992 Du Plessis & Spies 1992
<i>P. cirrhulosa</i>	7 14+0-2B	<i>Spies 4627.</i> <i>Spies 3243.</i>	Klopper <i>et al.</i> 1998 Du Plessis & Spies 1988
<i>P. colorata</i>	14	<i>Spies 5403.</i>	Klopper <i>et al.</i> 1998
<i>P. curvifolia</i>	7 7+0-2B 7+0-4B 7+1B	<i>Spies 3487, 3631, 3888, 4443, 5401.</i> <i>Spies 3486, 3488.</i> <i>Spies 6236.</i> <i>Spies 6215.</i>	Du Plessis & Spies 1992, Klopper <i>et al.</i> 1998 Du Plessis & Spies 1992 * *
<i>P. densifolia</i>	7 28	<i>Spies 3878a.</i> <i>Spies 6328</i>	Klopper <i>et al.</i> 1998 *
<i>P. eriostoma</i>	13 13+0-1B 13+1B 13+0-2B 13+0-3B 26 26+0-2B 26+2B 39+0-4B 91/2	<i>Davidse 33577, Spies 3479, 3528.</i> <i>Spies 5370.</i> <i>Spies 3581.</i> <i>Spies 3850, 5470.</i> <i>Spies 5372.</i> <i>Spies 3144, 5337.</i> <i>Spies 5339.</i> <i>Spies 3254.</i> <i>Spies 5319.</i> <i>Davidse 33348.</i>	Du Plessis & Spies 1992 Klopper <i>et al.</i> 1998 Du Plessis & Spies 1992 Klopper <i>et al.</i> 1998 Klopper <i>et al.</i> 1998 Spies & Du Plessis 1988, Klopper <i>et al.</i> 1998 Klopper <i>et al.</i> 1998 Du Plessis & Spies 1992 Klopper <i>et al.</i> 1998 Du Plessis & Spies 1992
<i>P. lima</i>	c42	<i>Ellis 5422.</i>	Klopper <i>et al.</i> 1998
<i>P. malouinensis</i>	7	<i>Spies 3259.</i>	Spies & Du Plessis 1988
<i>P. manni</i>	2n = 26 c39 2n = 40 2n = 52	<i>Hedberg 4208.</i> <i>Hedberg 2329.</i> <i>Hedberg 2329.</i>	Hedberg 1957, Hedberg 1977

<i>P. minor</i>	2n = 26	<i>Hedberg 4437, 4487.</i>	Hedberg 1957, Tateoka 1965
	2n = 52	<i>Hedberg 1773.</i>	
<i>P. natalensis</i>	7	<i>Linder 4717.</i>	Du Plessis & Spies 1992
	14	<i>Davidse 6558.</i>	Davidse <i>et al.</i> 1986
<i>P. pallida</i>	7	<i>Davidse 33837, Spies 3493, 3650, 3840, 4534, 5367, 5393, 5476.</i>	Klopper <i>et al.</i> 1998, Du Plessis & Spies 1992
	7+0-1B	<i>Spies 4406.</i>	Klopper <i>et al.</i> 1998
	7+0-3B	<i>Spies 5368.</i>	Klopper <i>et al.</i> 1998
	7+2B	<i>Spies 3658.</i>	Du Plessis & Spies 1992
	7+1-2B	<i>Spies 3652.</i>	Du Plessis & Spies 1992
	14	<i>Spies 3828, 3859, 3860, 5292, 5381, 3458, 3103, 3239, 6208.</i>	Klopper <i>et al.</i> 1998, Du Plessis & Spies 1992, Du Plessis & Spies 1988, *
<i>P. papillosa</i>	21	<i>Spies 3408, 4409, 3407.</i>	Du Plessis & Spies 1992
	7	<i>Spies 3440.</i>	Du Plessis & Spies 1992
	7+2B	<i>Spies 3611.</i>	Du Plessis & Spies 1992
<i>P. patula</i>	7+4B	<i>Spies 3651.</i>	Du Plessis & Spies 1992
	7	<i>Spies 3179, 3390, 3401, 3403, 3409, 3411, 4326, 4327, 4345, 4368.</i>	Spies <i>et al.</i> 1994
<i>P. pictigluma</i>	7+ 0-3B	<i>Spies 3391, 4346.</i>	Spies <i>et al.</i> 1994
	13	<i>Hedberg 5440, 5604.</i>	Hedberg 1977
<i>P. rigidissima</i>	7	<i>Spies 6243.</i>	*
	7+0-2B	<i>Spies 5431.</i>	Klopper <i>et al.</i> 1998
	21	<i>Spies 5458.</i>	Klopper <i>et al.</i> 1998
<i>P. rupestris</i>	28+0-1B	<i>Spies 5796.</i>	Klopper <i>et al.</i> 1998
	35	<i>Spies 6308, 6330.</i>	*
	49	<i>Spies 6309, 6310.</i>	*
<i>P. tomentella</i>	7	<i>Spies 2985, 3379, 3773, 4306, 6343.</i>	Du Plessis & Spies 1988, Klopper <i>et al.</i> 1998, *

	7+0-2B	<i>Spies 3379, 6344.</i>	Du Plessis & Spies 1992, *
	14	<i>Spies 3008, 3782, 3878b, 5765.</i>	Spies & Du Plessis 1988, Klopper <i>et al.</i> 1998
	14+2B	<i>Spies 2996.</i>	Du Plessis & Spies 1988
<i>P. tortuosa</i>	7	<i>Davidse 33645, Spies 3489, 3521.</i>	Du Plessis & Spies 1992
	28	<i>Spies 6214.</i>	*
<i>P. trisetata</i>	7	<i>Davidse 33416, 33430, 33445, Spies 3410, 3414, 4413.</i>	Du Plessis & Spies 1992, Klopper <i>et al.</i> 1998
<i>P. trisetidoides</i>	13	<i>Hedberg 5343.</i>	Hedberg 1977
<i>P. veneta</i>	14	<i>Spies 6327.</i>	*
<i>P. viscidula</i>	14 +0-2B	<i>Spies 6178.</i>	*
	21	<i>Spies 3520.</i>	Klopper <i>et al.</i> 1998
<i>P. species</i>	7+0-2B	<i>Spies 5336, 5768.</i>	Klopper <i>et al.</i> 1998

ern African species, whereas  $x = 13$  prevails in species from the remainder of Africa (Du Plessis & Spies 1992).

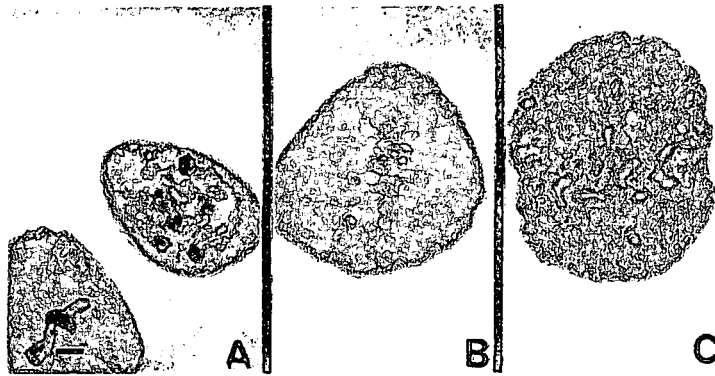
Polyploidy is a common phenomenon in the genus *Pentaschistis*, and ten of the seventeen species used in this study include polyploid specimens. The ploidy levels ranged from diploid to 14-ploid. Six specimens were diploid, three tetraploid, one hexaploid, two octaploid, two decaploid and two 14-ploid.

Seventeen specimens, representing ten species and the different groups in *Pentaschistis* (Table 2.1) have been studied. In this discussion data from this study will be compared with previous studies. The species of the genus *Pentaschistis* will be discussed in alphabetical order.

- *Pentaschistis acinosa* Stapf has never been cytogenetically studied.
- In previous studies on *P. airoides* subsp. *airoides* (Nees) Stapf four polyploid levels, diploidy, tetraploidy, hexaploidy and octaploidy have been observed (Spies & Du Plessis 1988, Du Plessis and Spies 1988, Spies *et al.* 1994, Klopper *et al.* 1998). All specimens were collected in the western Cape.

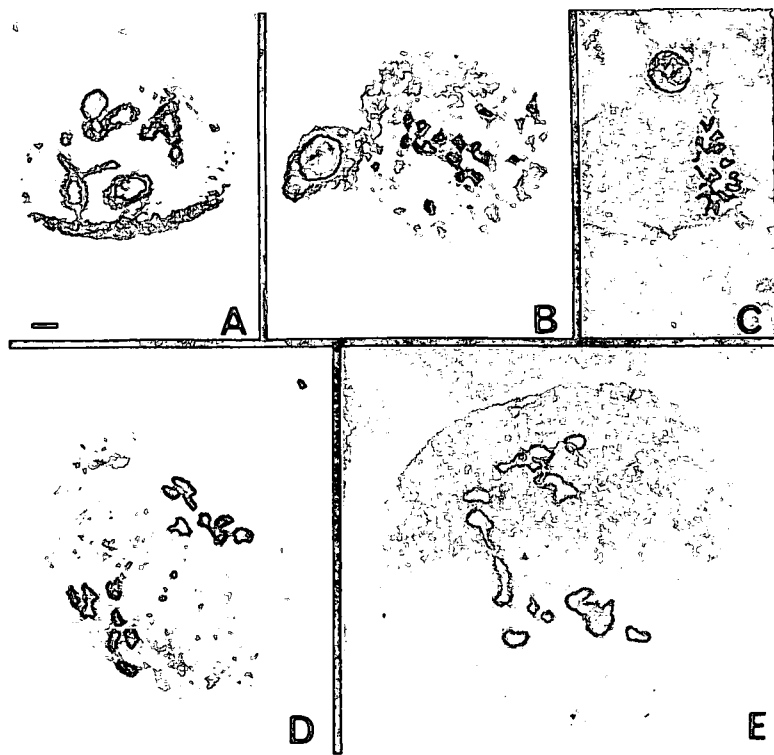
- In *P. aroides* subsp. *jugorum* (Stapf) Linder only tetraploidy has been observed (Klopper *et al.* 1998). Although this subspecies are distributed from the Drakensberg, to the Katberg and west to Bloemfontein (Linder & Ellis 1990a), the specimen used in this study was collected in the eastern Cape.
- *Pentaschistis alticola* Linder, *P. ampla* (Nees) McClean, *P. andringitrensis* A. Camus, *P. angulata* (Nees) Adamson and *P. angustifolia* (Nees) Stapf have never been studied.
- In *P. argentea* (Stapf) hexaploidy has been observed in the western Cape species.
- Diploidy has been observed in previous studies on *P. aristidoides* (Thunb.) Stapf (Du Plessis & Spies 1988, Spies & Du Plessis 1988).
- In *P. aristifolia* Schweick tetraploidy has been observed.
- *Pentaschistis aspera* (Thunb.) Stapf has never been studied.
- In *P. aurea* (Steud.) McClean subsp. *aurea* diploidy was observed in both specimens previously studied (Du Plessis & Spies 1992).
- *Pentaschistis aurea* (Steud.) McClean subsp. *pilosogluma* (McClean) Linder have never been studied.
- In *P. barbata* (Nees) Linder subsp. *barbata* three polyploid levels, namely tetraploidy, 12-ploidy and 13-ploidy have been observed.
- *Pentaschistis barbata* (Nees) Linder subsp. *orientalis* Linder and *P. basutorum* Stapf have never been studied.
- In *P. borussica* (K. Schum.) Pilg. two polyploid levels, namely diploidy and tetraploidy have previously been observed (Hedberg 1957 and Tateoka 1965b).
- *Pentaschistis calsicola* Linder var. *calsicola* and *P. calsicola* Linder var. *hirsuta* have never been cytogenetically studied.
- This is the first time that the chromosome number of *P. capensis* (Nees) Stapf is determined. *Pentaschistis capensis* is diploid ( $n = x = 7 + 0 - 2B$ ), with 0-2 B-chromosomes present (Figure 3.1A, B & C) and a normal meiosis.
- In *P. capillaris* (Thunb.) McClean only diploidy has been observed (Du Plessis & Spies 1988 and Klopper *et al.* 1998).
- *Pentaschistis caulescens* Linder, *P. chippindalliae* Linder and *P. chrysurus* (K. Schum.) Peter have never been studied.





**Figure 3.1** Photomicrographs of meiotic chromosomes in the genus *Pentaschistis*. A - C, *P. capensis* (Spies 6169): A, diakinesis with 7<sub>II</sub>; B, metaphase I with 7<sub>II</sub> + 2B; C, anaphase I. Scale bar = 4.8  $\mu$ m.

- *Pentaschistis cirrhulosa* (Nees) Linder produced diploid (Klopper *et al.* 1998), as well as tetraploid (Du Plessis & Spies 1988) specimens from the western Cape.
- In *P. colorata* only one specimen from the western Cape has been studied. This species proved to be tetraploid (Klopper *et al.* 1998).
- Both *P. curvifolia* (Schrad.) Stapf specimens examined were diploid (Spies 6215  $n = x = 7 + 1B$ , Spies 6236  $n = x = 7 + 0-4B$ ) (Figure 3.2A, B & D, E). Previously studied specimens were all diploid. In Spies 6236 an anaphase I bridge was observed, which might be due to parasentric inversion. B chromosomes are also common in *P. curvifolia*. The occurrence of 4B-chromosomes in one plant could indicate occasional non-disjunction of the B-chromosomes at a division prior to the second microspore division, thus giving a generative nucleus with 2B-chromosomes, and hence some male nuclei with 4B-chromosomes (Dawson 1962).
- One *P. densifolia* (Nees) Stapf specimen has been studied (Klopper *et al.* 1998), this specimen is octaploid ( $n = 4x = 28$ ), with no abnormalities during meiosis (Figure 3.2C). Previous studies indicate diploidy.
- *Pentaschistis ecklonii* (Nees) McClean and *P. elegans* (Nees) Stapf have never been studied.



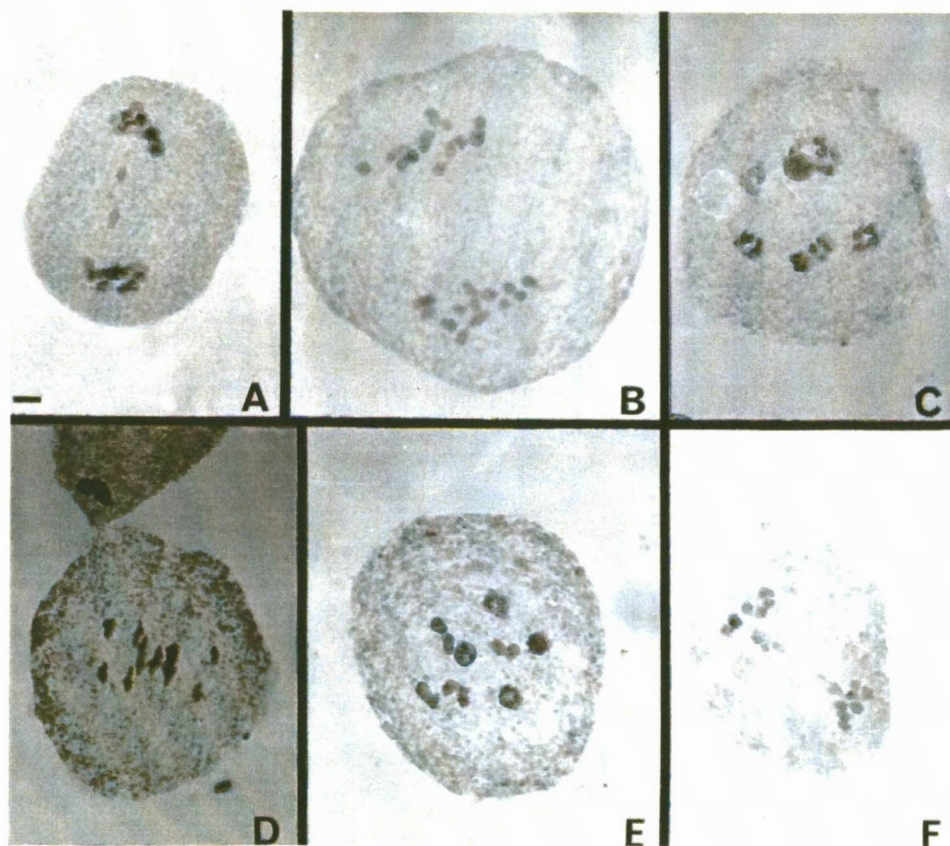
**Figure 3.2** Photomicrographs of meiotic chromosomes in the genus *Pentaschistis*. A - B, *P. curvifolia* (Spies 6315): A, diakinesis with 3II 2IV; B, anaphase I + 1B. C, *P. densifolia*, Spies 6329, diakinesis. D - E, *P. curvifolia* (Spies 6236): D, anaphase I + 4B; E, anaphase I bridge. Scale bar = 4.8  $\mu$ m.

- *Pentaschistis eriostoma* (Nees) Stapf, is the only species with a basic chromosome number of  $x = 13$ . Previous studies indicate diploidy (Du Plessis & Spies 1992, Klopper *et al.* 1998) tetraploidy (Du Plessis & Spies 1992, Du Plessis & Spies 1988 and Klopper *et al.* 1998), hexaploidy (Klopper *et al.* 1998) as well as heptaploidy (Du Plessis & Spies 1992).
- *Pentaschistis exerta* Linder, *P. galpinii* (Stapf) McClean, *P. glandulosa* (Schrad.) Linder, *P. gracilis* S. M. Phillips, *P. heptamera* (Nees) Stapf, *P. holciformis* (Nees) Linder, *P. humbertii* A. Camus. and *P. imatongensis* C. E. Hubb. have never been studied.
- In *P. lima* only one specimen from the western Cape was examined and showed a somatic chromosome number of 84.
- *Pentaschistis longipes* Stapf has never been studied.
- *Pentaschistis malouinensis* was diploid (Spies & Du Plessis 1988).
- In *P. manni* a somatic chromosome number of 78, with a basic chromosome

number of  $x = 13$ , as well as diploidy and octaploidy were observed (Hedberg 1957, Hedberg & Hedberg 1977).

- *Pentaschistis microphylla* (Nees) McClean has never been studied.
- In *P. minor* (Ballard & C. E. Hubb.) Ballard & C. E. Hubb. two polyploid levels, diploidy and octaploidy, were observed (Hedberg 1957, Tateoka 1965).
- *Pentaschistis montana* Linder has never been studied.
- In *P. natalensis* Stapf diploidy (Du Plessis & Spies 1992), as well as tetraploidy (Davidse *et al.* 1986) were observed.
- *Pentaschistis oreodoxa* Schweick and *P. pallescens* (Schrad.) Stapf have never been studied.
- In *P. pallida* (Thunb.) Linder: The only specimen examined is tetraploid ( $n = 2x = 14$ ). This was also observed in earlier studies and Du Plessis & Spies (1992) observed diploidy and hexaploidy in this species. Univalents (metaphase I) and laggards (anaphase I) were observed in some of the cells (Figure 3.3A, B).
- In *P. papillosa* (Steud.) Linder diploidy (Du Plessis & Spies 1992) were observed and all specimens were collected in the same geographical territory.
- In *P. patula* (Nees) Stapf all the specimens previously studied, were diploid and all specimens were collected in the western Cape.
- *Pentaschistis pictigluma* (Steud.) Pilg. diploidy was observed (Hedberg & Hedberg 1977).
- *Pentaschistis praecox* Linder, *P. pseudopallescens* Linder, *P. pungens* Linder, *P. pusilla* (Nees) Linder, *P. pyrophila* Linder and *P. reflexa* Linder have never been studied.
- *Pentaschistis rigidissima* Pilg. ex. Linder was diploid ( $n = x = 7$ ) (Figure 3.3C & D) as described by Klopper *et al.* (1998). In previous studies hexaploidy is also observed. More ring than rod bivalents were observed and the number of chiasmata per bivalent was 1.7.
- *Pentaschistis rosea* Linder has never been studied.
- The highest chromosome number so far known in *Pentaschistis* is that of *P. angulata*, with  $2n = 13x = 91$ . In this study *P. rupestris* (Nees) Stapf, however, has a chromosome number of  $2n = 14x = 98$  (Figure 3.4A, B & C) (Spies 6309, 6310). Univalents are observed, which are expected in a plant with such a high polyploid

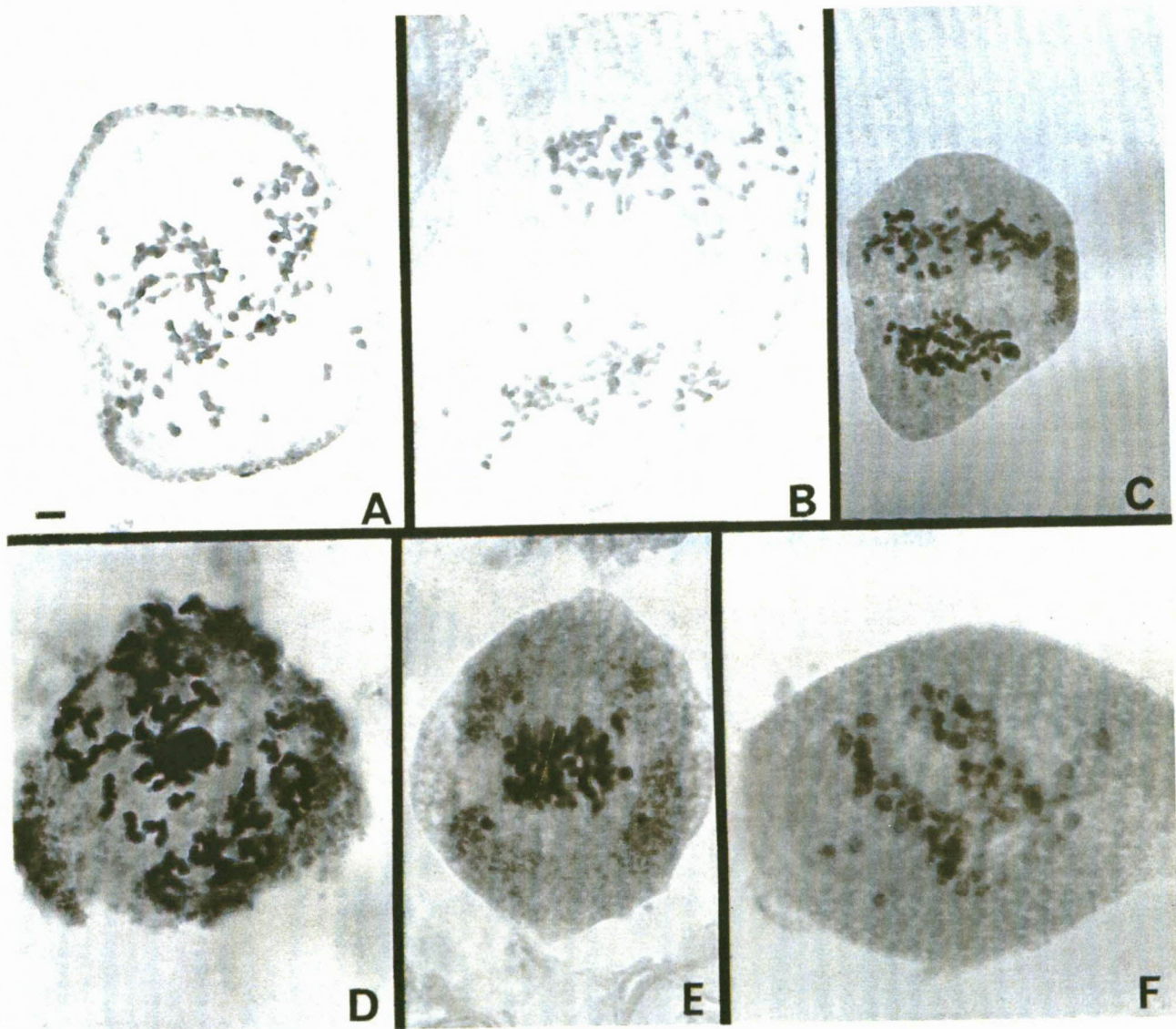
level, due to competition during pairing (Spies & Du Plessis 1988). Because no multivalents are present, autopoloidy is not expected, as this plant has such a high polyploid level that it would have led to meiotic instability and consequently to sterility (Swanson *et al.* 1969, Stebbins 1971). Another polyploid level namely decaploidy ( $n = 5x = 35$ ), has also been observed (Figure 3.4D, E & F) (Spies 6308, 6330). Previous studies also indicates octaploidy (Klopper *et al.* 1998).



**Figure 3.3** Photomicrographs of meiotic chromosomes in the genus *Pentaschistis*. **A - B**, *P. pallida* (Spies 6208): **A**, anaphase I with two laggards; **B**, anaphase I. **C - D**, *P. rigidissima* (Spies 6243): **C**, diakinesis with  $7_{II}$ ; **D**, anaphase I. **E - F**, *P. tomentella*: **E**, Spies 6344, diakinesis with  $7_{II} + 2B$ ; **F**, Spies 6343, anaphase I. Scale bar = 4.8  $\mu$ m.

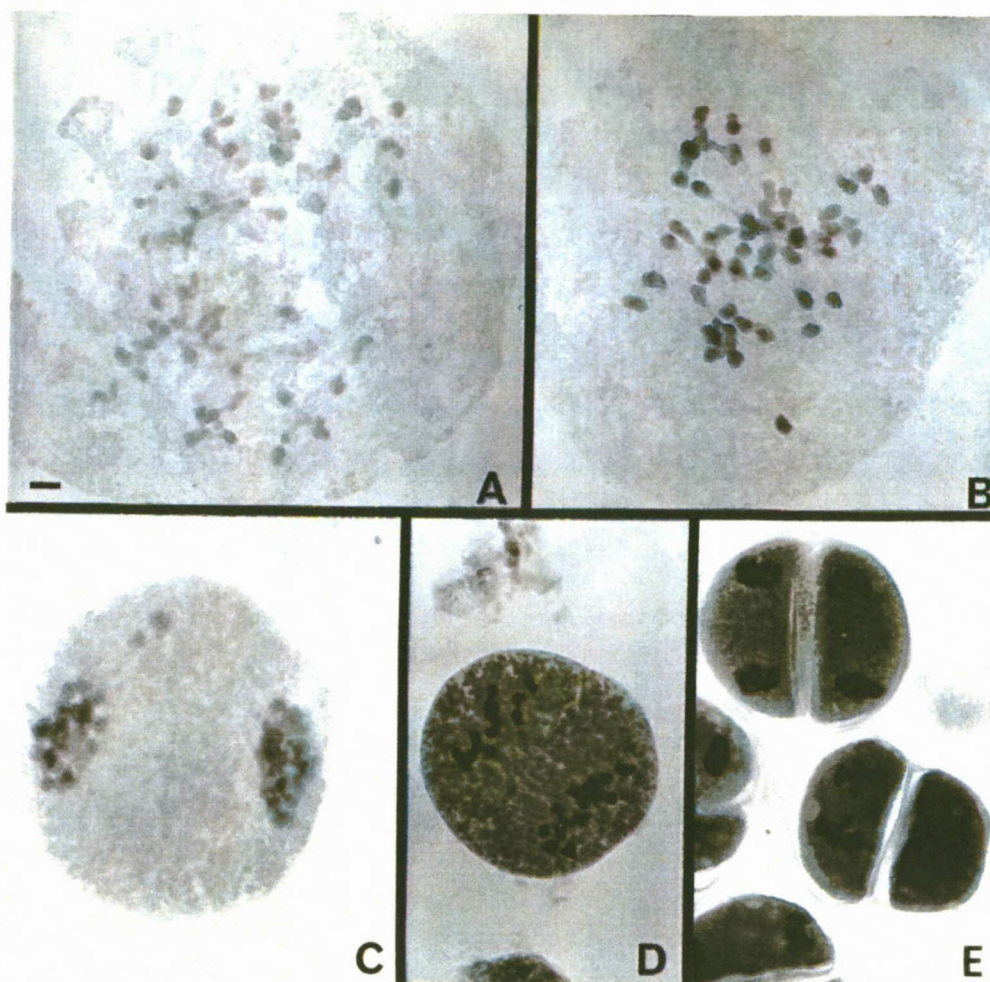
- *Pentaschistis scandens* Linder has never been studied.
- *Pentaschistis setifolia* (Thunb.) McClean has never been studied.
- *Pentaschistis tomentella* Stapf: Both specimens studied were diploid ( $n = x = 7$ ), with more ring than rod bivalents present (Figure 3.3E & F). Spies 6344 contains 0-2B chromosomes per cell. Du Plessis & Spies (1988) observed univalents in 30%

of the studied cells, as well as anaphase I bridges. Tetraploidy is also common in this species, as described by Klopper *et al.* (1996), Du Plessis & Spies (1988) and Spies & Du Plessis (1988).



**Figure 3.4** Photomicrographs of meiotic chromosomes in *Pentaschistis rupestris*. A - B, Spies 6309: A, anaphase I; B, anaphase I. C, Spies 6310, anaphase I; D, Spies 6308, diakinesis; E, Spies 6308, anaphase I. F, Spies 6330, anaphase I. Scale bar = 4.8  $\mu\text{m}$ .

- Previously described specimens of *P. tortuosa* (Trin.) Stapf indicated diploidy, but in this study octaploidy ( $n = 4x = 28$ ) was observed. Two univalents were observed during metaphase I in 50% of the cells, as well as two micronuclei during telophase I in 50% of the cells studied (Figure 3.5A, B & C).



**Figure 3.5** Photomicrographs of meiotic chromosomes in the genus *Pentaschistis*. **A -C**, *P. tortuosa* (Spies 6214): **A & B**, anaphase I; **C**, telophase I with two micronuclei. **D - G**, *P. viscidula*: **D**, Spies 6178, anaphase I; **E**, Spies 6178, anaphase II and telophase II. Scale bar = 4.8  $\mu\text{m}$

- In *P. trisetata* (Thunb.) Stapf only diploidy was observed from specimens collected in the western Cape.
- In *P. trisetoides* (Hochst. ex Steud.) Pilg. diploidy was observed.
- *Pentaschistis tysonii* Stapf, *P. velutina* Linder and have never been examined.
- In *P. veneta* Linder the only specimen studied was tetraploid ( $n = 2x = 14$ ). During this study we also observed univalents during metaphase I, as well as laggards during anaphase I (Figure 3.6A & B) in some cells.

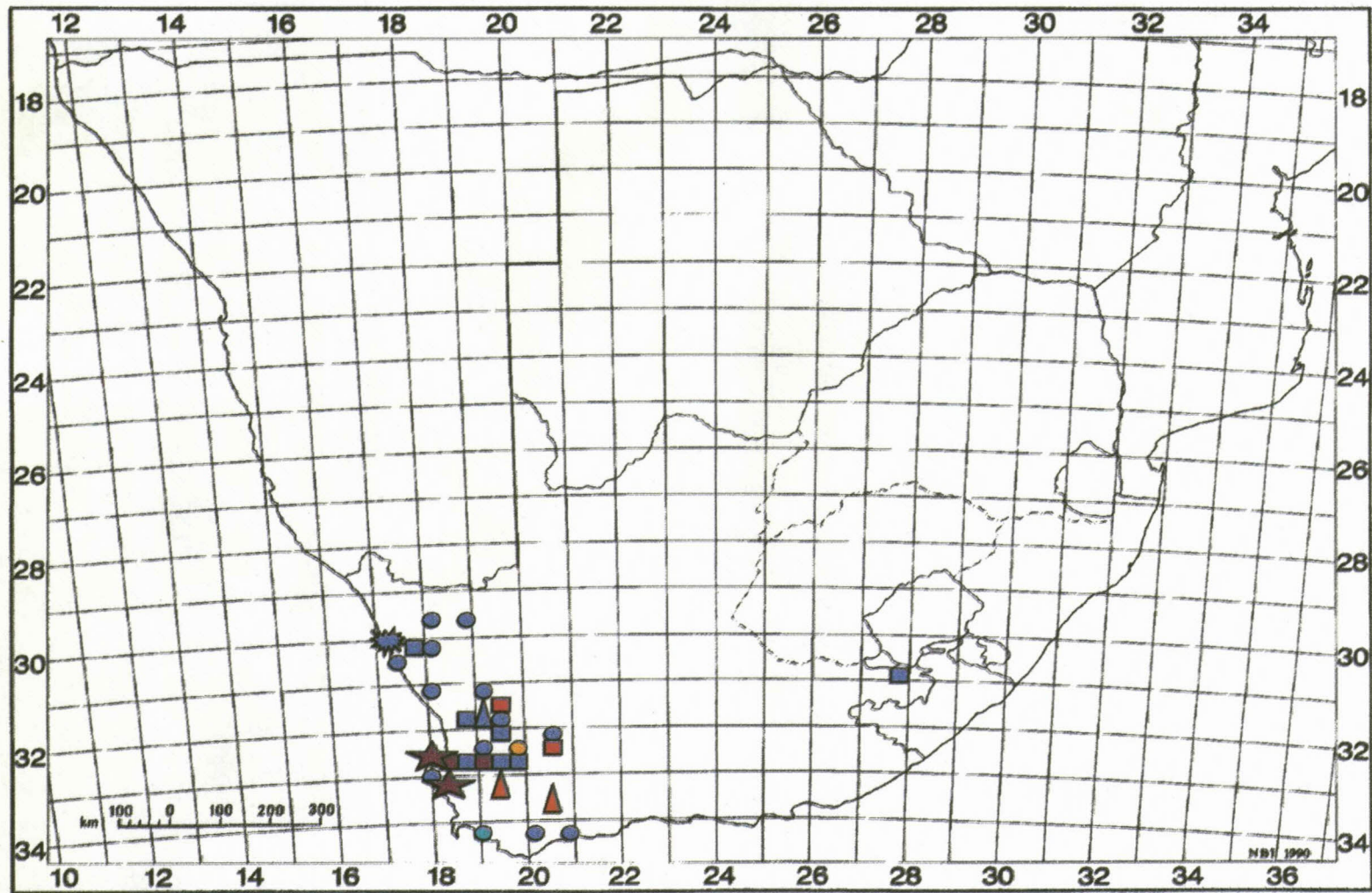


**Figure 3.6** Photomicrographs of meiotic chromosomes in the genus *Pentaschistis*. **A - B**, *P. veneta* (Spies 6327): **A**, metaphase I with two univalents; **B**, anaphase I with 4 laggards. Scale bar = 4.8  $\mu$ m

- A specimen of *P. viscidula* (Nees) Stapf was examined. This species, which is described as being an old polyploid complex (Klopper 1996) has one polyploid level. Spies 6178 is a tetraploid ( $n = 2x = 14 + 0-2B$ ) specimen. The only previously studied specimen also indicates a polyploid level of  $n = 3x = 21$  (Klopper *et al.* 1998). However, more specimens must be examined to confirm whether it is part of an old polyploid complex. B-chromosomes (0-2) are also present, but no size differences were observed (Figure 3.5D & E).

All the existing chromosome data (Table 3.1) and the geographical distribution of the different polyploid levels (Figure 3.7.1-6) were compared to determine whether there is any correlation between ploidy level and geographical distribution. All polyploid levels were scattered throughout the Cape Province. Certain species, however, were restricted to certain parts of the Cape Province (Figure 3.7.1), for instance all *P. airoides* specimens were located in the western Cape.

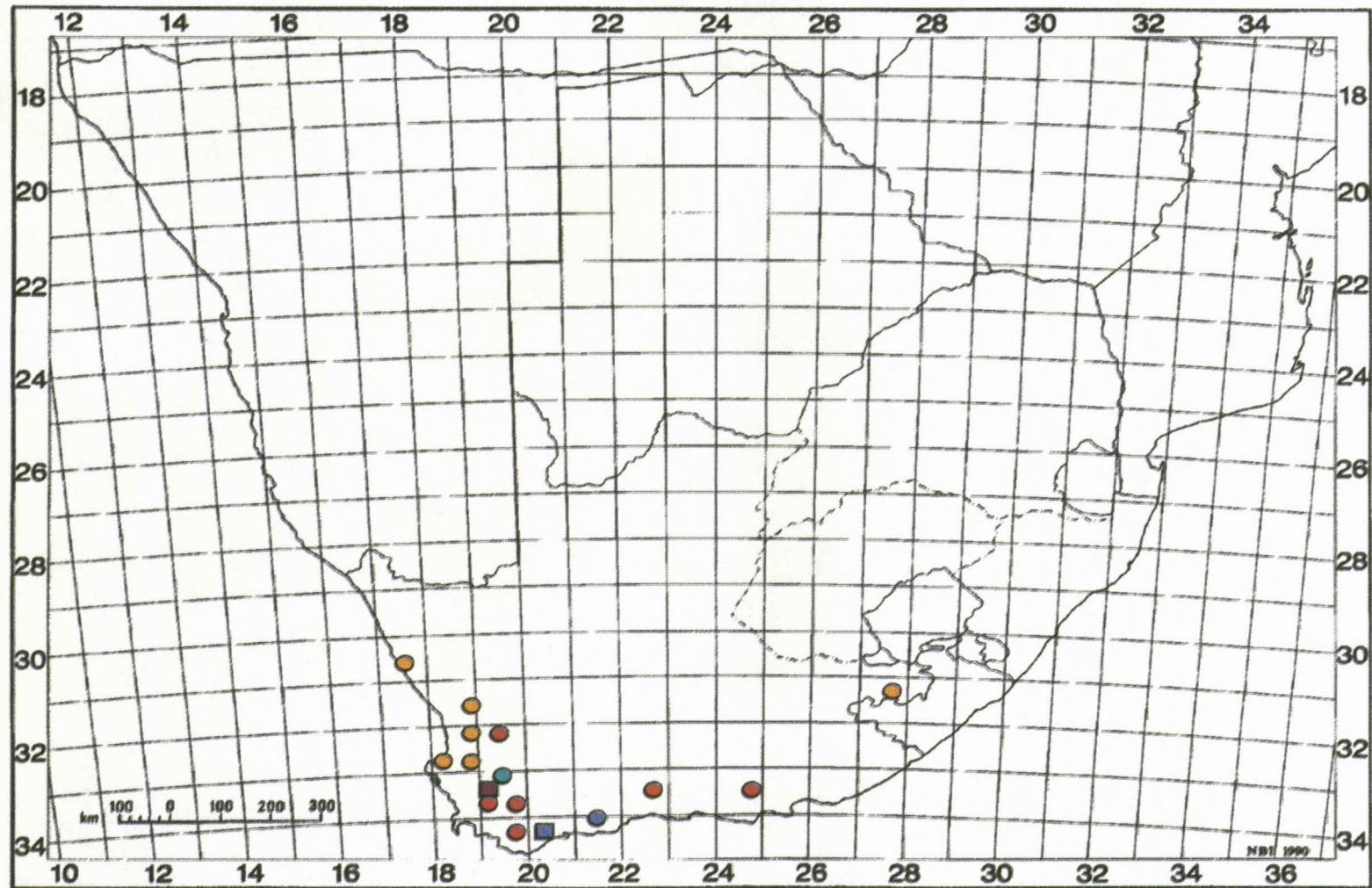
In *P. barbata* an increase in ploidy level was observed in the southern parts of the Cape province, from the Clanwilliam towards Worcester. In both *P. cirrhulosa* and *P. rigidissima* an increase was observed towards the southern part, De Hoop, but more specimens of this species must be examined before supporting this conclusion. In *P. tomentella*, the tetraploid specimens were restricted to the Cedar Mountains, whereas the diploid specimens were found on a different habitat, towards the sea. In *P. tortuosa* the number of diploids increase from the Klein Winterhoek Mountains towards the western Cape and in *P. pallida* the higher polyploid levels are situated in the northern parts, in



**Figure 3.7.1** Geographical distribution of the different ploidy levels of: *P. airoides*, *P. argentea*, *P. aristidoides*, *P. aristifolia*, *P. aurea* and *P. barbata*.

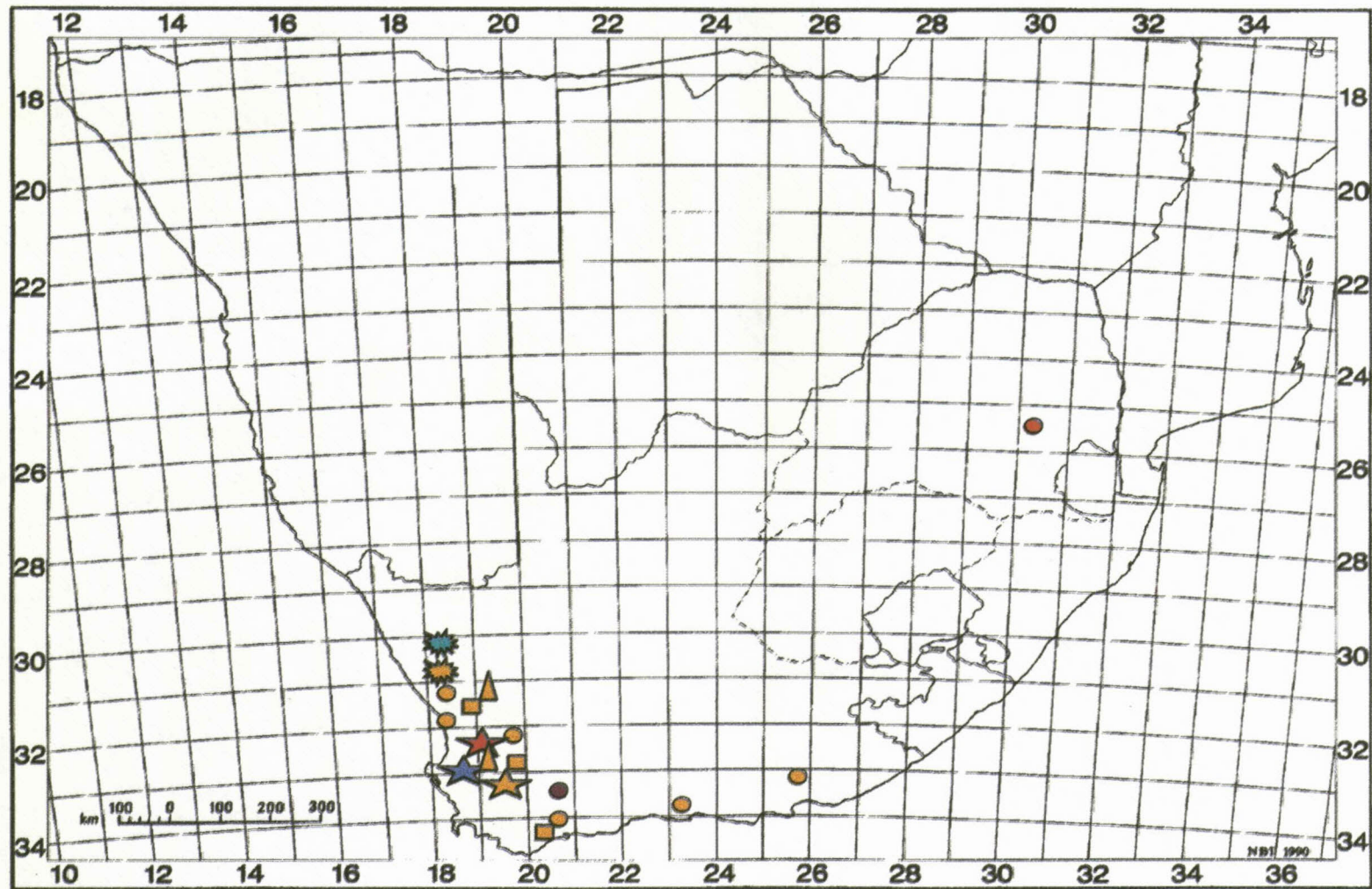
- diploidy
- tetraploidy
- △ hexaploidy
- ⊛ octaploidy
- ☆ decaploidy and higher





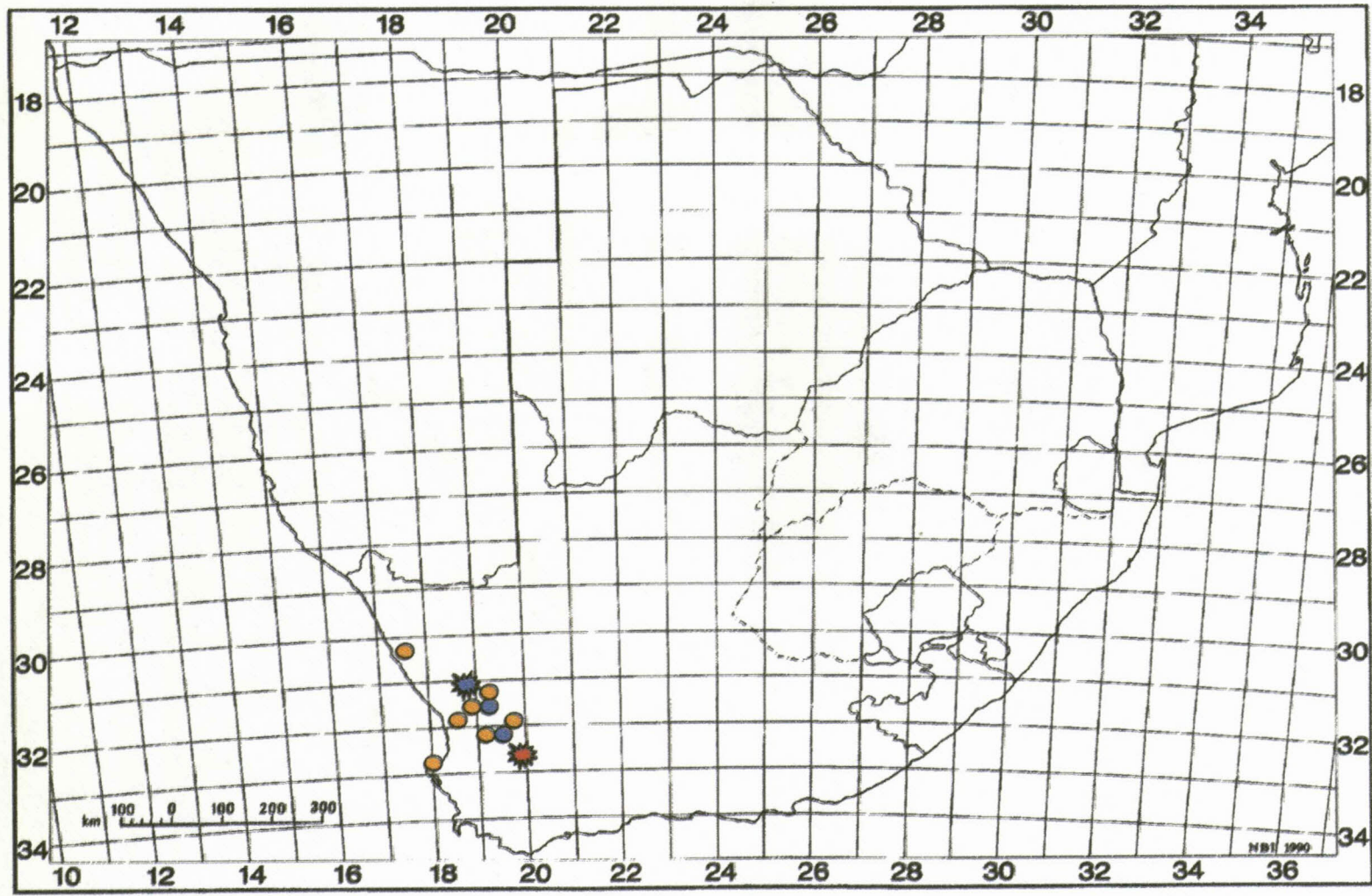
**Figure 3.7.2** Geographical distribution of the different polyploid levels of: *P. cirrhulosa*, *P. capensis*, *P. cappilaris*, *P. curvifolia*, *P. densifolia* and *P. colorata*.

- |               |                         |
|---------------|-------------------------|
| ○ diploidy    | ✱ octaploidy            |
| □ tetraploidy | ☆ decaploidy and higher |
| △ hexaploidy  |                         |



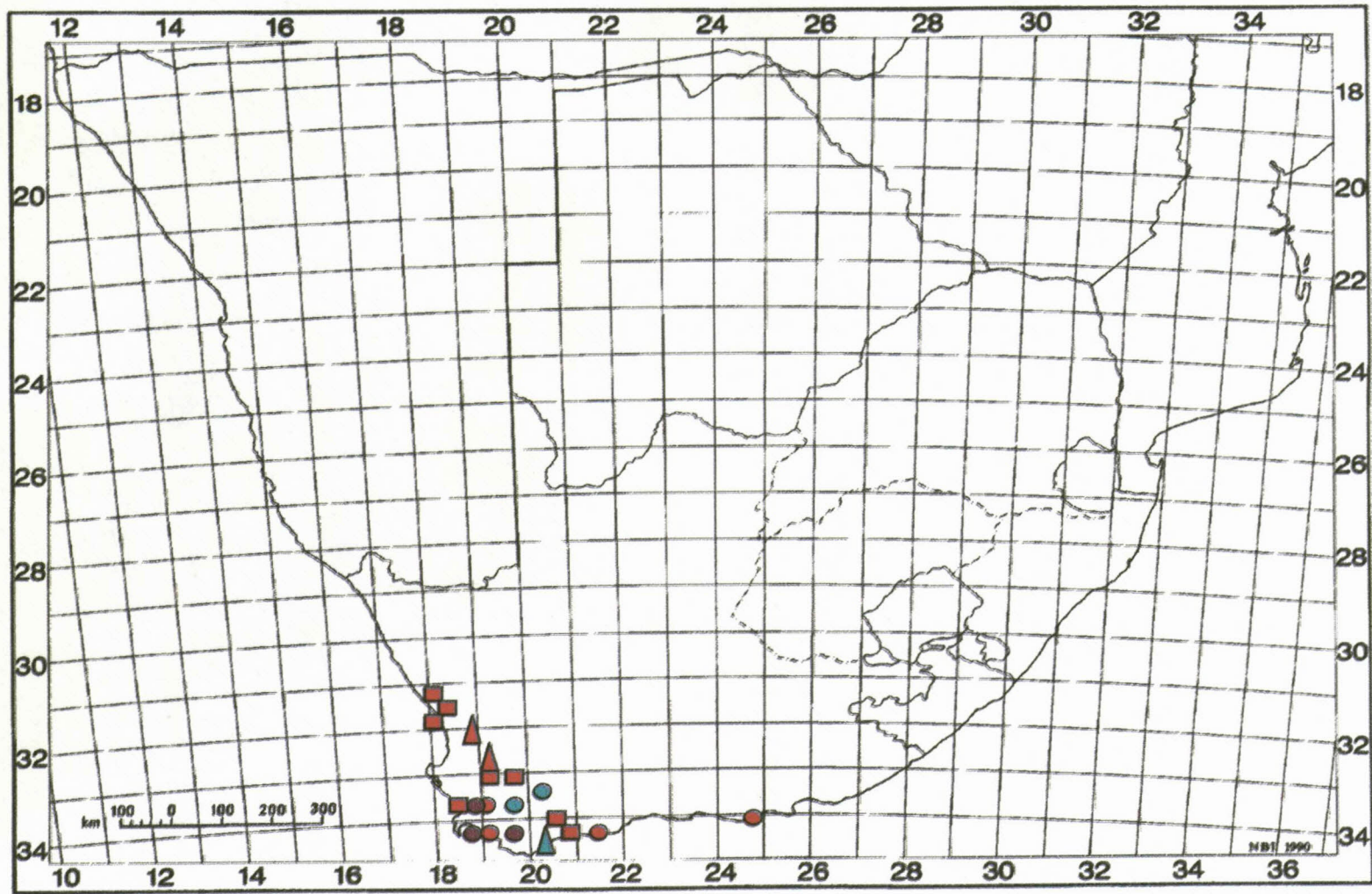
**Figure 3.7.3** Geographical distribution of the different ploidy levels of: *P. elegans*, *P. natalensis*, *P. eriostoma*, *P. pallescens*, *P. lima* and *P. malouinensis*

○ diploidy	☼ octaploidy
□ tetraploidy	☆ decaploidy and higher
△ hexaploidy	



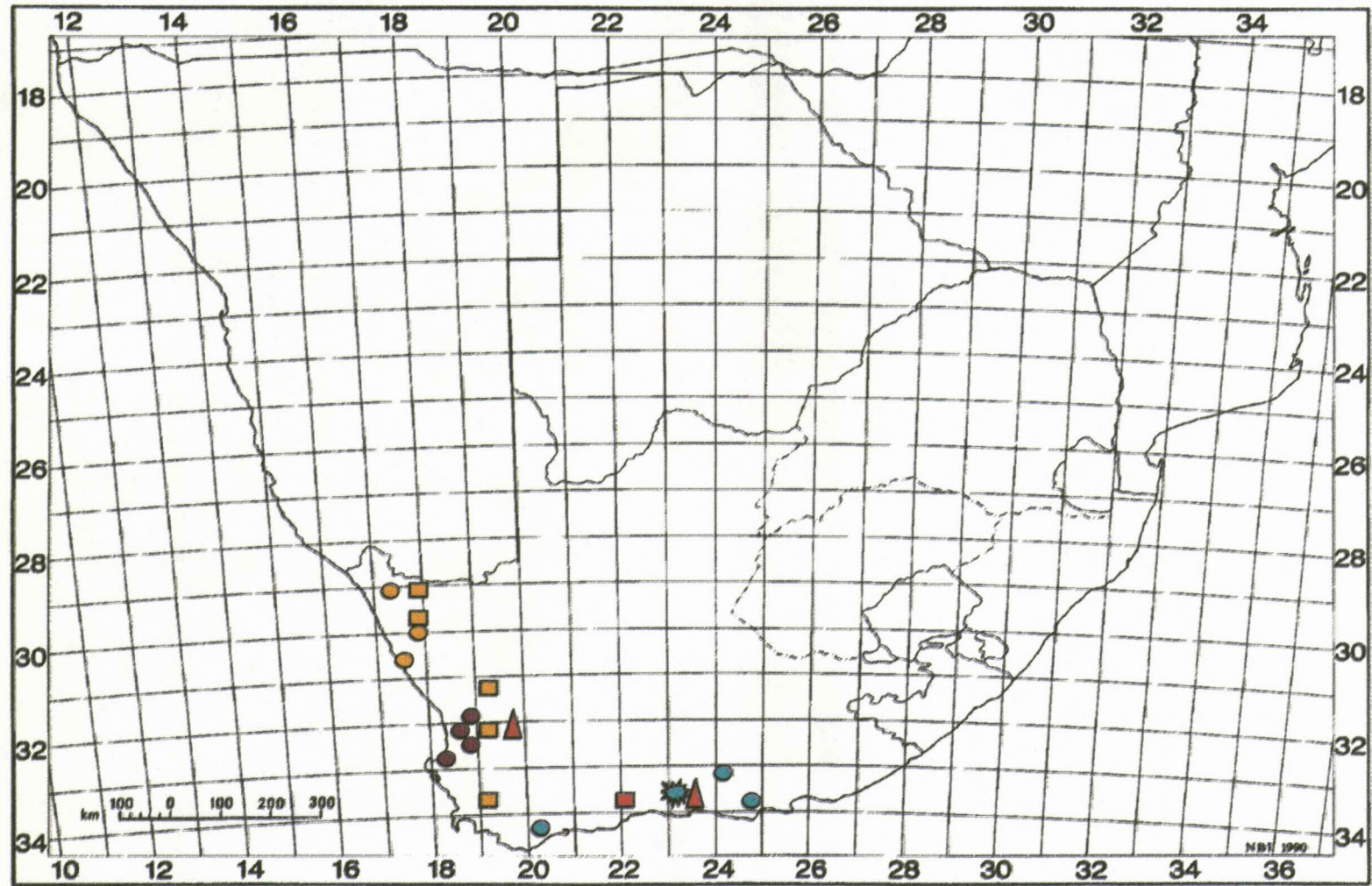
**Figure 3.7.4** Geographical distribution of the different polyploid levels of: *P. densifolia*, *P. rupestris*, *P. patula*.

- diploidy
- tetraploidy
- △ hexaploidy
- ★ octaploidy
- ☆ decaploidy and higher



**Figure 3.7.5** Geographical distribution of the different polyploid levels of: *P. pallida*, *P. rigidissima* and *P. papilosa*

- diploidy
- tetraploidy
- △ hexaploidy
- ☆ octaploidy
- ☆ decaploidy and higher



**Figure 3.7.6** Geographical distribution of the different ploidy levels of: *P. tomentella*, *P. viscidula*, *P. tortuosa* and *P. trisetata*.

- diploidy
- tetraploidy
- △ hexaploidy
- ☼ octaploidy
- ☆ decaploidy and higher

Steinkopf, with diploidy in the south western Cape, towards Kleinriviers Mountains, as far as Cape St. Francis. Diploid specimens are mainly found in the Eastern Cape. This could be because of the polyploid organisms adapting to another habitat in order to avoid competition.

Earlier studies on the genus *Pentaschistis* indicated that *Pentaschistis* is a young polyploid hybrid complex (Klopper 1996). Due to the fact that no, or very few multivalents were observed we concluded that the species are allopolyploids or segmental allopolyploid tending towards allopolyploidy.

Univalents were observed in seven of the seventeen specimens studied (*P. pallida*, *P. veneta*, *P. rupestris*, *P. tortuosa*, and *P. curvifolia*). An univalent may arise from one of two possible origins: it may either be a chromosome which has failed to pair at zygotene or it might have failed to form a chiasmata in the paired regions. Univalents may pass to one pole without dividing at the first division of meiosis and divide normally at the second, but there is a strong tendency for them to lag at the first division. The lagging chromosomes are usually not included in the nuclei resulting from meiosis, but appear as micronuclei in telophase II. This was clearly the case in *P. tortuosa* (Figure 3.5C).

An anaphase I bridge was observed in 10% of the cells in *P. curvifolia* (Figure 3.2E), which is most probably the result of a paracentric inversion. The frequency of these abnormalities was so low that they should not affect the fertility of the specimens and because all material in our laboratory is fixed during collection, no pollen germination fertility tests could be done.

B-chromosomes were present in a third of the species studied. They were morphologically identical to the A-chromosomes. No correlation exists between the phylogenetic development of the species and the presence of the B-chromosomes.

### **3.3 Conclusion.**

A total number of 166 specimens and 32 species have now been studied. Of this number, seventeen specimens were examined in this study. The number of diploids was 58% of the total amount with 25% tetraploid, 7% hexaploid, 6% octaploid and the rest representing higher levels. The polyploid levels of *P. veneta* (tetraploid), *P. capensis* (diploid) were observed for the first time. New polyploid levels were also observed in *P. densifolia* (octaploidy), *P. rupestris* (decaploid & 14-ploid), *P. tortuosa* (octaploidy) and *P.*

*viscidula* (tetraploidy). There are no correlations between the polyploid levels and geographical distribution, but a clear correlation between the species and the geographical distribution. Although only Cape species were collected, it appears as if the *Pentaschistis* species are geographically isolated to the western Cape and adapted to a certain habitat.

The aim of this study was to determine the polyploid levels, mainly to interpret the fingerprint analysis. These results, as well as previously published data, will be used in conjunction with molecular data to determine the relationships between the species.

## CHAPTER 4

### RANDOM AMPLIFIED POLYMORPHIC DNA

#### 4.1 Introduction.

The genus *Pentaschistis* consists of 68 species (Linder & Ellis 1990a) and is one of the largest genera in the tribe Arundineae. It is endemic to Africa and 57 species are indigenous to South Africa, with at least 40 of them being endemic.

Since the development of RAPD analysis (Welsh & McClelland 1990, Williams *et al.* 1990), many applications for this technique were found, i.e. population genetics (Haig *et al.* 1994), strain identification (Welsh *et al.* 1991), genetic mapping (Williams *et al.* 1993) and phytogeography (Van Oppen *et al.* 1994). Although RAPDs can be used for the reconstruction of phylogenies (Wilkie *et al.* 1993, Borowsky *et al.* 1995, Hoey *et al.* 1996), its use in a phylogenetic context is not widely accepted. Heun & Helentjaris (1993) indicated that the variation in RAPD markers may not be caused by point mutations, but rather by competition between PCR products and primers. Due to the fact that RAPD fragment polymorphisms are not yet completely understood, the reliability of RAPD fragments in phylogenetic studies is still debated (Kump & Javarnik 1996).

RAPD data can be analysed by a cladistic framework (Borowsky *et al.* 1995), which considers each RAPD fragment as an independent character, coded as either being present or absent for every specimen. A data matrix is assembled and a cladistic analysis performed.

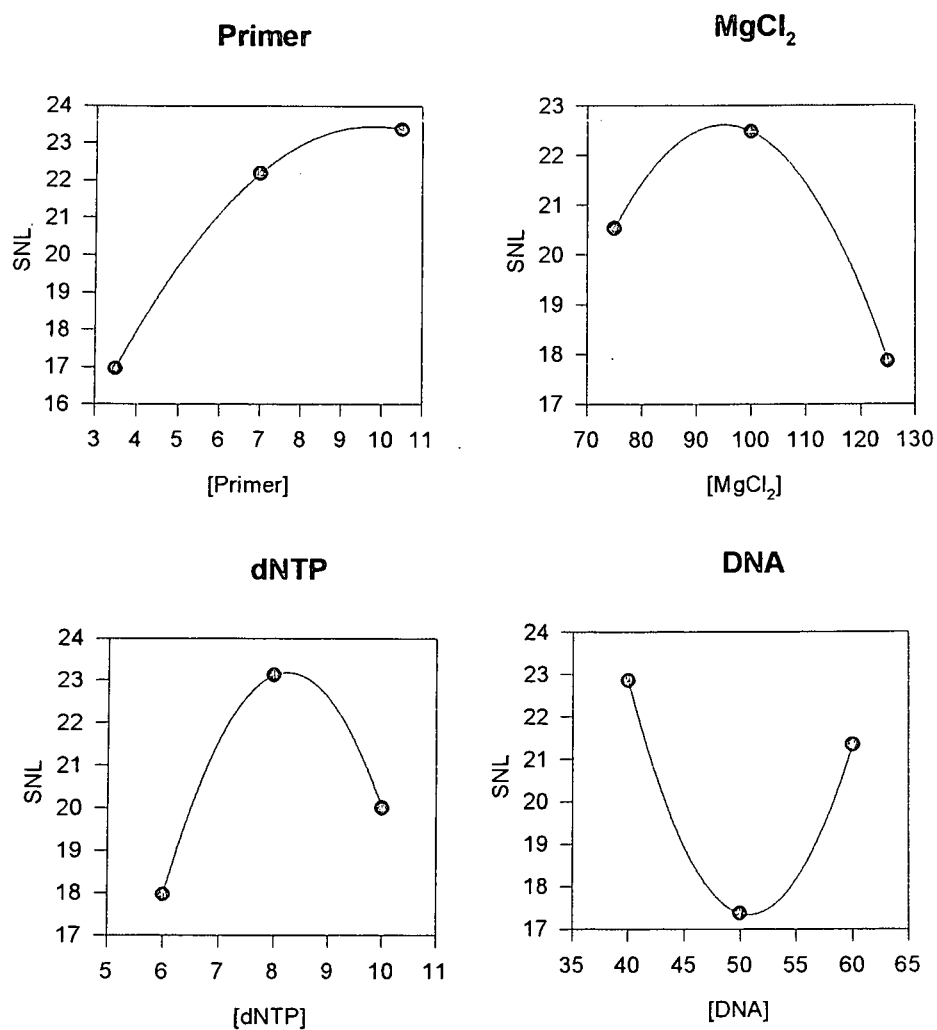
Klopper (1996) used RAPDs on ten *Pentaschistis* species and found the genetic variation to be very high. The RAPD data suggested a close relationship between *P. eriostoma* and *P. curvifolia*. She also suggested that more species, and more specimens, should be studied.

Because almost a third of the world's representatives of the tribe Arundineae are indigenous to South Africa, it gives us an ideal opportunity to study phylogenetic relationships in the genus *Pentaschistis*. The aim of this study was to extend Klopper's (1996) research and to use more species, and more specimens per species, to determine the genetic variation within, and between, the morphological groups.



## 4.2 Results.

The SNL values for the optimal RAPD conditions were calculated (Table 4.1) and plotted to determine the optimal amplification conditions (Figure 4.1).



**Figure 4.1** The SNL values plotted against the different concentrations of the primer (OPA 16) in pmol, dNTPs in mM, MgCl<sub>2</sub> in mM and DNA according to the dilutions. The optimal volumes for each component can be seen as the peak values on the graphs.

**TABLE 4.1** The SNL values as calculated for the different concentrations of the variables (A, B & C), according to the Taguschi method (Cobb and Clarkson 1994) as described in paragraph 2.2.5 of the materials and methods.

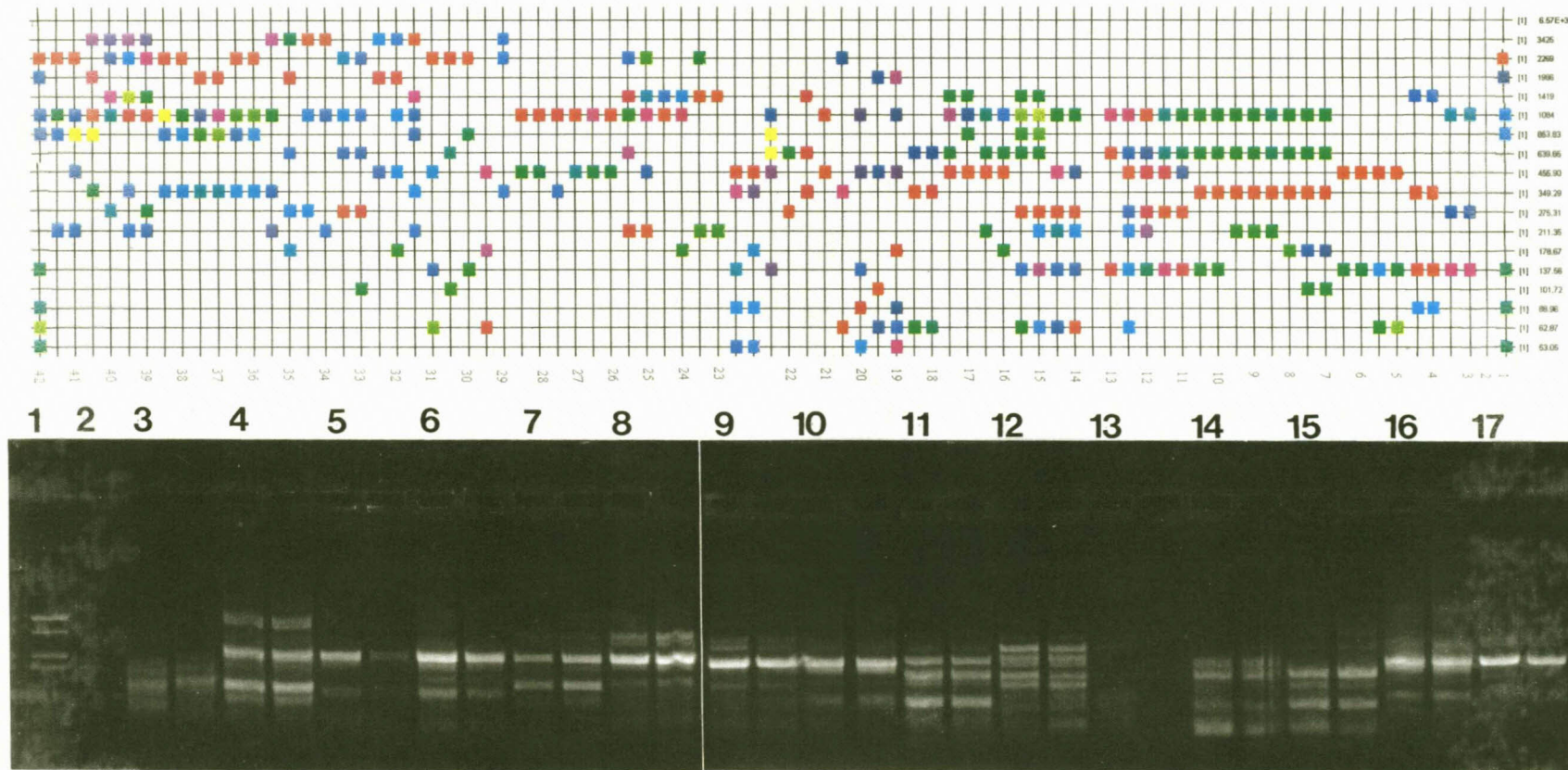
	A	B	C
<b>Primer</b>	16.95	22.18	23.37
<b>MgCl<sub>2</sub></b>	20.53	22.48	17.86
<b>dNTPs</b>	23.12	20.00	17.96
<b>DNA</b>	22.85	17.37	21.34

RAPD fragments are represented by schematic representations of the gel profiles (Figures. 4.2.1 - 5). There were an average of 18 fragments per specimen for OPA 7 and OPA 11, 19 fragments per specimen for OPA 20 and OPC 4 and 20 fragments for OPA 16.

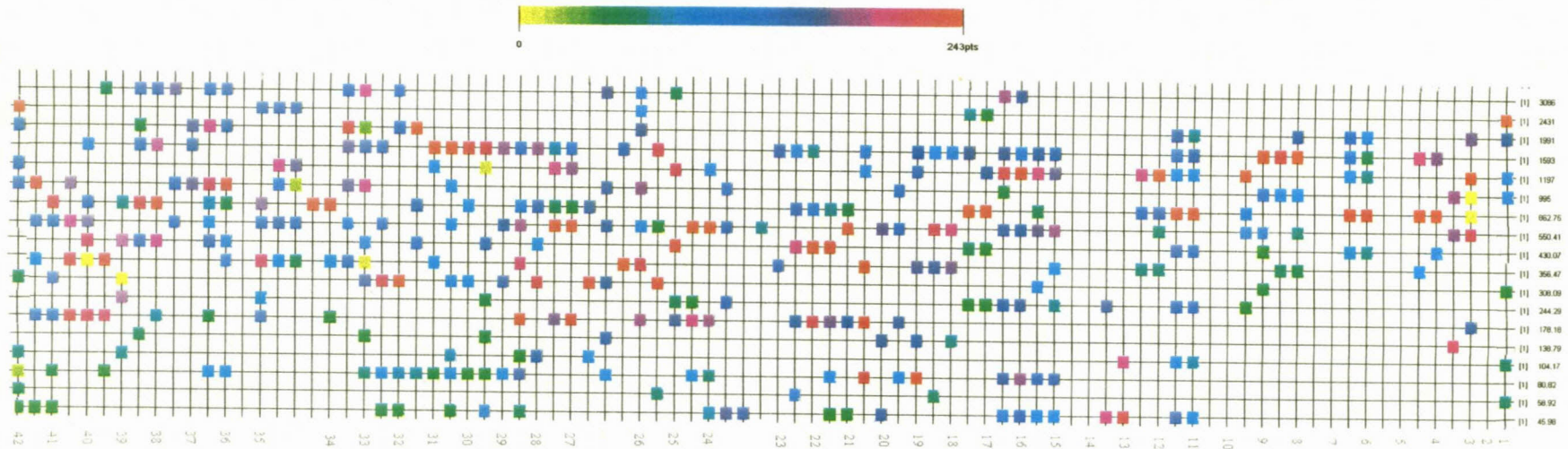
An average of ninety-four fragments per specimen were scored, which gave a total of 3666 fragments scored for all the primers used for *Pentasthitis*. The lengths of the fragments ranged from 220bp to 2100bp. The reproducibility of RAPD analysis for Primer OPA 11 (Table 4.2) was the highest. A comparison of the average of 18 fragments per specimen from the two replicate OPA 11 amplifications, resulted in 96% reproducibility. Results differed for nine scores only, primarily due to fragments scored as absent in one replication and weakly visible in the second. Slightly lower levels of reproducibility were observed in the replicated amplifications of the other four primers examined.

**TABLE 4.2** The reproducibility for each primer examined in this study, with the number of fragments observed with each primer.

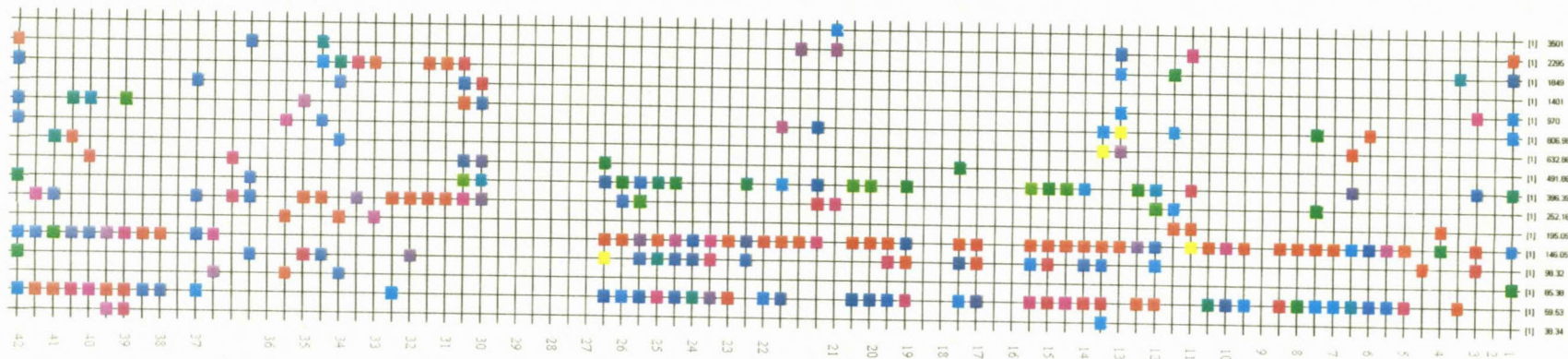
<b>PRIMER</b>	<b>AVERAGE # FRAGMENTS/ PRIMER</b>	<b>REPRODUCIBILITY</b>
<b>OPA 7</b>	18	95 %
<b>OPA 11</b>	18	96 %
<b>OPA 16</b>	20	94 %
<b>OPA 20</b>	19	93 %
<b>OPC 4</b>	19	92 %



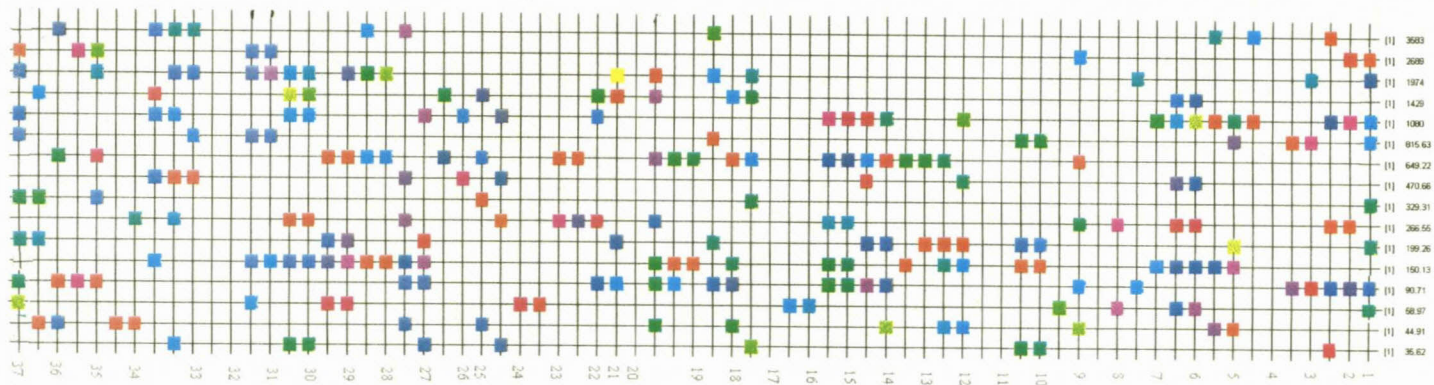
**Figure 4.2.1** Schematic representation (A) and photomicrograph (B) of the RAPD fragment pattern on the genus *Pentaschistis*, using Operon primer OPA<sub>11</sub>. 1 - DNA marker VI, 2 - negative control, 3 - *P. aristifolia* (Spies 6295), 4 - *P. airoides* (Spies 6205), 5 - *P. barbata* (Spies 6267), 6 - *P. veneta* (Spies 6327), 7 - *P. pallida* (Spies 6208), 8 - *P. tomentella* (Spies 6345), 9 - *P. tomentella* (Spies 6342), 10 - *P. rupestris* (Spies 6330), 11 - *P. eriostoma* (Spies 6173), 12 - *P. densifolia* (Spies 6328), 13 - *P. calcicola* (Spies 6210), 14 - *P. tortuosa* (Spies 6179), 15 - *P. colorata* (Spies 6313), 16 - *P. rupestris* (Spies 6309), 17 - *P. tomentella* (Spies 6356), 18 - *P. tortuosa* (Spies 6102), 19 - *P. barbata* (Spies 6086), 20 - *P. eriostoma* (Spies 5919), 21 - *P. curvifolia* (Spies 6026), 22 - *P. curvifolia* (Spies 6073), 23 - *P. rigidissima* (Spies 6243), 24 - *P. tomentella* (Spies 6277), 25 - *P. curvifolia* (Spies 6215), 26 - *P. pallida* (Spies 6281), 27 - *P. rupestris* (Spies 6308), 28 - *P. rupestris* (Spies 6310), 29 - *P. veneta* (Spies 6001), 30 - *P. lima* (Spies 5961), 31 - *P. pallida* (Spies 5922), 32 - *P. pallida* (Spies 5924), 33 - *P. patula* (Spies 6076), 34 - *P. tomentella* (Spies 5960), 35 - *P. tomentella* (Spies 5898), 36 - *P. viscidula* (Spies 6059), 37 - *P. patula* (Spies 6036), 38 - *P. elegans* (Spies 5927), 39 - *P. eriostoma* (Spies 6055), 40 - *P. rigidissima* (Spies 6109), 41 - *P. eriostoma* (Spies 6041), 42 - DNA marker VI.



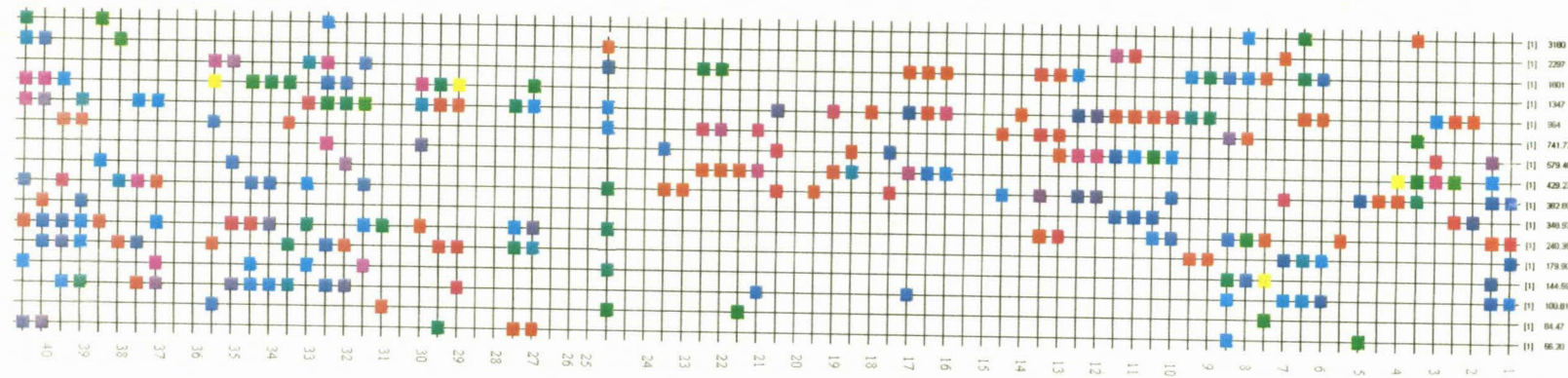
**Figure 4.2.2** Schematic representation of the RAPD fragment pattern on the genus *Pentaschistis*, using Operon primer OPA<sub>16</sub>. 1 - DNA marker VI, 2 - negative control, 3 - *P. aristifolia* (Spies 6295), 4 - *P. airoides* (Spies 6205), 5 - *P. barbata* (Spies 6267), 6 - *P. veneta* (Spies 6327), 7 - *P. pallida* (Spies 6208), 8 - *P. tomentella* (Spies 6345), 9 - *P. tomentella* (Spies 6342), 10 - *P. rupestris* (Spies 6330), 11 - *P. eriostoma* (Spies 6173), 12 - *P. densifolia* (Spies 6328), 13 - *P. calcicola* (Spies 6210), 14 - *P. tortuosa* (Spies 6179), 15 - *P. colorata* (Spies 6313), 16 - *P. rupestris* (Spies 6309), 17 - *P. tomentella* (Spies 6356), 18 - *P. veneta* (Spies 6001), 19 - *P. lima* (Spies 5961), 20 - *P. pallida* (Spies 5922), 21 - *P. patula* (Spies 6076), 22 - *P. tomentella* (Spies 5960), 23 - *P. tomentella* (Spies 5898), 24 - *P. viscidula* (Spies 6059), 25 - *P. patula* (Spies 6036), 26 - *P. elegans* (Spies 5927), 27 - *P. eriostoma* (Spies 6055), 28 - *P. rigidissima* (Spies 6109), 29 - *P. eriostoma* (Spies 6041), 30 - *P. tortuosa* (Spies 6102), 31 - *P. rigidissima* (Spies 6243), 32 - *P. tomentella* (Spies 6277), 33 - *P. curvifolia* (Spies 6215), 34 - *P. pallida* (Spies 6281), 35 - *P. rupestris* (Spies 6308), 36 - *P. rupestris* (Spies 6310), 37 - *P. tortuosa* (Spies 6102), 38 - *P. barbata* (Spies 6086), 39 - *P. eriostoma* (Spies 5919), 40 - *P. curvifolia* (Spies 6026), 41 - *P. curvifolia* (Spies 6073), 42 - DNA marker VI.



**Figure 4.2.3** Schematic representation of the RAPD fragment pattern on the genus *Pentaschistis*, using Operon primer OPA<sub>20</sub>. 1 - DNA marker VI, 2 - negative control, 3 - *P. aristifolia* (Spies 6295), 4 - *P. airoides* (Spies 6205), 5 - *P. barbata* (Spies 6267), 6 - *P. veneta* (Spies 6327), 7 - *P. pallida* (Spies 6208), 8 - *P. tomentella* (Spies 6345), 9 - *P. tomentella* (Spies 6342), 10 - *P. rupestris* (Spies 6330), 11 - *P. eriostoma* (Spies 6173), 12 - *P. veneta* (Spies 6001), 13 - *P. lima* (Spies 5961), 14 - *P. pallida* (Spies 5922), 15 - *P. pallida* (Spies 5924), 16 - *P. patula* (Spies 6076), 17 - *P. tomentella* (Spies 5960), 18 - *P. tomentella* (Spies 5898), 19 - *P. viscidula* (Spies 6059), 20 - *P. patula* (Spies 6036), 21 - *P. elegans* (Spies 5927), 22 - *P. eriostoma* (Spies 6055), 23 - *P. rigidissima* (Spies 6109), 24 - *P. eriostoma* (Spies 6041), 25 - *P. tortuosa* (Spies 6102), 26 - *P. barbata* (Spies 6086), 27 - *P. densifolia* (Spies 6328), 28 - *P. calcicola* (Spies 6210), 29 - *P. tortuosa* (Spies 6179), 30 - *P. colorata* (Spies 6313), 31 - *P. rupestris* (Spies 6309), 32 - *P. tomentella* (Spies 6356), 33 - *P. rigidissima* (Spies 6243), 34 - *P. tomentella* (Spies 6277), 35 - *P. curvifolia* (Spies 6215), 36 - *P. pallida* (Spies 6281), 37 - *P. rupestris* (Spies 6308), 38 - *P. rupestris* (Spies 6310), 39 - *P. eriostoma* (Spies 5919), 40 - *P. curvifolia* (Spies 6026), 41 - *P. curvifolia* (Spies 6073), 42 - DNA marker VI.



**Figure 4.2.4** Schematic representation of the RAPD fragment pattern on the genus *Pentaschistis*, using Operon primer OPA<sub>7</sub>. 1 - *P. rigidissima* (Spies 6243), 2 - *P. tomentella* (Spies 6277), 3 - *P. curvifolia* (Spies 6215), 4 - *P. pallida* (Spies 6281), 5 - *P. rupestris* (Spies 6308), 6 - *P. rupestris* (Spies 6310), 7 - *P. veneta* (Spies 6001), 8 - *P. lima* (Spies 5961), 9 - *P. pallida* (Spies 5922), 10 - *P. viscidula* (Spies 6059), 11 - *P. patula* (Spies 6036), 12 - *P. elegans* (Spies 5927), 13 - *P. eriostoma* (Spies 6055), 14 - *P. rigidissima* (Spies 6109), 15 - *P. tortuosa* (Spies 6102), 16 - *P. barbata* (Spies 6086), 17 - *P. eriostoma* (Spies 5919), 18 - *P. curvifolia* (Spies 6026), 19 - *P. curvifolia* (Spies 6073), 20 - DNA marker VI, 21 - negative control, 22 - *P. aristifolia* (Spies 6295), 23 - *P. airoides* (Spies 6205), 24 - *P. barbata* (Spies 6267), 25 - *P. veneta* (Spies 6327), 26 - *P. pallida* (Spies 6208), 27 - *P. tomentella* (Spies 6345), 28 - *P. tomentella* (Spies 6342), 29 - *P. rupestris* (Spies 6330), 30 - *P. eriostoma* (Spies 6173), 31 - *P. densifolia* (Spies 6328), 32 - *P. calcicola* (Spies 6210), 33 - *P. tortuosa* (Spies 6179), 34 - *P. colorata* (Spies 6313), 35 - *P. rupestris* (Spies 6309), 36 - *P. tomentella* (Spies 6356), 37 - DNA marker VI.



**Figure 4.2.5** Schematic representation of the RAPD fragment pattern on the genus *Pentaschistis*, using Operon primer OPC<sub>4</sub>. 1 - *P. tortuosa* (Spies 6102), 2 - *P. barbata* (Spies 6086), 3 - *P. eriostoma* (Spies 5919), 4 - *P. curvifolia* (Spies 6026), 5 - *P. curvifolia* (Spies 6073), 6 - *P. rigidissima* (Spies 6243), 7 - *P. tomentella* (Spies 6277), 8 - *P. curvifolia* (Spies 6215), 9 - *P. pallida* (Spies 6281), 10 - *P. rupestris* (Spies 6308), 11 - *P. rupestris* (Spies 6310), 12 - *P. veneta* (Spies 6001), 13 - *P. lima* (Spies 5961), 14 - *P. pallida* (Spies 5922), 15 - *P. pallida* (Spies 5924), 16 - *P. patula* (Spies 6076), 17 - *P. tomentella* (Spies 5960), 18 - *P. tomentella* (Spies 5898), 19 - *P. viscidula* (Spies 6059), 20 - *P. patula* (Spies 6036), 21 - *P. elegans* (Spies 5927), 22 - *P. eriostoma* (Spies 6055), 23 - *P. rigidissima* (Spies 6109), 24 - *P. eriostoma* (Spies 6041), 25 - DNA marker VI, 26 - negative control, 27 - *P. aristifolia* (Spies 6295), 28 - *P. airoides* (Spies 6205), 29 - *P. barbata* (Spies 6267), 30 - *P. veneta* (Spies 6327), 31 - *P. pallida* (Spies 6208), 32 - *P. tomentella* (Spies 6345), 33 - *P. tomentella* (Spies 6342), 34 - *P. rupestris* (Spies 6330), 35 - *P. eriostoma* (Spies 6173), 36 - *P. densifolia* (Spies 6328), 37 - *P. calcicola* (Spies 6210), 38 - *P. tortuosa* (Spies 6179), 39 - *P. colorata* (Spies 6313), 40 - *P. rupestris* (Spies 6309), 41 - *P. tomentella* (Spies 6356).

Cladograms were obtained by using PAUP and Hennig86 (paragraph 2.3.3) (Appendix A). The branch lengths between the species were determined by choosing the phylogram option. The coefficients of similarity (Appendix B) and the genetic distances (paragraph 2.3.2) within and between the species were calculated and the average coefficient of similarity, and the genetic distances within and between groups, were calculated (Table 4.3 & 4.4).

### **4.3 Discussion.**

The PCR based RAPD procedure is a very sensitive technique (Sambrook *et al.* 1989), and control reactions should be included. Fragments present in both the negative control reaction and the reactions containing DNA, should not be used for analysis (Yu *et al.* 1993). Some primers will give fragment patterns without DNA templates, resulting from the formation of primer multimers (Williams *et al.* 1990).

High concentrations of primers can result in the formation of primer-dimers, as they are substrates for amplification and competitors for the other constituents of the reaction (Innis & Gelfond 1990). In other literature optimal primer concentrations of 10 - 50 pmol were suggested (Klopper 1996), although 9.5 pmol primer gave optimal results during this study.

**Table 4.3** The average genetic distances between and within the different morphological groups of 39 specimens.

	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 6
GROUP 1	0.94	0.87	0.92	0.88	0.90
GROUP 2		—	0.93	0.83	0.80
GROUP 3			1.06	0.87	1.00
GROUP 4				0.93	0.79
GROUP 6					1.03



**Table 4.4** The genetic distances within and between the species in the genus *Pentascistis*.

1 = *P. airoides*, 2 = *P. aristifolia*, 3 = *P. barbata*, 4 = *P. densifolia*, 5 = *P. lima*, 6 = *P. pallida*, 7 = *P. patula*, 8 = *P. rupestris*, 9 = *P. tomentella*, 10 = *P. veneta*, 11 = *P. viscidula*, 12 = *P. calcicola*, 13 = *P. colorata*, 14 = *P. elegans*, 15 = *P. rigidissima*, 16 = *P. tortuosa*, 17 = *P. curvifolia*, and 18 = *P. eriostoma*.

	GROUP 1										GROUP 2	GROUP 3					GROUP 4	GROUP 6	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
1	1.06	0.94	0.84	1.05	0.95	1.19	1.05	1.06	0.86	1.11	1.51	0.92	1.27	0.94	0.84	0.88	0.99		
2		0.88	0.92	0.99	0.96	1.35	0.98	0.77	1.16	1.15	0.69	0.87	0.86	0.90	0.76	0.84	1.00		
3			0.60	1.10	1.21	0.69	0.69	0.90	0.83	0.84	0.48	1.15	0.93	0.79	0.60	0.83	1.00	0.87	
4				0.99	0.82	1.00	0.80	1.00	1.01	1.20	0.60	0.70	1.20	1.28	0.92	0.86	0.90		
5					0.87	0.97	0.82	1.08	0.98	0.99	1.39	1.13	0.90	0.92	1.18	0.88	0.91		
6						0.82	0.89	0.73	0.86	0.82	0.58	1.08	0.89	0.79	0.81	0.83	0.96	0.73	
7							0.79	0.84	0.96	1.08	0.56	1.33	0.81	0.75	0.82	1.10	0.84	0.81	
8								0.92	0.68	0.93	0.77	1.06	1.03	0.83	0.91	0.78	0.80	0.80	
9									0.93	0.89	0.88	1.10	0.84	0.96	0.92	0.73	0.87	0.96	
10										1.41	0.97	-	0.89	1.00	0.93	0.92	0.91	0.98	
11												1.19	1.02	0.70	0.84	0.92	0.83	0.80	
12													1.20	1.56	1.71	1.32	0.99	1.25	
13														1.02	0.87	0.92	0.85	0.99	
14															0.78	0.86	0.85	0.82	
15																0.74	0.91	0.80	1.03
16																	0.79	0.86	0.91
17																		0.93	0.79
18																			1.03

High concentrations of dNTP substrates generally increase the polymerase error rate by driving the enzymatic reaction in the direction of DNA synthesis (Eckert *et al.* 1991). Equal concentrations of all four dNTPs should be used to minimise misincorporation (Innis & Gelfond 1990). In this study a dNTP concentration of 8 mM was used. Primer annealing and enzyme activity may be influenced by the magnesium concentrations (Innis & Gelfond 1990). The *Taq* polymerase activity can be improved by decreasing the magnesium chloride concentration relative to the total concentration of dNTPs present in the reaction. Since DNA, dNTPs and proteins all bind  $Mg^{2+}$  ions, high DNA synthesis by *Taq* polymerase must be determined empirically for any protocol. In this study a  $MgCl_2$  concentration of 100 mM was used. Too high concentrations of DNA template result in smears, without distinct amplified fragments (Williams *et al.* 1990). DNA concentrations are dependent on the size of the genome and may vary from

picograms to nanograms (Yu *et al.* 1993). The DNA concentrations could not be determined by spectrophotometry, because higher values were obtained for degraded DNA than for intact DNA. Therefore, the DNA concentrations were optimised by dilution and the concentrations were visually obtained (~100ng).

The optimal reaction concentrations were used to amplify the DNA templates of 18 species (Table 2.1). The DNA fragment patterns were converted into data matrices for each primer (Appendix A). These data sets were used for cladistical analysis.

Klopper (1996) described the genus *Pentaschistis* as having a high degree of genetic variation. This was also observed in this study where the genetic distances were high in different species. In *P. barbata* the genetic distance is 0.60 (Table 4.4), which is expected for specimens of the same species. Morphologically *Spies 6267* and *Spies 6086* are similar. Earlier studies indicated chromosome numbers of  $2n = 12x = 84$  and  $2n = 13x = 91$ . These high polyploid levels indicate that variation within this species is quite likely. This species was also described as being a "rather variable group" by Linder & Ellis (1990a). The relative low genetic distance in this species can, consequently, be attributed to only two specimens being studied. *Pentaschistis viscidula* is the closest related species to *P. barbata*, with a genetic distance of 0.48. This could be explained by the tetraploid level observed in both species. The morphological similar species *P. barbata*, *P. rupestris* and *P. veneta* are however not closely related to one another, according to the genetic distances. This can be explained by the high polyploid levels, specifically observed in *P. rupestris*, which indicates that high genetic distances in this species is quite likely.

The genetic distance within *P. pallida* is 0.82 (Table 4.4), but this species is well known for its variability and many synonyms have been described (Linder & Ellis 1990a). An investigation into the morphology of the studied specimens, revealed variation in the leaf blades and inflorescence. The polyploid levels ranged from diploidy to hexaploidy. Thus, the preservation of the subdivisions (forms rather than subspecies) of this species by Linder & Ellis (1990a), are corroborated by a relative low genetic distance. The genetic distances also indicate a low genetic distance between *P. pallida* and *P. viscidula* and can be explained by the tetraploid level observed in both species.

*Pentaschistis patula* has also been described as a species with an enormous range of variation, which is reflected in this study by a genetic distance of 0.79 (Table 4.4), between these specimens. These specimens differ morphologically. Due to the variation in morphology, we would suggest that the groupings of *P. patula* should be reviewed and

rather be grouped into different subspecies, than into different forms. According to Spies *et al.* (1994a) this species should be closely related to *P. airoides*, but the genetic distance between these two species is too high to support the grouping of these two species. According to the genetic distance the closest relative to *P. patula* is *P. viscidula* (0.56). The fact that only diploidy have been observed in *P. patula* could indicate that *P. viscidula* (tetraploid), developed through polyploidisation of *P. patula*, therefore, the low genetic distance between these species. This could also mean that *P. patula* should be closely related to *P. barbata*, because *P. barbata* & *P. viscidula* are closely related. The genetic distance confirms this expectation.

The morphological variability in *P. rupestris* is underlined by a genetic distance of 0.92 (Table 4.4). The high polyploid levels (decaploid & 14-ploid), also support this genetic distance.

The wide geographical distribution of *P. tomentella* is linked to the morphological variation present in this species and this is underlined by the intraspecific genetic distance (0.93). On the morphological level, differences were observed in the size of the plants and their inflorescences, supporting the variation. Although *P. tomentella* is difficult to separate morphologically from *P. pallida* form F, the genetic distance between these species is relatively high and we would not recommend a change in the groupings. According to the genetic distances, *P. tortuosa* is the closest related to *P. tomentella*, even though they differ morphologically. Both specimens are however, diploid and thus it explains the close relationship.

*P. veneta* which is difficult to distinguish from *P. barbata* has a genetic distance of 1.41 within itself, but have a genetic distance of 0.84. Thus, this grouping is not well supported.

All of the above mentioned species belong to morphological group 1, but the genetic distances between these species (0.94) does not support this grouping. Morphological and cytogenetic data do, however, support this grouping in most cases. Klopper (1996) indicated a genetic distance of 1.2, but 12 specimens were used, in comparison with the 24 specimens used in this study.

In the morphological group 2, only one species has been studied.

The average genetic distance within *P. rigidissima* is 0.74. This can be explained by a polyploid level of  $n = 3x = 21$ , which indicates that a high genetic distance within this species is quite likely. Linder & Ellis (1990a) speculated that some of these specimens

should, however, be placed with *P. colorata*, but the genetic distance between these species is 1.71 and this hypothesis is not supported.

*Pentaschistis tortuosa* (0.79) is also difficult to distinguish from *P. colorata*, but the genetic distance (0.92) does not support this relationship.

The species belonging to morphological group 3, have a genetic distance of 1. This is, however, explained by the morphological variability within this group, as well as the fact that only seven specimens were examined.

Even though an average genetic distance of 0.93 is observed in *P. curvifolia*, the *P. curvifolia* specimens, *Spies 6026* and *Spies 6073*, have a genetic distance of 0.61 and are not closely related to *Spies 6215*, with a genetic distance of respectively 0.94 and 0.91 between them. This is substantiated by their morphology, i.e. the different inflorescence of these specimens. These species have a contracted inflorescence, whereas *Spies 6215* has an open inflorescence.

The *P. eriostoma* specimens from group 6 have low genetic distances (1.03), which is substantiated by high polyploid levels.

A further observation is that, according to the genetic distances, morphological group 4 is the closest to morphological group 6. This could be expected as some morphological features are shared. Morphological group 3 is the furthest from morphological group 6. The genetic distances between the other groups have an average of 0.91, which indicates a high degree of variation in the genus *Pentaschistis*.

Linder & Ellis (1990a) postulated that *P. curvifolia* and *P. eriostoma* should be closely related, and the genetic distances support this postulation.

What is interesting is the low genetic distance between *P. rigidissima* and *P. barbata*, in spite of morphological differences. *Pentaschistis densifolia* and *P. calcicola* have a genetic distance of 0.60 and were collected in the same geographical territory. On morphological level almost no similarities were observed and no cytogenetic material was available for *P. calcicola*.

Even though Landry and Laponte (1997) indicated that RAPDs are not reliable for phylogenetic studies, we decided to use phylogenetic trees to support the genetic distances and to compare these results with other molecular techniques.

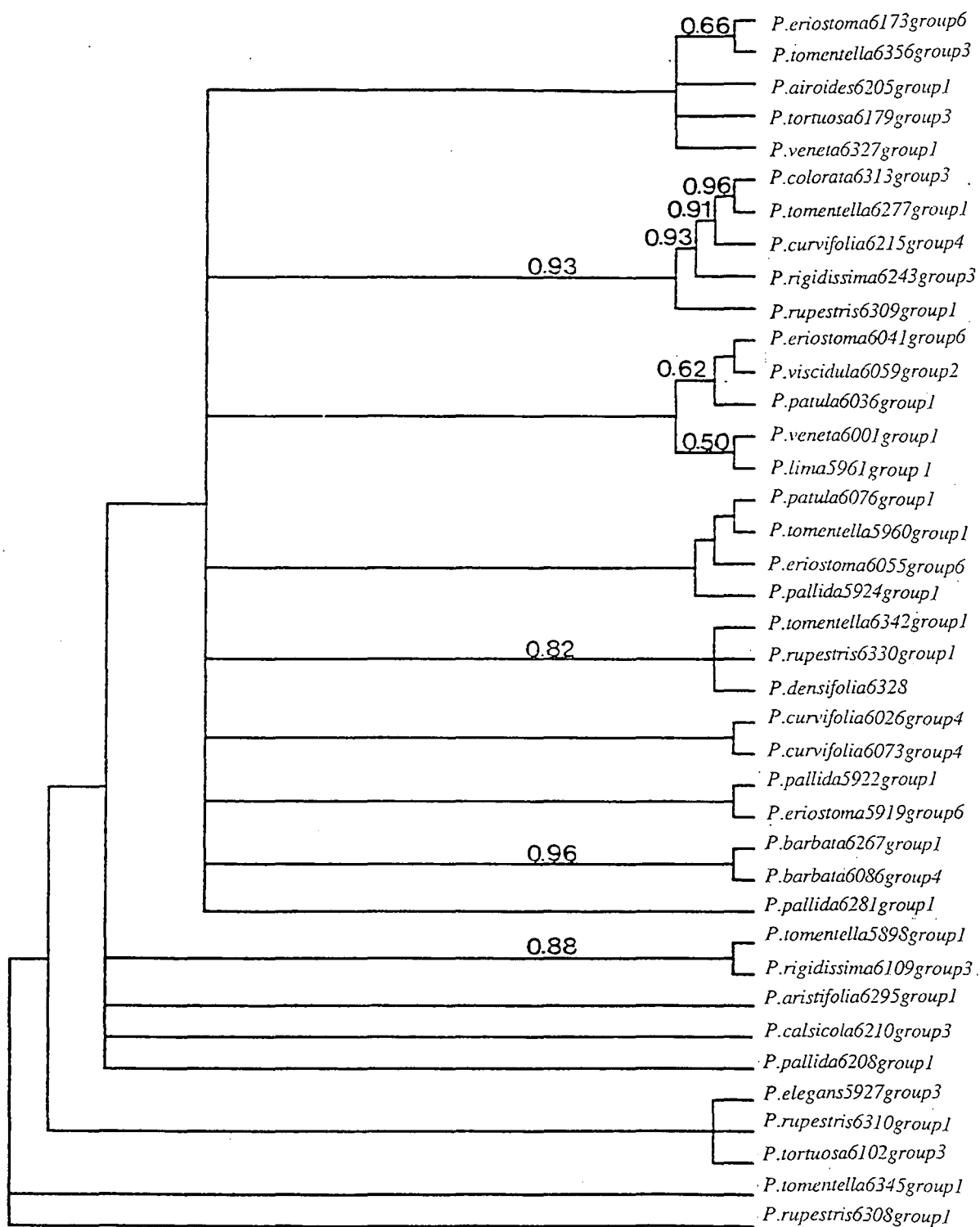
*Pentaschistis eriostoma* is used as the outgroup because of the basic chromosome number,  $x = 13$ , that is present in this species, but it differs from all the other species. The topology of the cladograms between the different primer data were in general agreement.

In the first cladogram with all the RAPD data and *P. eriostoma* as the outgroup, the sister alliance of *P. airoides* (Spies 6205), *P. veneta* (Spies 6327) and *P. tortuosa* (Spies 6179) are supported by both cladograms. The alliance between *P. airoides* & *P. veneta* can be attributed to the morphology, habitat and tetraploidy observed in these specimens. *P. tortuosa*'s alliance is, however, not supported by the genetic distances, but it can be possible that *P. airoides* & *P. veneta* are the tetraploid ancestors of *P. tortuosa*. The sister clade of *P. barbata* (Spies 6267 & Spies 6086) are well supported in both cladograms.

*Pentastichis rupestris* (Spies 6330) is a sister species to *P. densifolia*, both were collected in the same geographical territory. Both species does, however, have a high polyploid level. It can thus be possible that they share the same genome. Both cladograms indicate that *P. viscidula*, *P. patula* (Spies 6036) and *P. eriostoma* aff. (Spies 6041) are sister species. The genetic distances (Table 4.4) support this relationship. The clade marked I (Figure 4.3 & 4.4) is well supported in both cladograms, with morphological concord, as well as diploidy observed within these specimens. Clade II is also well supported by both cladograms and, with the exception of *P. curvifolia* & *P. rupestris*, belongs to the same morphological group 3. However, *P. curvifolia* is very variable. *Pentastichis rigidissima* (Spies 6109) is a sister species to *P. tomentella*. This is supported by their polyploid levels and some similarities in their inflorescences i.e. contracted, pedicels mostly erect, glabrous and ultimate pedicel segments larger or shorter than the spikelets.

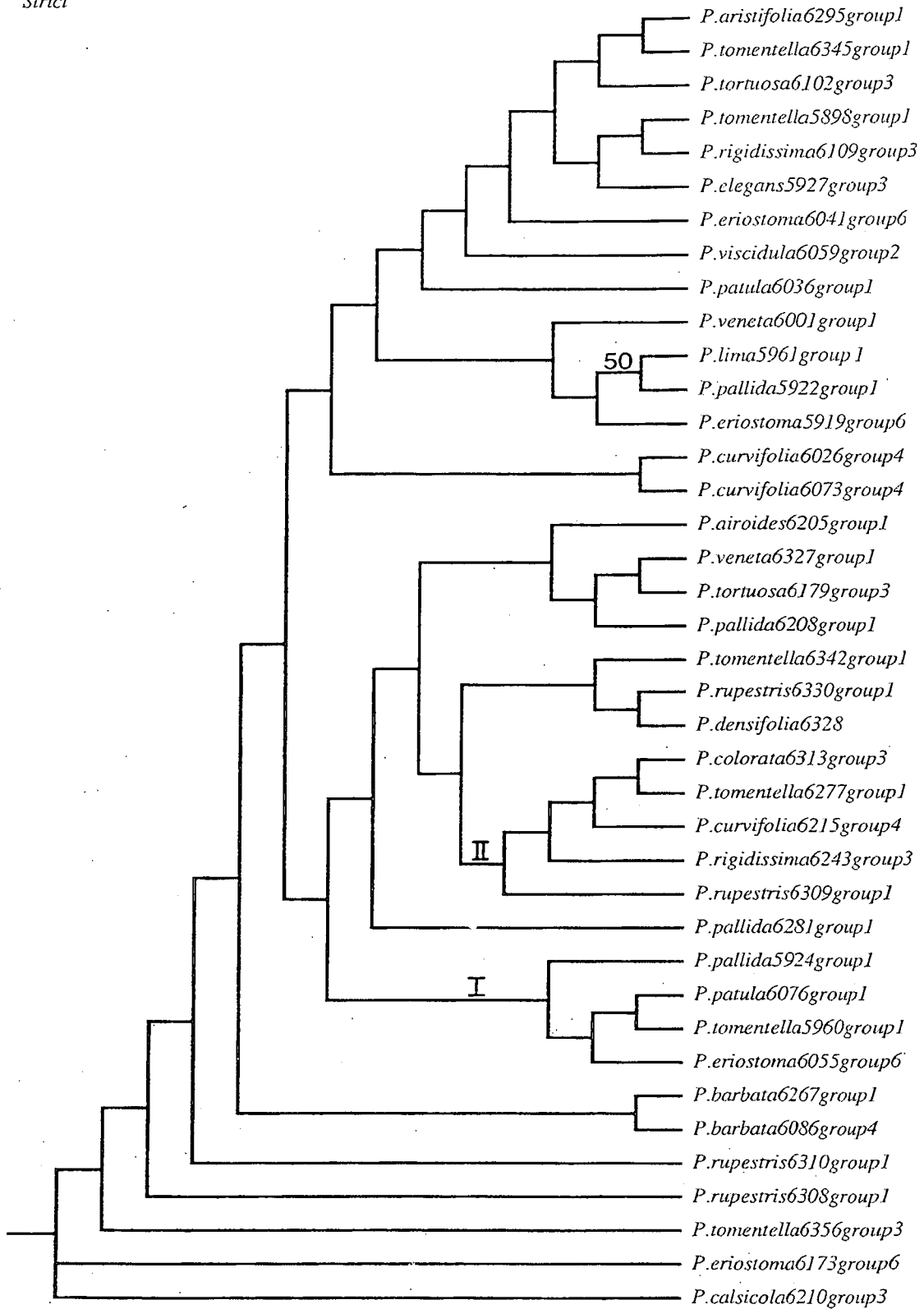
It is clear that even though many of the clades in the two cladograms are in general agreement, there are a few differences. According to Lipscomb (1998) characters with a higher retention index than a consistency index are more informative in the phylogenetic tree, therefore, we excluded characters having a lower retention index than a consistency index.

In the cladogram obtained after character exclusion (Figure 4.5), *P. patula* and *P. rupestris* are unresolved. In Figure 4.6, a strict consensus tree, which is largely congruent with the consensus tree from the complete data set, *P. patula* (Spies 6345) is sister to *P. aristifolia* (Spies 6295). This is supported by their genetic distances. Spies *et al.* (1994a) suggested that *P. airoides* should be closely related to *P. patula*, but neither the genetic distances nor the cladograms indicated such a relationship. *Pentastichis aristifolia* is, however, morphologically very similar to *P. airoides*, but the genetic distances do not indicate a close affinity.



**Figure 4.3** Consensus (*nelsen*) of 100 parsimonious trees (CI = 0.16, RI = 0.37), with length 563, containing 39 *Pentaschistis* specimens, with *P. eriostoma* as outgroup. Numbers above the branches are Jackknife values.

Strict



**Figure 4.4** Strict consensus of two most parsimonious trees (CI = 0.16, RI = 0.38), with length 547, containing 39 *Pentaschistis* specimens, with *P. eriostoma* as outgroup. Number above the branches are % bootstrap values of 200 replicates. Nodes without numbers have bootstrap values of < 50%.

There are a few areas of conflict between Figures 4.4 and 4.6. *Pentaschistis veneta* (Spies 6001) is now sister to clade IV (Figure 4.6). Morphologically *P. veneta* is very similar to *P. rupestris*. Unfortunately this *P. veneta* specimen has not been studied cytogenetically. *Pentaschistis eriostoma* (Spies 6173) and *P. tomentella* (Spies 6356) are sister taxa to *P. tortuosa* (Spies 6179), which was also observed in the genetic distances, and they are sister taxa to *P. veneta* (Spies 6327). All these specimens are tetraploids. In Figure 4.6 *P. pallida* (Spies 5924) emerges in a clade with *P. eriostoma*, and *P. patula* (Spies 6076) in a clade with *P. calcicola* (Spies 6210).

In both Figures 4.5 and 4.6 *P. airoides*, *P. veneta*, *P. eriostoma*, *P. tomentella* and *P. tortuosa* are sister groups to *P. rupestris*, *P. colorata*, *P. tomentella*, *P. curvifolia* and *P. rigidissima*. *Pentaschistis airoides*, *P. veneta*, *P. eriostoma*, *P. tomentella* and *P. tortuosa* are all tetraploid. The difference between these two cladograms is the position of *P. tortuosa* (Spies 6102) and *P. elegans* (Spies 5927). These two specimens are grouped with *P. rupestris* (Spies 6310) in Figure 4.4, but in Figure 4.6 they emerge in clade III.

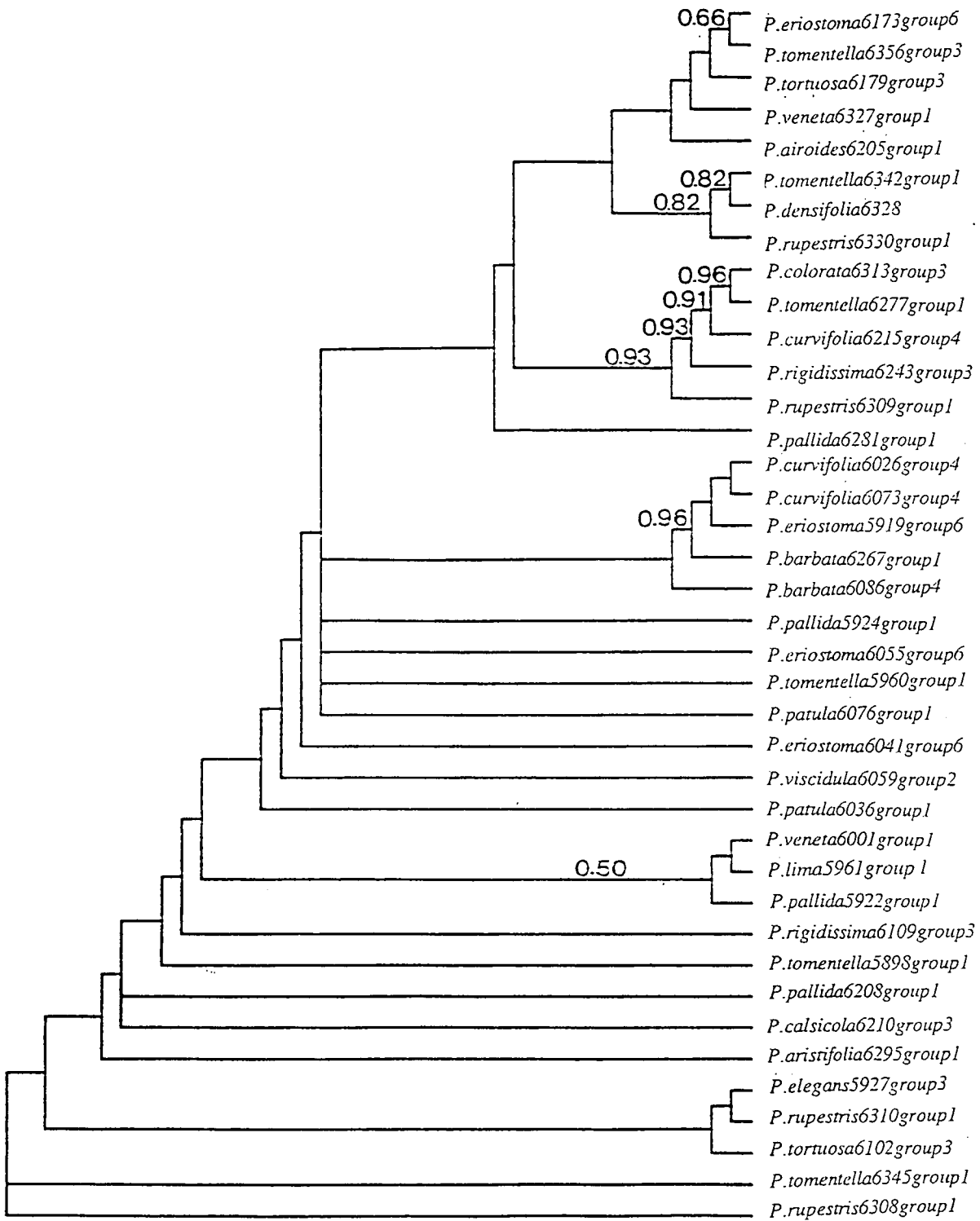
*Pentaschistis veneta* (Spies 6001) is a sister group to *P. lima* in Figure 4.5 but in Figure 4.6 *P. veneta* is a sister group to clade IV. Figure 4.6 is, however, better supported by a shorter tree length.

The cladograms did not support the hypothesis of Linder & Ellis (1990a), that the hybridisation of *P. curvifolia* led to the existence of *P. eriostoma*.

Although the same data sets were used for Hennig86 & PAUP, we obtained different results with the cladograms. This may be because Hennig86, considers the information provided by each character one at a time, whereas PAUP connects taxa together one at a time until all the taxa have been added (Wagner). Because the Hennig86 trees have a higher tree length than PAUP trees, we consider the cladograms obtained by using PAUP (Figures 4.4 and 4.6) to be more reliable.

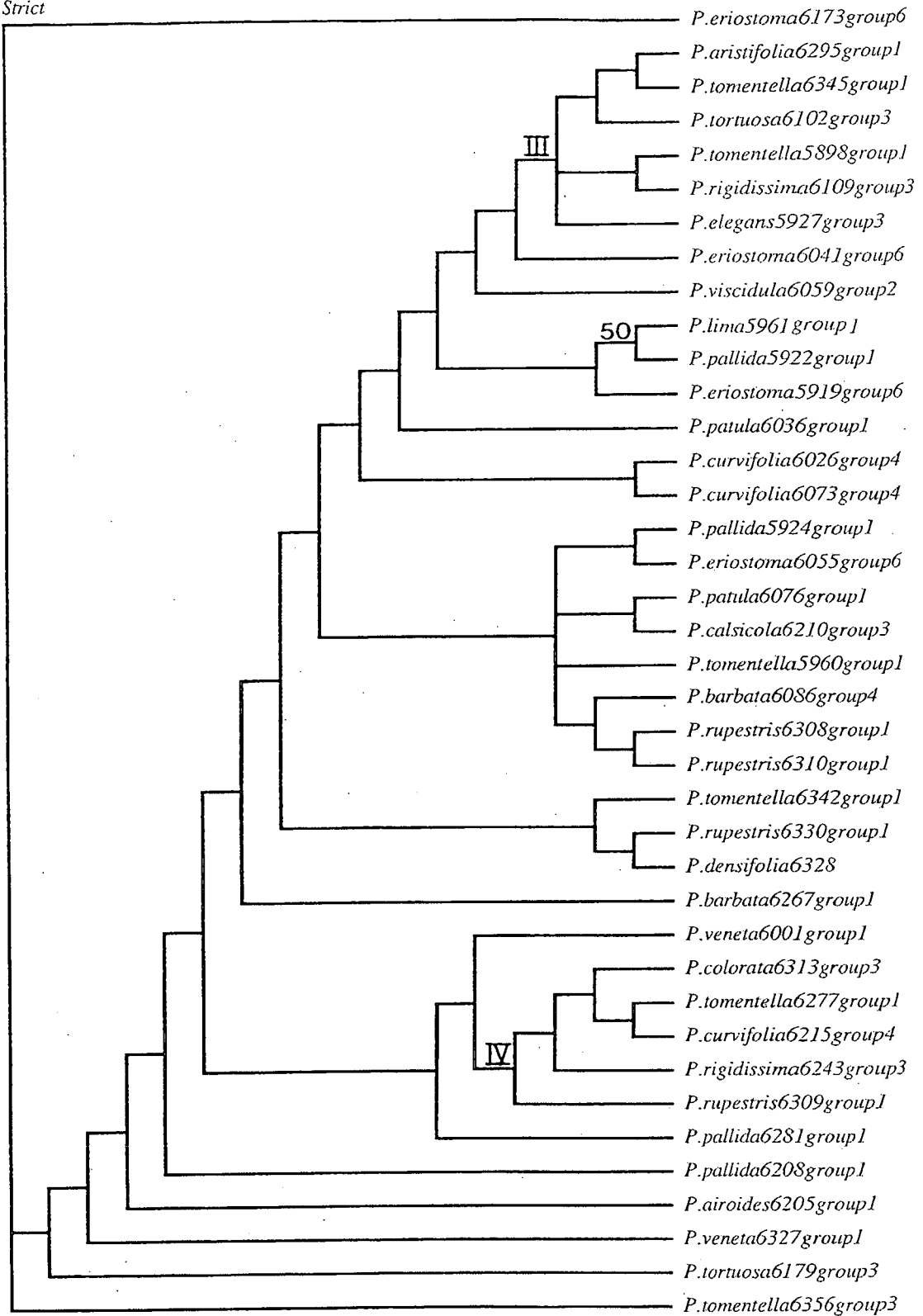
Landry and Laponte (1997) also found the interpretation of an absent RAPD fragment problematic. They indicated two ways of interpreting these results, i) either the fragment was present in an ancestral organism and it was lost or ii) the particular fragment was never present in any ancestor. Two individuals lacking one fragment as a result of shared loss should thus be considered more similar, but this case is far less likely than the second possibility. The more related the taxa the more likely shared loss is true. RAPD analysis is thus more accurate in investigating relationships among populations of a single species, or very closely related species, than between less related species (Gonzalès *et al.*,





**Figure 4.5** Consensus (*nelsen*) of 12 most parsimonious trees (CI = 0.15, RI = 0.46), with length 347, with some characters temporarily removed. Numbers above the branches are Jackknife values.

Strict



**Figure 4.6** Strict consensus of nine most parsimonious trees (CI = 0.15, RI = 0.48), with length 314 with some characters temporarily removed. Numbers above branches are % bootstrap values of 200 replicates. Nodes without numbers have bootstrap values of <50%.

1992). More species of groups 2, 4, 5 and 6 should be studied to determine the reliability of the genetic variations. No relationship within any of the groups could be determined because of the high degree of variation within and between the morphological groups (Table 4.2). The distribution of the specimens of the species can probably be attributed to the polyphyletic nature of this genus. Linder & Ellis (1990) made the statement that the groupings are for classification and do not have any phylogenetic significance, but according to RAPD data some of the groupings are supported by genetic distances as well as by cytogenetic data.

In conclusion it can be stated that RAPD-analyses could be used in a phylogenetic study of the genus *Pentasthitis* if more than 11 primers are screened, as suggested by Landry & Laporte 1997, but it seems as if it is better to use this technique to test for the variation within a species. This could be because of the co-migration of the fragments, which result in wrong interpretation of a specific fragment. More molecular studies are being undertaken to indicate accurately the relationships between the different groups.

## CHAPTER 5

### DNA AMPLIFICATION FINGERPRINTING

#### 5.1. Introduction.

DNA amplification fingerprinting was first used by Caetano-Anolles *et al.* (1991a, b). This Multiple Arbitrary Amplicon Profiling (MAAP) (Caetano-Anolles *et al.* 1992a, 1994) method utilizes very short primers, 5 to 15 nucleotides in length. DAF products are separated by thin polyacrylamide gels and stained by the silver staining method (Bassam *et al.* 1991). In general, the DAF procedure generates scoreable polymorphisms in the molecular size range from 100 to 800 bp and about 20 to 40 scoreable fragments. DAF markers detect both nuclear and cytoplasmic DNA. The ability to generate many amplification products means that DAF analysis is highly efficient in scanning the genome of an organism for variable sites.

DAF analysis helps to detect genetic differences in a wide variety of organisms, including animals, plants and bacteria. Although it is relatively simple to find differences between organisms at the species level, DAF analysis also differentiates between those that are closely related, such as bacterial isolates, plant cultivars, near isogenic lines and human individuals (Caetano-Anolles *et al.* 1991a, Caetano-Anolles 1993). DAFs can also be used to fingerprint small segments of DNA, either cloned in suitable vectors or resulting from PCR amplification (Caetano-Anolles & Gresshoff 1994).

DNA amplification fingerprinting generates relatively complex amplification profiles (Caetano-Anolles *et al.* 1991a, b). In contrast, other techniques such as RAPD analysis produce simple profiles (Williams *et al.* 1990). DAF has been coupled to restriction endonuclease digestion of template DNA (Caetano-Anolles *et al.* 1993), or to the use of structured primers containing mini-hairpin sequences at their 5' terminus (Caetano-Anolles & Gresshoff 1994b), to increase the detection of polymorphic DNA and allow the fingerprinting of small fragments of DNA generated by cloning or PCR amplification. Intensity differences within one pattern remain constant, as does the mobility of fragments, supporting the claim that amplification is accurate, repeatable and non-random. Whether DAF is used for fingerprinting or gene mapping, it is essential that the degree of quantitative variation is assessed (Lewontin & Hartl 1991, Maddox 1992).

The aim of this study is to use DAFs to compare the genetic variation as observed with RAPDs with the genetic variation observed with DAFs and to use the DAF profiles to determine the relationships between different *Pentastichis* species.

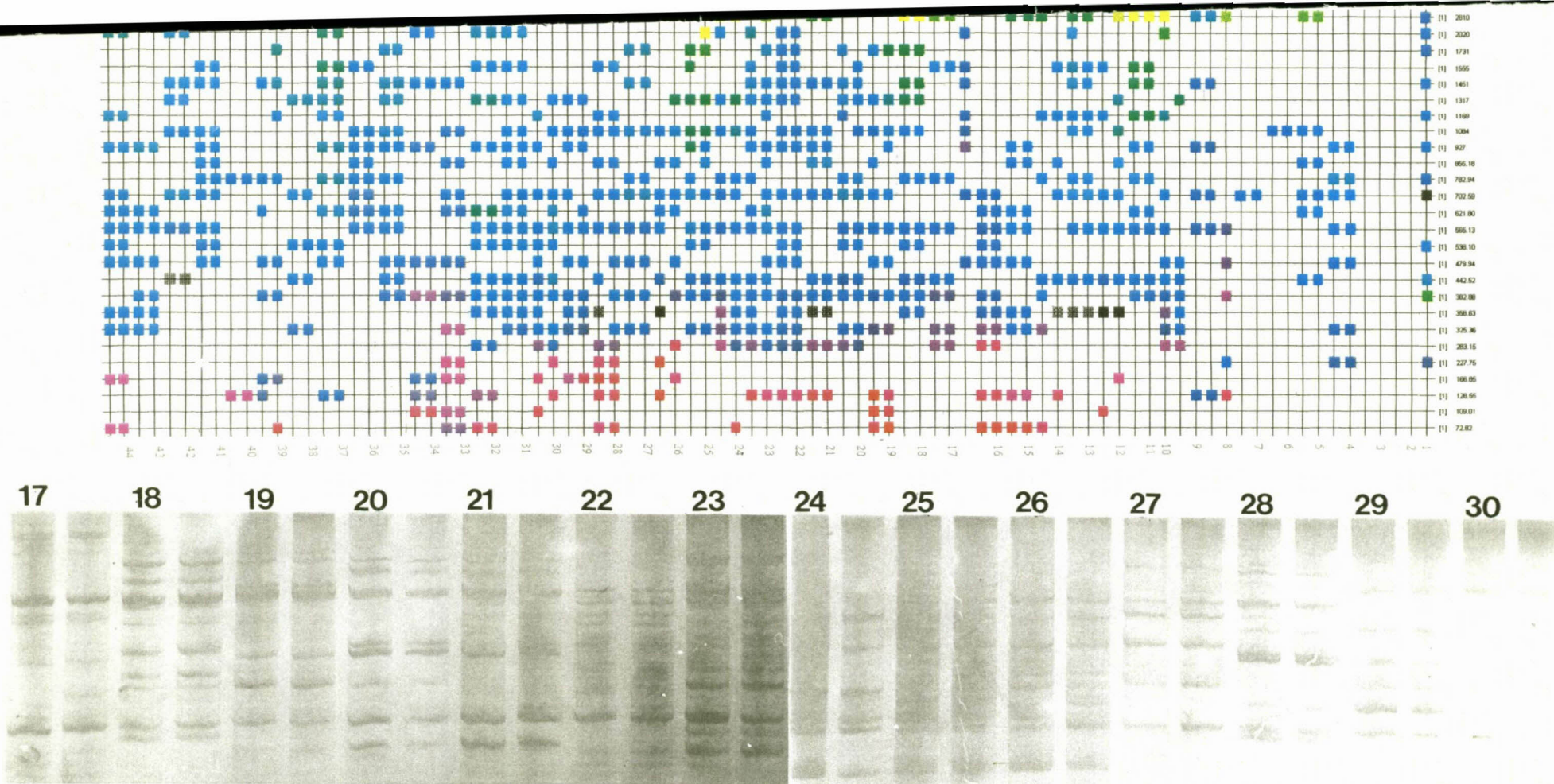
## 5.2. Results.

The value of DAF analysis for species identification and phylogenetic analysis depends largely on its reproducibility. In order to apply DAF analysis to *Pentastichis*, the optimum conditions for DNA amplification were determined (Table 5.1). The reproducibility of DNA patterns produced, using the optimal reaction conditions, was experimentally confirmed (Figure 5.1.1). Under the chosen conditions DNA amplification fragments ranging from 67 bp through several kilobase pairs were produced. When more than one sample of the same DNA preparation is amplified in a particular experiment, the banding patterns obtained are usually identical.

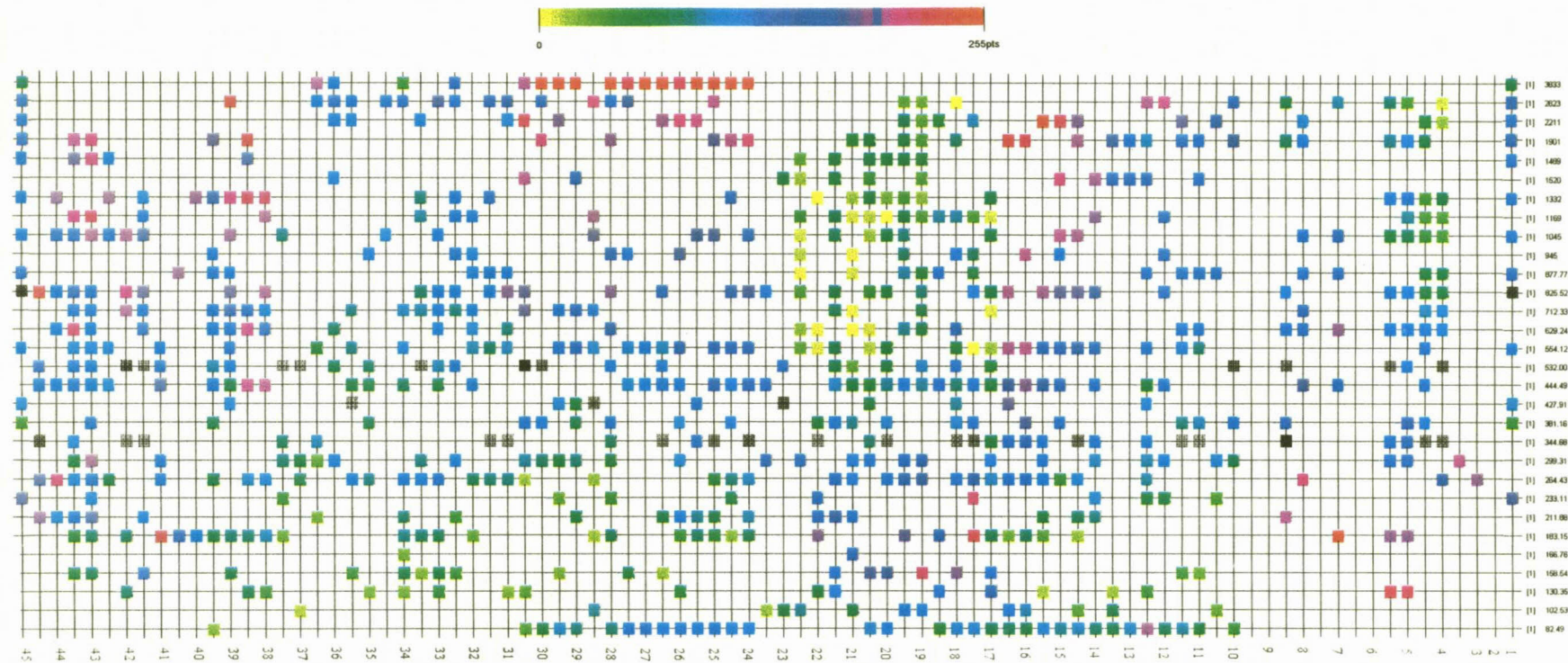
**TABLE 5.1** The range of concentrations used for each variable in order to obtain the optimal conditions for DNA amplification fingerprinting.

	Range studied	Optimal concentration
<b>Buffer</b>	10 x	10 x
<b>MgCl<sub>2</sub></b>	75 mM - 125 mM	75 mM
<b>Template</b>	6 ng/μl - 10 ng/μl	10 ng/μl
<b>Primer</b>	50 pmol - 150 pmol	150 pmol
<b>Enzyme</b>	0.8 Units	0.8 Units
<b>dNTPs</b>	6 mM - 10 mM	10 mM

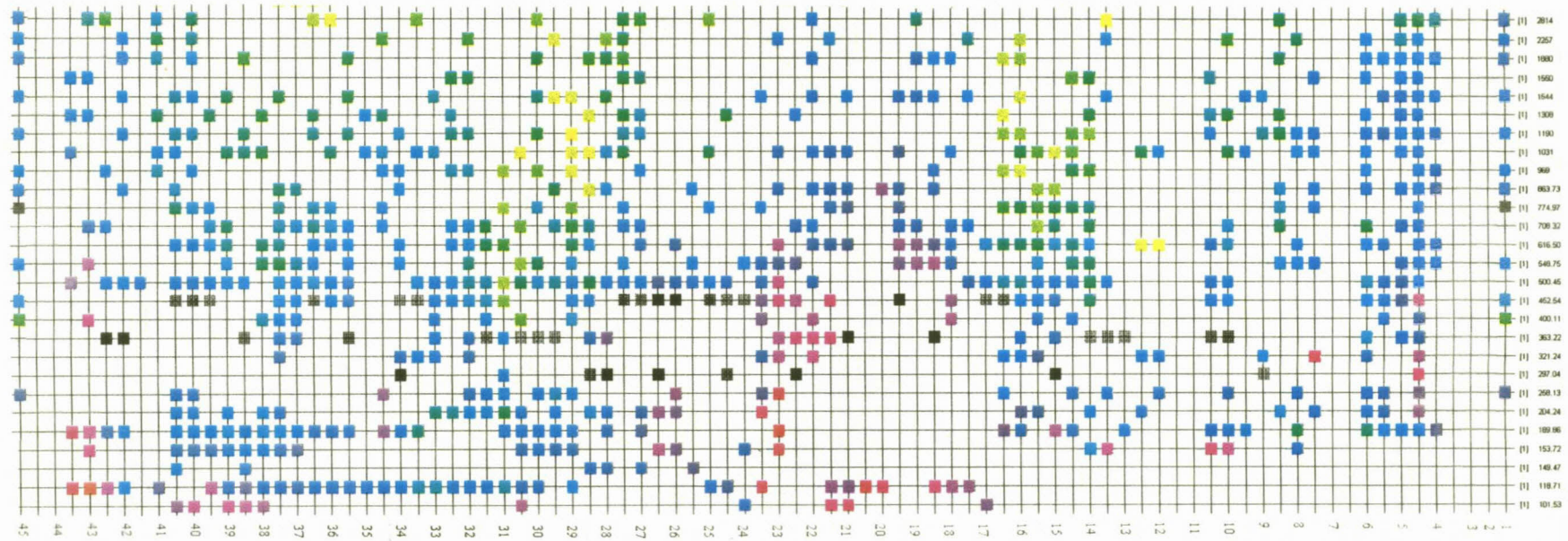
Seventeen *Pentastichis* species and a total of 42 specimens were included in this study. Fingerprints were obtained with 11 of the 12 arbitrary octamer primers tested. The profiles obtained allowed different degrees of species discrimination. These fingerprints are represented by schematic representations of the gel profiles (Figures 5.1.1-11). The primers revealed between 18 and 34 fragments per specimen. An average of 316 fragments



**Figure 5.1.1** Schematic representation (A) and photomicrograph (B) of the DAF fragment profile in the genus *Pentaschistis*, using DAF primer DAF<sub>2</sub>. 1 - DNA marker VI, 2 - negative control, 3 - *P. airoides* (Spies 6152), 4 - *P. airoides* (Spies 6205), 5 - *P. aristifolia* (Spies 6295), 6 - *P. airoies* (Spies 6311), 7 - *P. veneta* (Spies 6001), 8 - *P. barbata* (Spies 6267), 9 - *P. barbata* (Spies 6321), 10 - *P. veneta* (Spies 6327), 11 - *P. eriostoma* (Spies 6000), 12 - *P. pallida* (Spies 6189), 13 - *P. pallida* (Spies 6276), 14 - *P. pallida* (Spies 6276), 15 - *P. tomentella* (Spies 6337), 16 - *P. tomentella* (Spies 6345), 17 - *P. patula* (Spies 6272), 18 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 21 - *P. tomentella* (Spies 6344), 22 - *P. papillosa* (Spies 6226), 23 - *P. rupestris* (Spies 6330), 24 - *P. eriostoma* (Spies 6144), 25 - *P. eriostoma* (Spies 6173), 26 - *P. eriostoma* (Spies 6269), 27 - *P. viscidula* (Spies 6178), 28 - *P. densifolia* (Spies 6328), 29 - *P. tortuosa* (Spies 6179), 30 - *P. colorata* (Spies 6313), 31 - *P. rupestris* (Spies 6309), 32 - *P. tomentella* (Spies 6356), 33 - *P. rigidissima* (Spies 6243), 34 - *P. rigidissima* (Spies 6109), 35 - *P. tortuosa* (Spies 6214), 36 - *P. tomentella* (Spies 6277), 37 - *P. curvifolia* (Spies 6215), 38 - *P. curvifolia* (Spies 6221), 39 - *P. curvifolia* (Spies 6232), 40 - *P. curvifolia* (Spies 6270), 41 - *P. capensis* (Spies 6169), 42 - *P. pallida* (Spies 6281), 43 - *P. rupestris* (Spies 6308), 44 - *P. rupestris* (Spies 6310), 45 - DNA marker VI.

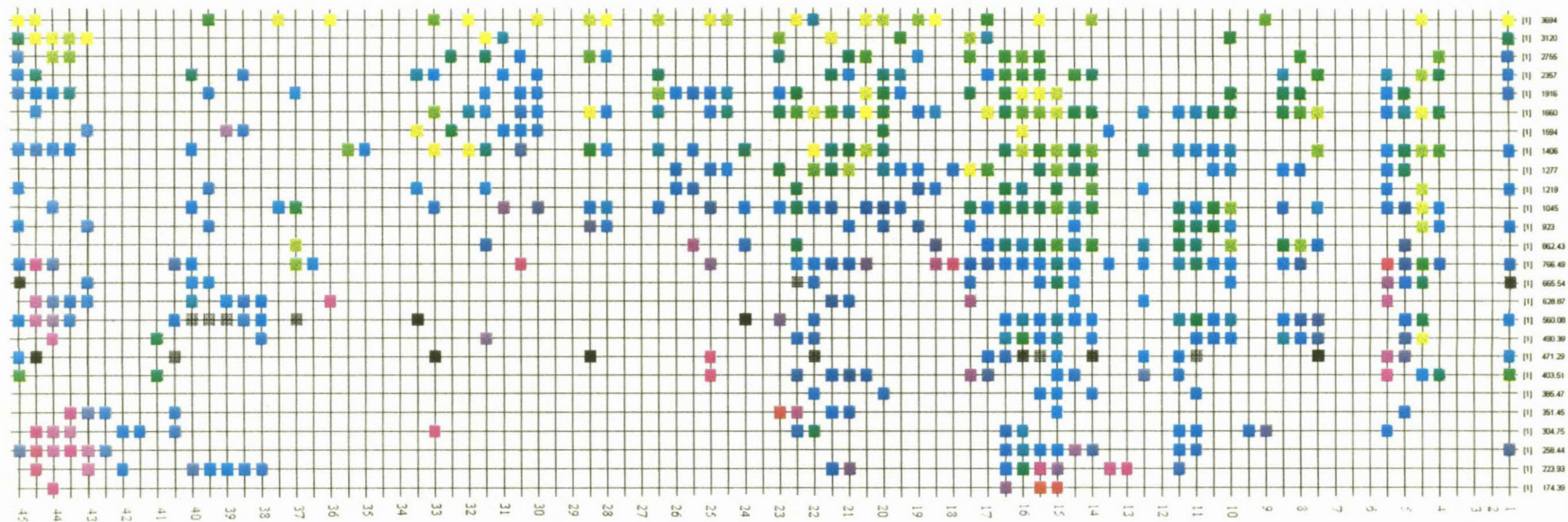


**Figure 5.1.2** Schematic representation of the DAF fragment profile in the genus *Pentaschistis*, using DAF primer DAF<sub>1</sub>. 1 - DNA marker VI, 2 - negative control, 3 - *P. airoides* (Spies 6152), 4 - *P. airoides* (Spies 6205), 5 - *P. aristifolia* (Spies 6295), 6 - *P. airoides* (Spies 6311), 7 - *P. veneta* (Spies 6001), 8 - *P. barbata* (Spies 6267), 9 - *P. barbata* (Spies 6321), 10 - *P. veneta* (Spies 6327), 11 - *P. eriostoma* (Spies 6000), 12 - *P. pallida* (Spies 6189), 13 - *P. pallida* (Spies 6276), 14 - *P. pallida* (Spies 6276), 15 - *P. tomentella* (Spies 6337), 16 - *P. tomentella* (Spies 6345), 17 - *P. patula* (Spies 6272), 18 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 21 - *P. tomentella* (Spies 6344), 22 - *P. papillosa* (Spies 6226), 23 - *P. rupestris* (Spies 6330), 24 - *P. eriostoma* (Spies 6144), 25 - *P. eriostoma* (Spies 6173), 26 - *P. eriostoma* (Spies 6269), 27 - *P. viscidula* (Spies 6178), 28 - *P. densifolia* (Spies 6328), 29 - *P. tortuosa* (Spies 6179), 30 - *P. colorata* (Spies 6313), 31 - *P. rupestris* (Spies 6309), 32 - *P. tomentella* (Spies 6356), 33 - *P. rigidissima* (Spies 6243), 34 - *P. rigidissima* (Spies 6109), 35 - *P. tortuosa* (Spies 6214), 36 - *P. tomentella* (Spies 6277), 37 - *P. curvifolia* (Spies 6215), 38 - *P. curvifolia* (Spies 6221), 39 - *P. curvifolia* (Spies 6232), 40 - *P. curvifolia* (Spies 6270), 41 - *P. capensis* (Spies 6169), 42 - *P. pallida* (Spies 6281), 43 - *P. rupestris* (Spies 6308), 44 - *P. rupestris* (Spies 6310), 45 - DNA marker VI.

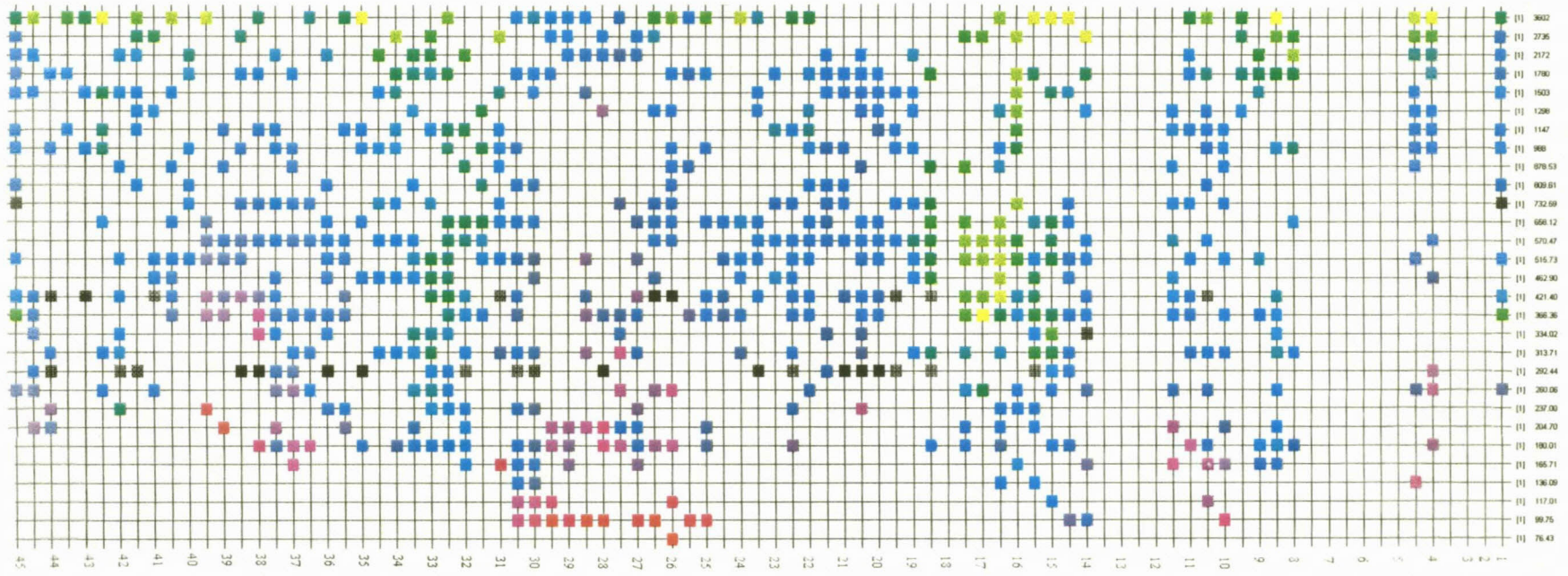


**Figure 5.1.3** Schematic representation of the DAF fragment profile in the genus *Pentaschistis*, using DAF primer DAF<sub>3</sub>. 1 - DNA marker VI, 2 - negative control, 3 - *P. airoides* (Spies 6152), 4 - *P. airoides* (Spies 6205), 5 - *P. aristifolia* (Spies 6295), 6 - *P. airoides* (Spies 6311), 7 - *P. veneta* (Spies 6001), 8 - *P. barbata* (Spies 6267), 9 - *P. barbata* (Spies 6321), 10 - *P. veneta* (Spies 6327), 11 - *P. eriostoma* (Spies 6000), 12 - *P. pallida* (Spies 6189), 13 - *P. pallida* (Spies 6276), 14 - *P. pallida* (Spies 6276), 15 - *P. tomentella* (Spies 6337), 16 - *P. tomentella* (Spies 6345), 17 - *P. patula* (Spies 6272), 18 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 21 - *P. tomentella* (Spies 6344), 22 - *P. papillosa* (Spies 6226), 23 - *P. rupestris* (Spies 6330), 24 - *P. eriostoma* (Spies 6144), 25 - *P. eriostoma* (Spies 6173), 26 - *P. eriostoma* (Spies 6269), 27 - *P. viscidula* (Spies 6178), 28 - *P. densifolia* (Spies 6328), 29 - *P. tortuosa* (Spies 6179), 30 - *P. colorata* (Spies 6313), 31 - *P. rupestris* (Spies 6309), 32 - *P. tomentella* (Spies 6356), 33 - *P. rigidissima* (Spies 6243), 34 - *P. rigidissima* (Spies 6109), 35 - *P. tortuosa* (Spies 6214), 36 - *P. tomentella* (Spies 6277), 37 - *P. curvifolia* (Spies 6215), 38 - *P. curvifolia* (Spies 6221), 39 - *P. curvifolia* (Spies 6232), 40 - *P. curvifolia* (Spies 6270), 41 - *P. capensis* (Spies 6169), 42 - *P. pallida* (Spies 6281), 43 - *P. rupestris* (Spies 6308), 44 - *P. rupestris* (Spies 6310), 45 - DNA marker VI.

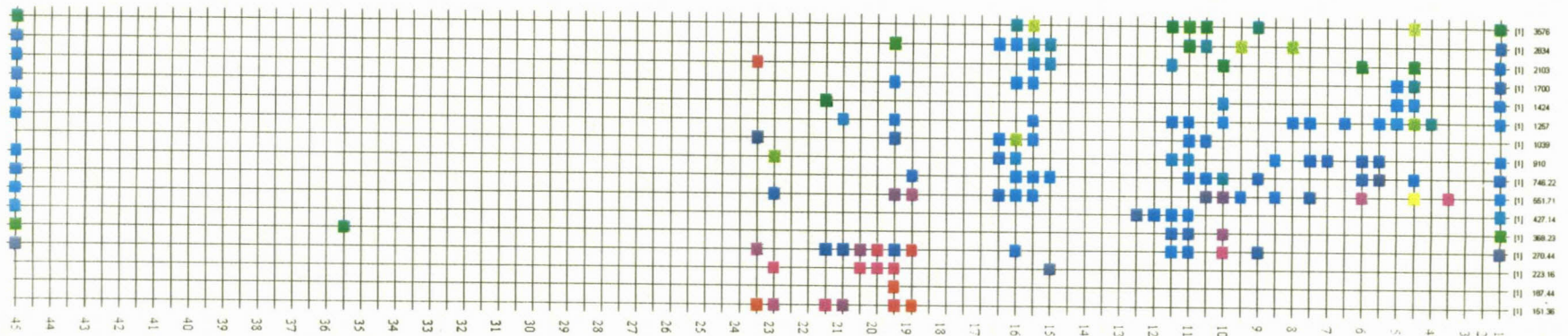




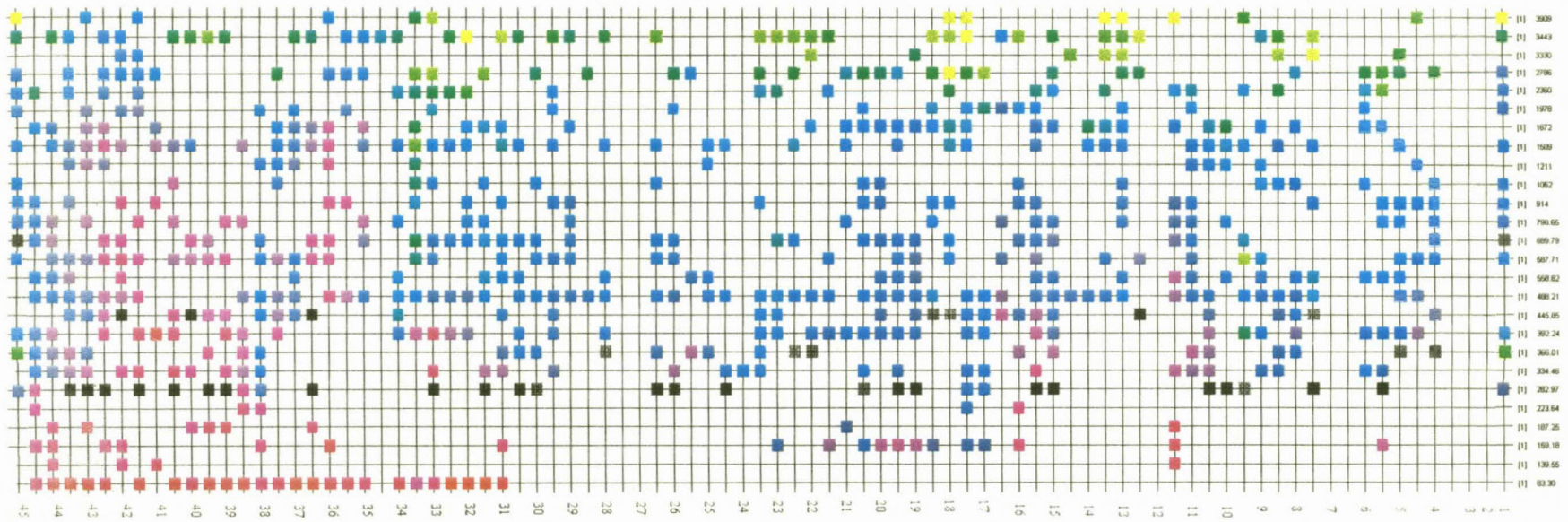
**Figure 5.1.4** Schematic representation of the DAF fragment profile in the genus *Pentaschistis*, using DAF primer DAF<sub>5</sub>. 1 - DNA marker VI, 2 - negative control, 3 - *P. airoides* (Spies 6152), 4 - *P. airoides* (Spies 6205), 5 - *P. aristifolia* (Spies 6295), 6 - *P. airoies* (Spies 6311), 7 - *P. veneta* (Spies 6001), 8 - *P. barbata* (Spies 6267), 9 - *P. barbata* (Spies 6321), 10 - *P. veneta* (Spies 6327), 11 - *P. eriostoma* (Spies 6000), 12 - *P. pallida* (Spies 6189), 13 - *P. pallida* (Spies 6276), 14 - *P. pallida* (Spies 6276), 15 - *P. tomentella* (Spies 6337), 16 - *P. tomentella* (Spies 6345), 17 - *P. patula* (Spies 6272), 18 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 21 - *P. tomentella* (Spies 6344), 22 - *P. papillosa* (Spies 6226), 23 - *P. rupestris* (Spies 6330), 24 - *P. eriostoma* (Spies 6144), 25 - *P. eriostoma* (Spies 6173), 26 - *P. eriostoma* (Spies 6269), 27 - *P. viscidula* (Spies 6178), 28 - *P. densifolia* (Spies 6328), 29 - *P. tortuosa* (Spies 6179), 30 - *P. colorata* (Spies 6313), 31 - *P. rupestris* (Spies 6309), 32 - *P. tomentella* (Spies 6356), 33 - *P. rigidissima* (Spies 6243), 34 - *P. rigidissima* (Spies 6109), 35 - *P. tortuosa* (Spies 6214), 36 - *P. tomentella* (Spies 6277), 37 - *P. curvifolia* (Spies 6215), 38 - *P. curvifolia* (Spies 6221), 39 - *P. curvifolia* (Spies 6232), 40 - *P. curvifolia* (Spies 6270), 41 - *P. capensis* (Spies 6169), 42 - *P. pallida* (Spies 6281), 43 - *P. rupestris* (Spies 6308), 44 - *P. rupestris* (Spies 6310), 45 - DNA marker VI.



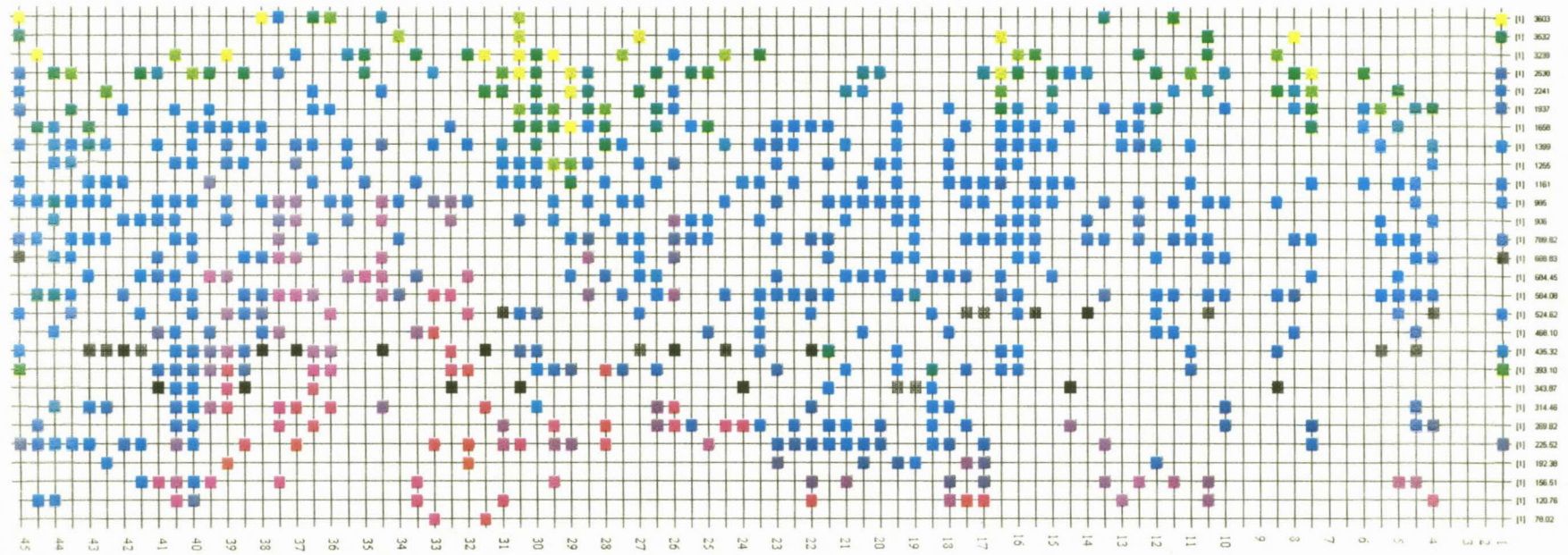
**Figure 5.1.5** Schematic representation of the DAF fragment profile in the genus *Pentaschistis*, using DAF primer DAF<sub>6</sub>. 1 - DNA marker VI, 2 - negative control, 3 - *P. airoides* (Spies 6152), 4 - *P. airoides* (Spies 6205), 5 - *P. aristifolia* (Spies 6295), 6 - *P. airoies* (Spies 6311), 7 - *P. veneta* (Spies 6001), 8 - *P. barbata* (Spies 6267), 9 - *P. barbata* (Spies 6321), 10 - *P. veneta* (Spies 6327), 11 - *P. eriostoma* (Spies 6000), 12 - *P. pallida* (Spies 6189), 13 - *P. pallida* (Spies 6276), 14 - *P. pallida* (Spies 6276), 15 - *P. tomentella* (Spies 6337), 16 - *P. tomentella* (Spies 6345), 17 - *P. patula* (Spies 6272), 18 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 21 - *P. tomentella* (Spies 6344), 22 - *P. papillosa* (Spies 6226), 23 - *P. rupestris* (Spies 6330), 24 - *P. tomentella* (Spies 6346), 25 - *P. eriostoma* (Spies 6173), 26 - *P. eriostoma* (Spies 6269), 27 - *P. viscidula* (Spies 6178), 28 - *P. densifolia* (Spies 6328), 29 - *P. tortuosa* (Spies 6179), 30 - *P. colorata* (Spies 6313), 31 - *P. rupestris* (Spies 6309), 32 - *P. tomentella* (Spies 6356), 33 - *P. rigidissima* (Spies 6243), 34 - *P. rigidissima* (Spies 6109), 35 - *P. tortuosa* (Spies 6214), 36 - *P. tomentella* (Spies 6277), 37 - *P. curvifolia* (Spies 6215), 38 - *P. curvifolia* (Spies 6221), 39 - *P. curvifolia* (Spies 6232), 40 - *P. curvifolia* (Spies 6270), 41 - *P. capensis* (Spies 6169), 42 - *P. pallida* (Spies 6281), 43 - *P. rupestris* (Spies 6308), 44 - *P. rupestris* (Spies 6310), 45 - DNA marker VI.



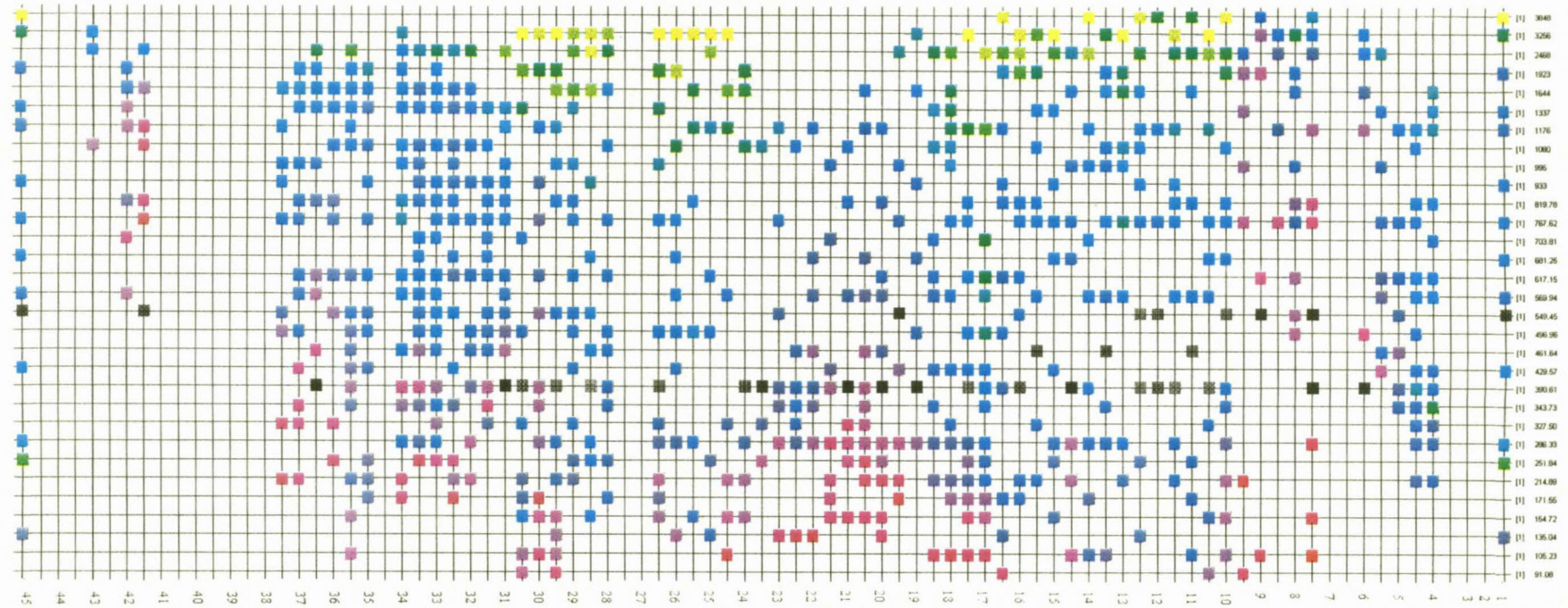
**Figure 5.1.6** Schematic representation of the DAF fragment profile in the genus *Pentaschistis*, using DAF primer DAF7. 1 - DNA marker VI, 2 - negative control, 3 - *P. airoides* (Spies 6152), 4 - *P. airoides* (Spies 6205), 5 - *P. aristifolia* (Spies 6295), 6 - *P. airoies* (Spies 6311), 7 - *P. veneta* (Spies 6001), 8 - *P. barbata* (Spies 6267), 9 - *P. barbata* (Spies 6321), 10 - *P. veneta* (Spies 6327), 11 - *P. eriostoma* (Spies 6000), 12 - *P. pallida* (Spies 6189), 13 - *P. pallida* (Spies 6276), 14 - *P. pallida* (Spies 6276), 15 - *P. tomentella* (Spies 6337), 16 - *P. tomentella* (Spies 6345), 17 - *P. patula* (Spies 6272), 18 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 21 - *P. tomentella* (Spies 6344), 22 - *P. papillosa* (Spies 6226), 23 - *P. rupestris* (Spies 6330), 24 - *P. eriostoma* (Spies 6144), 25 - *P. eriostoma* (Spies 6173), 26 - *P. eriostoma* (Spies 6269), 27 - *P. viscidula* (Spies 6178), 28 - *P. densifolia* (Spies 6328), 29 - *P. tortuosa* (Spies 6179), 30 - *P. colorata* (Spies 6313), 31 - *P. rupestris* (Spies 6309), 32 - *P. tomentella* (Spies 6356), 33 - *P. rigidissima* (Spies 6243), 34 - *P. rigidissima* (Spies 6109), 35 - *P. tortuosa* (Spies 6214), 36 - *P. tomentella* (Spies 6277), 37 - *P. curvifolia* (Spies 6215), 38 - *P. curvifolia* (Spies 6221), 39 - *P. curvifolia* (Spies 6232), 40 - *P. curvifolia* (Spies 6270), 41 - *P. capensis* (Spies 6169), 42 - *P. pallida* (Spies 6281), 43 - *P. rupestris* (Spies 6308), 44 - *P. rupestris* (Spies 6310), 45 - DNA marker VI.



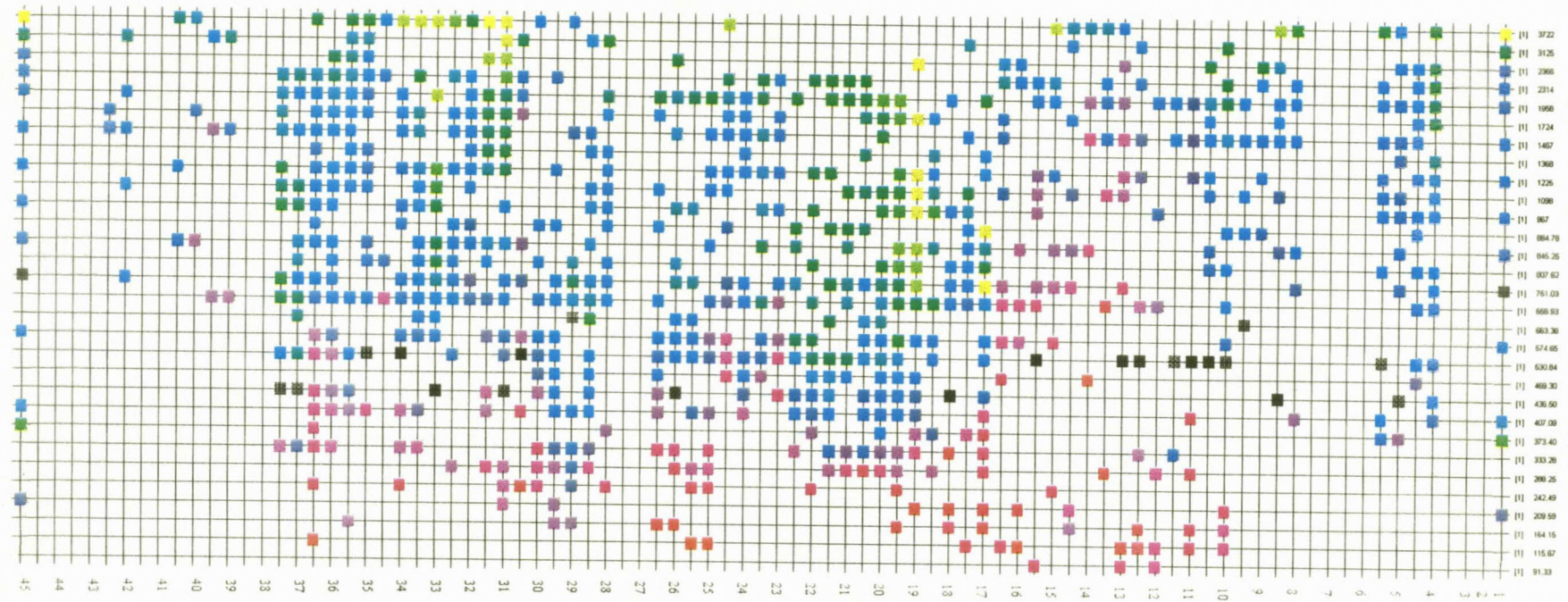
**Figure 5.1.7** Schematic representation of the DAF fragment profile in the genus *Pentaschistis*, using DAF primer DAF<sub>8</sub>. 1 - DNA marker VI, 2 - negative control, 3 - *P. airoides* (Spies 6152), 4 - *P. airoides* (Spies 6205), 5 - *P. aristifolia* (Spies 6295), 6 - *P. airoies* (Spies 6311), 7 - *P. veneta* (Spies 6001), 8 - *P. barbata* (Spies 6267), 9 - *P. barbata* (Spies 6321), 10 - *P. veneta* (Spies 6327), 11 - *P. eriostoma* (Spies 6000), 12 - *P. pallida* (Spies 6189), 13 - *P. pallida* (Spies 6276), 14 - *P. pallida* (Spies 6276), 15 - *P. tomentella* (Spies 6337), 16 - *P. tomentella* (Spies 6345), 17 - *P. patula* (Spies 6272), 18 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 21 - *P. tomentella* (Spies 6344), 22 - *P. papillosa* (Spies 6226), 23 - *P. rupestris* (Spies 6330), 24 - *P. eriostoma* (Spies 6144), 25 - *P. eriostoma* (Spies 6173), 26 - *P. eriostoma* (Spies 6269), 27 - *P. viscidula* (Spies 6178), 28 - *P. densifolia* (Spies 6328), 29 - *P. tortuosa* (Spies 6179), 30 - *P. colorata* (Spies 6313), 31 - *P. rupestris* (Spies 6309), 32 - *P. tomentella* (Spies 6356), 33 - *P. rigidissima* (Spies 6243), 34 - *P. rigidissima* (Spies 6109), 35 - *P. tortuosa* (Spies 6214), 36 - *P. tomentella* (Spies 6277), 37 - *P. curvifolia* (Spies 6215), 38 - *P. curvifolia* (Spies 6221), 39 - *P. curvifolia* (Spies 6232), 40 - *P. curvifolia* (Spies 6270), 41 - *P. capensis* (Spies 6169), 42 - *P. pallida* (Spies 6281), 43 - *P. rupestris* (Spies 6308), 44 - *P. rupestris* (Spies 6310), 45 - DNA marker VI.



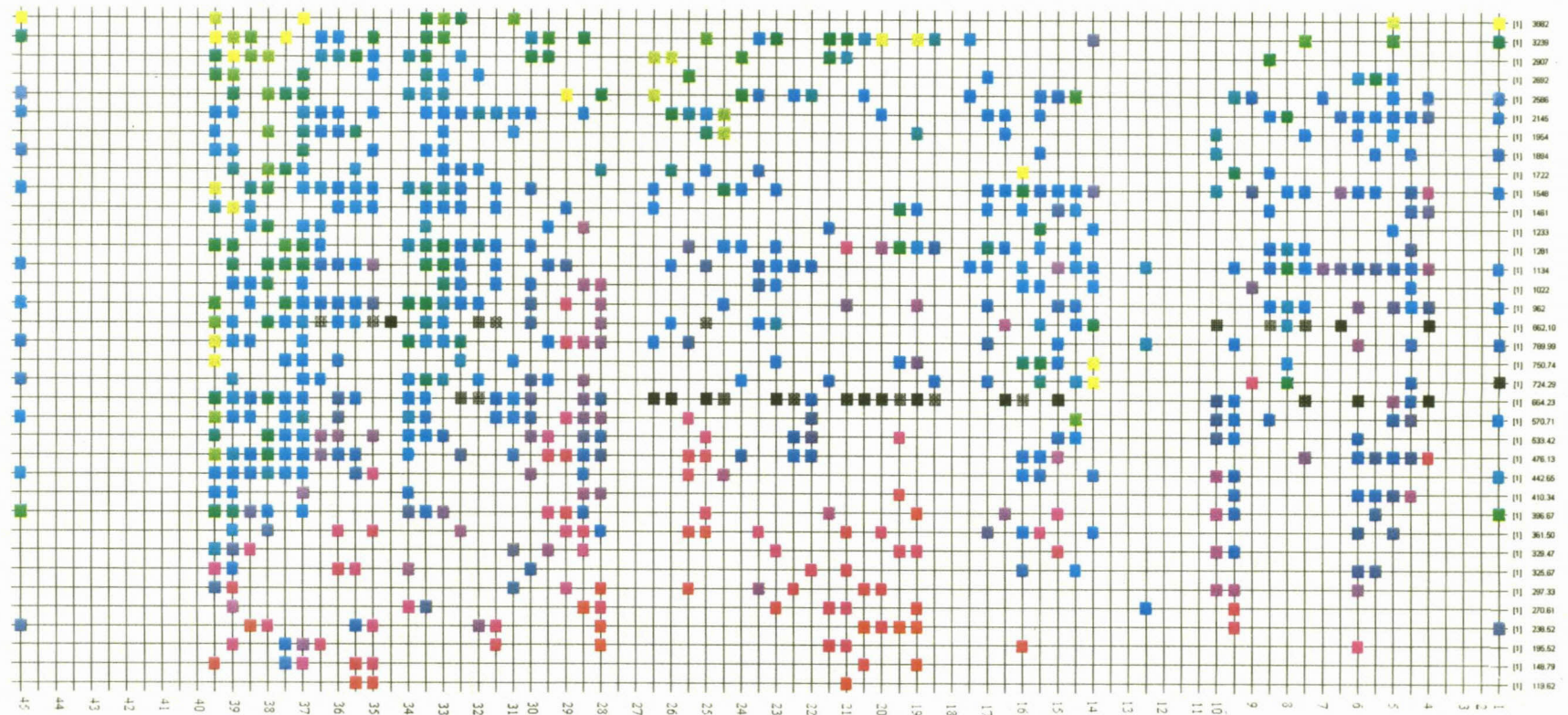
**Figure 5.1.8** Schematic representation of the DAF fragment profile in the genus *Pentaschistis*, using DAF primer DAF<sub>9</sub>. 1 - DNA marker VI, 2 - negative control, 3 - *P. airoides* (Spies 6152), 4 - *P. airoides* (Spies 6205), 5 - *P. aristifolia* (Spies 6295), 6 - *P. airoies* (Spies 6311), 7 - *P. veneta* (Spies 6001), 8 - *P. barbata* (Spies 6267), 9 - *P. barbata* (Spies 6321), 10 - *P. veneta* (Spies 6327), 11 - *P. eriostoma* (Spies 6000), 12 - *P. pallida* (Spies 6189), 13 - *P. pallida* (Spies 6276), 14 - *P. pallida* (Spies 6276), 15 - *P. tomentella* (Spies 6337), 16 - *P. tomentella* (Spies 6345), 17 - *P. patula* (Spies 6272), 18 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 21 - *P. tomentella* (Spies 6344), 22 - *P. papillosa* (Spies 6226), 23 - *P. rupestris* (Spies 6330), 24 - *P. eriostoma* (Spies 6144), 25 - *P. eriostoma* (Spies 6173), 26 - *P. eriostoma* (Spies 6269), 27 - *P. viscidula* (Spies 6178), 28 - *P. densifolia* (Spies 6328), 29 - *P. tortuosa* (Spies 6179), 30 - *P. colorata* (Spies 6313), 31 - *P. rupestris* (Spies 6309), 32 - *P. tomentella* (Spies 6356), 33 - *P. rigidissima* (Spies 6243), 34 - *P. rigidissima* (Spies 6109), 35 - *P. tortuosa* (Spies 6214), 36 - *P. tomentella* (Spies 6277), 37 - *P. curvifolia* (Spies 6215), 38 - *P. curvifolia* (Spies 6221), 39 - *P. curvifolia* (Spies 6232), 40 - *P. curvifolia* (Spies 6270), 41 - *P. capensis* (Spies 6169), 42 - *P. pallida* (Spies 6281), 43 - *P. rupestris* (Spies 6308), 44 - *P. rupestris* (Spies 6310), 45 - DNA marker VI.



**Figure 5.1.9** Schematic representation of the DAF fragment profile in the genus *Pentaschistis*, using DAF primer DAF<sub>10</sub>. 1 - DNA marker VI, 2 - negative control, 3 - *P. airoides* (Spies 6152), 4 - *P. airoides* (Spies 6205), 5 - *P. aristifolia* (Spies 6295), 6 - *P. airoies* (Spies 6311), 7 - *P. veneta* (Spies 6001), 8 - *P. barbata* (Spies 6267), 9 - *P. barbata* (Spies 6321), 10 - *P. veneta* (Spies 6327), 11 - *P. eriostoma* (Spies 6000), 12 - *P. pallida* (Spies 6189), 13 - *P. pallida* (Spies 6276), 14 - *P. pallida* (Spies 6276), 15 - *P. tomentella* (Spies 6337), 16 - *P. tomentella* (Spies 6345), 17 - *P. patula* (Spies 6272), 18 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 21 - *P. tomentella* (Spies 6344), 22 - *P. papillosa* (Spies 6226), 23 - *P. rupestris* (Spies 6330), 24 - *P. eriostoma* (Spies 6144), 25 - *P. eriostoma* (Spies 6173), 26 - *P. eriostoma* (Spies 6269), 27 - *P. viscidula* (Spies 6178), 28 - *P. densifolia* (Spies 6328), 29 - *P. tortuosa* (Spies 6179), 30 - *P. colorata* (Spies 6313), 31 - *P. rupestris* (Spies 6309), 32 - *P. tomentella* (Spies 6356), 33 - *P. rigidissima* (Spies 6243), 34 - *P. rigidissima* (Spies 6109), 35 - *P. tortuosa* (Spies 6214), 36 - *P. tomentella* (Spies 6277), 37 - *P. curvifolia* (Spies 6215), 38 - *P. curvifolia* (Spies 6221), 39 - *P. curvifolia* (Spies 6232), 40 - *P. curvifolia* (Spies 6270), 41 - *P. capensis* (Spies 6169), 42 - *P. pallida* (Spies 6281), 43 - *P. rupestris* (Spies 6308), 44 - *P. rupestris* (Spies 6310), 45 - DNA marker VI.



**Figure 5.1.10** Schematic representation of the DAF fragment profile in the genus *Pentaschistis*, using DAF primer DAF<sub>11</sub>. 1 - DNA marker VI, 2 - negative control, 3 - *P. airoides* (Spies 6152), 4 - *P. airoides* (Spies 6205), 5 - *P. aristifolia* (Spies 6295), 6 - *P. airoides* (Spies 6311), 7 - *P. veneta* (Spies 6001), 8 - *P. barbata* (Spies 6267), 9 - *P. barbata* (Spies 6321), 10 - *P. veneta* (Spies 6327), 11 - *P. eriostoma* (Spies 6000), 12 - *P. pallida* (Spies 6189), 13 - *P. pallida* (Spies 6276), 14 - *P. pallida* (Spies 6276), 15 - *P. tomentella* (Spies 6337), 16 - *P. tomentella* (Spies 6345), 17 - *P. patula* (Spies 6272), 18 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 21 - *P. tomentella* (Spies 6344), 22 - *P. papillosa* (Spies 6226), 23 - *P. rupestris* (Spies 6330), 24 - *P. eriostoma* (Spies 6144), 25 - *P. eriostoma* (Spies 6173), 26 - *P. eriostoma* (Spies 6269), 27 - *P. viscidula* (Spies 6178), 28 - *P. densifolia* (Spies 6328), 29 - *P. tortuosa* (Spies 6179), 30 - *P. colorata* (Spies 6313), 31 - *P. rupestris* (Spies 6309), 32 - *P. tomentella* (Spies 6356), 33 - *P. rigidissima* (Spies 6243), 34 - *P. rigidissima* (Spies 6109), 35 - *P. tortuosa* (Spies 6214), 36 - *P. tomentella* (Spies 6277), 37 - *P. curvifolia* (Spies 6215), 38 - *P. curvifolia* (Spies 6221), 39 - *P. curvifolia* (Spies 6232), 40 - *P. curvifolia* (Spies 6270), 41 - *P. capensis* (Spies 6169), 42 - *P. pallida* (Spies 6281), 43 - *P. rupestris* (Spies 6308), 44 - *P. rupestris* (Spies 6310), 45 - DNA marker VI.



**Figure 5.1.11** Schematic representation of the DAF fragment profile in the genus *Pentaschistis*, using DAF primer DAF<sub>12</sub>. 1 - DNA marker VI, 2 - negative control, 3 - *P. airoides* (Spies 6152), 4 - *P. airoides* (Spies 6205), 5 - *P. aristifolia* (Spies 6295), 6 - *P. airoides* (Spies 6311), 7 - *P. veneta* (Spies 6001), 8 - *P. barbata* (Spies 6267), 9 - *P. barbata* (Spies 6321), 10 - *P. veneta* (Spies 6327), 11 - *P. eriostoma* (Spies 6000), 12 - *P. pallida* (Spies 6189), 13 - *P. pallida* (Spies 6276), 14 - *P. pallida* (Spies 6276), 15 - *P. tomentella* (Spies 6337), 16 - *P. tomentella* (Spies 6345), 17 - *P. patula* (Spies 6272), 18 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 21 - *P. tomentella* (Spies 6344), 22 - *P. papillosa* (Spies 6226), 23 - *P. rupestris* (Spies 6330), 24 - *P. eriostoma* (Spies 6144), 25 - *P. eriostoma* (Spies 6173), 26 - *P. eriostoma* (Spies 6269), 27 - *P. viscidula* (Spies 6178), 28 - *P. densifolia* (Spies 6328), 29 - *P. tortuosa* (Spies 6179), 30 - *P. colorata* (Spies 6313), 31 - *P. rupestris* (Spies 6309), 32 - *P. tomentella* (Spies 6356), 33 - *P. rigidissima* (Spies 6243), 34 - *P. rigidissima* (Spies 6109), 35 - *P. tortuosa* (Spies 6214), 36 - *P. tomentella* (Spies 6277), 37 - *P. curvifolia* (Spies 6215), 38 - *P. curvifolia* (Spies 6221), 39 - *P. curvifolia* (Spies 6232), 40 - *P. curvifolia* (Spies 6270), 41 - *P. capensis* (Spies 6169), 42 - *P. pallida* (Spies 6281), 43 - *P. rupestris* (Spies 6308), 44 - *P. rupestris* (Spies 6310), 45 - DNA marker VI.



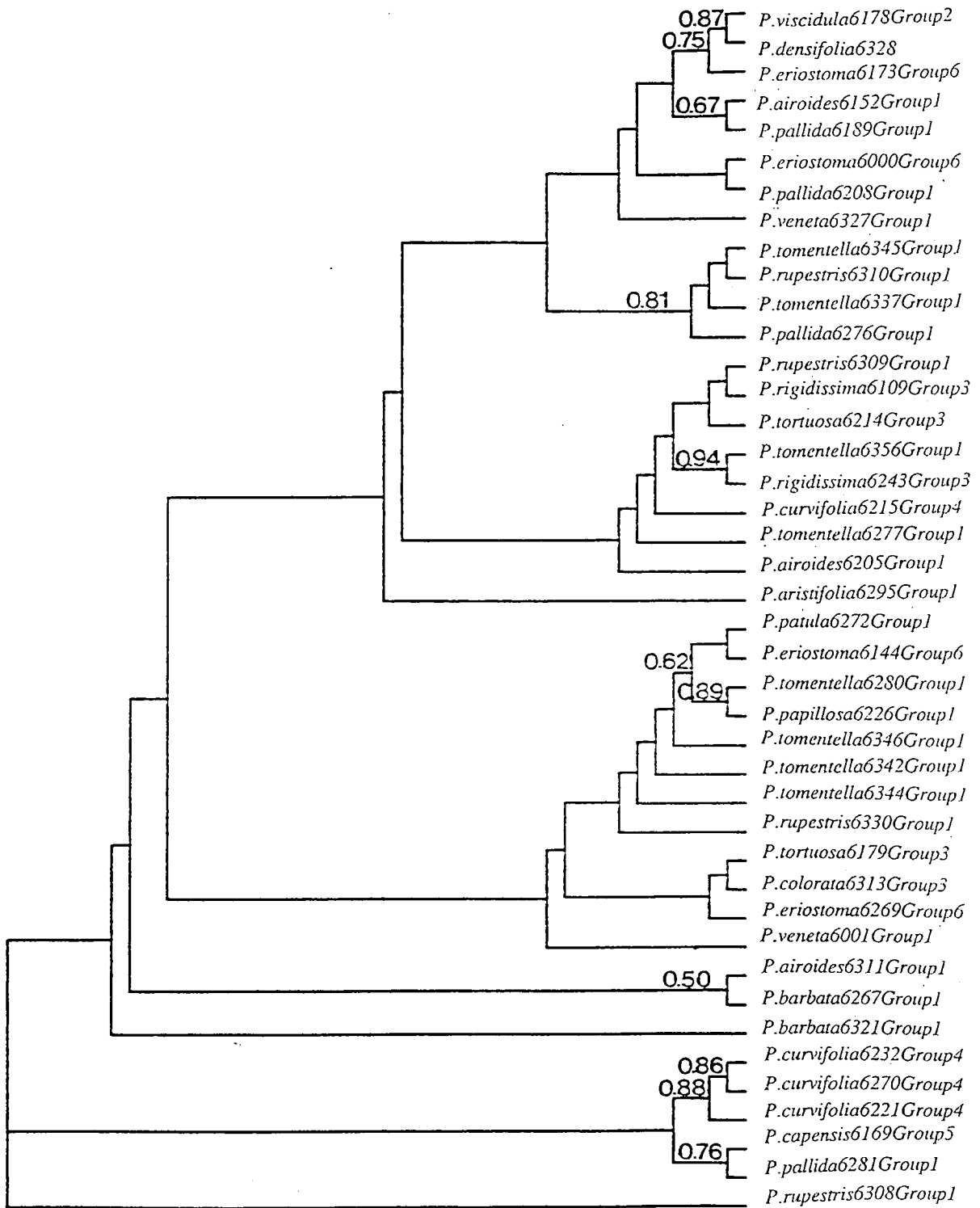
was scored per specimen, which gave a total of 13272 fragments scored for all the primers used (Appendix C). The reproducibility of the DAF primers varied between 68% and 90%, with DAF 1 being the highest. Fingerprint patterns were subjected to phylogenetic analysis. Scoring the presence or absence of fragments in DAF gels was used to create a data matrix. Phylogenetic analysis of the data matrix by the computer program Hennig86, which considers each character one at a time, produced the parsimonious phylogenetic trees (Figure 5.2 & 5.4) and the Wagner parsimony method of PAUP (Phylogenetic Analysis Using Parsimony) 3.1.1 resulted in the parsimonious phylogenetic trees (Figure 5.3 & 5.5). The genetic distances and coefficients of similarity (Appendix D) within, and between, the species and within, and between, the morphological groups were determined (Table 5.2 & 5.3).

**TABLE 5.2** The average genetic distances within and between the morphological groups of the genus *Pentastichis*.

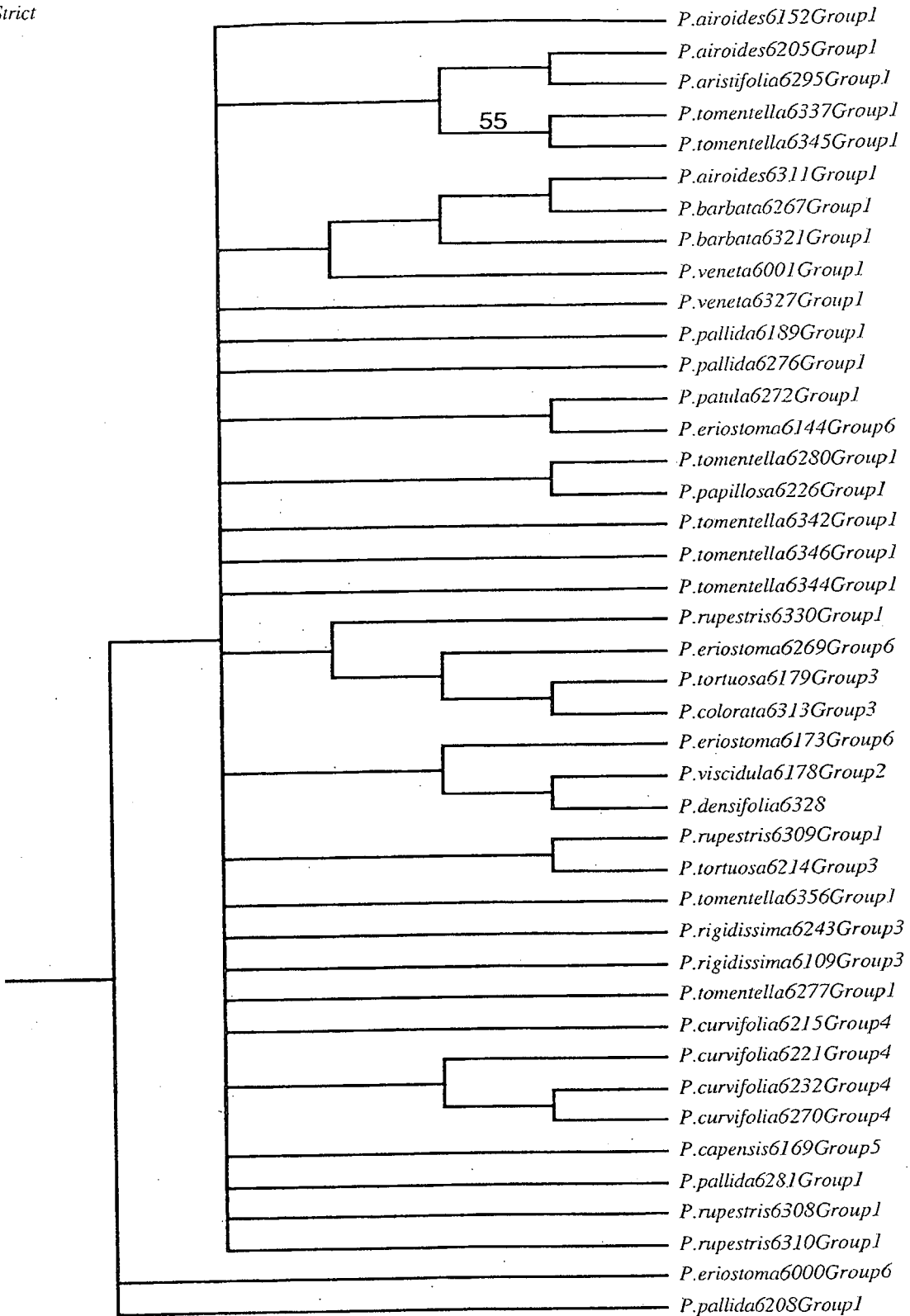
	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
GROUP 1	0.82	1.13	0.72	0.86	0.98	0.85
GROUP 2		—	0.94	0.75	1.05	1.13
GROUP 3			0.56	0.80	0.96	0.75
GROUP 4				0.71	0.89	1.17
GROUP 5					—	1.08
GROUP 6						0.82

### **5.3 Discussion.**

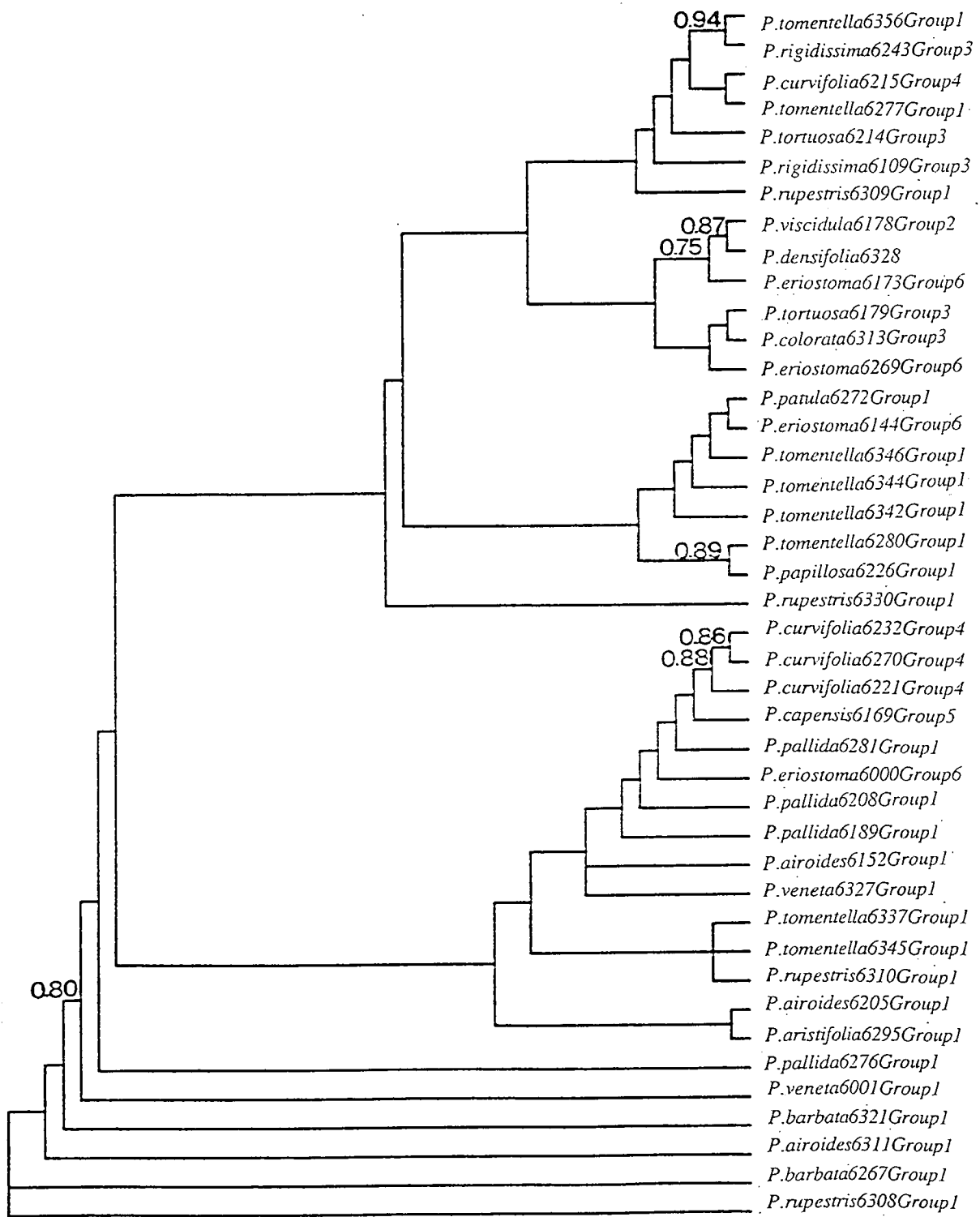
While the information basis of nucleotide sequences is growing exponentially, methods are needed to investigate plant genomes at a level of complexity "above" the primary sequence, but "below" the cytogenetic, karyotypic arrangement. In recent years PCR based techniques (Arnheim & Erlich 1992), have revolutionised genome characterization for all living organisms, including higher plants (Caetano-Anollès *et al.* 1991a, Schaal *et al.* 1991). DNA amplification using PCR has become a key procedure in



**Figure 5.2** Consensus of two parsimonious trees, (CI = 0.11, RI = 0.34), with length 2699, containing 42 *Pentaschistis* specimens with *P. eriostoma* as outgroup. Numbers above branches are Jackknife values.

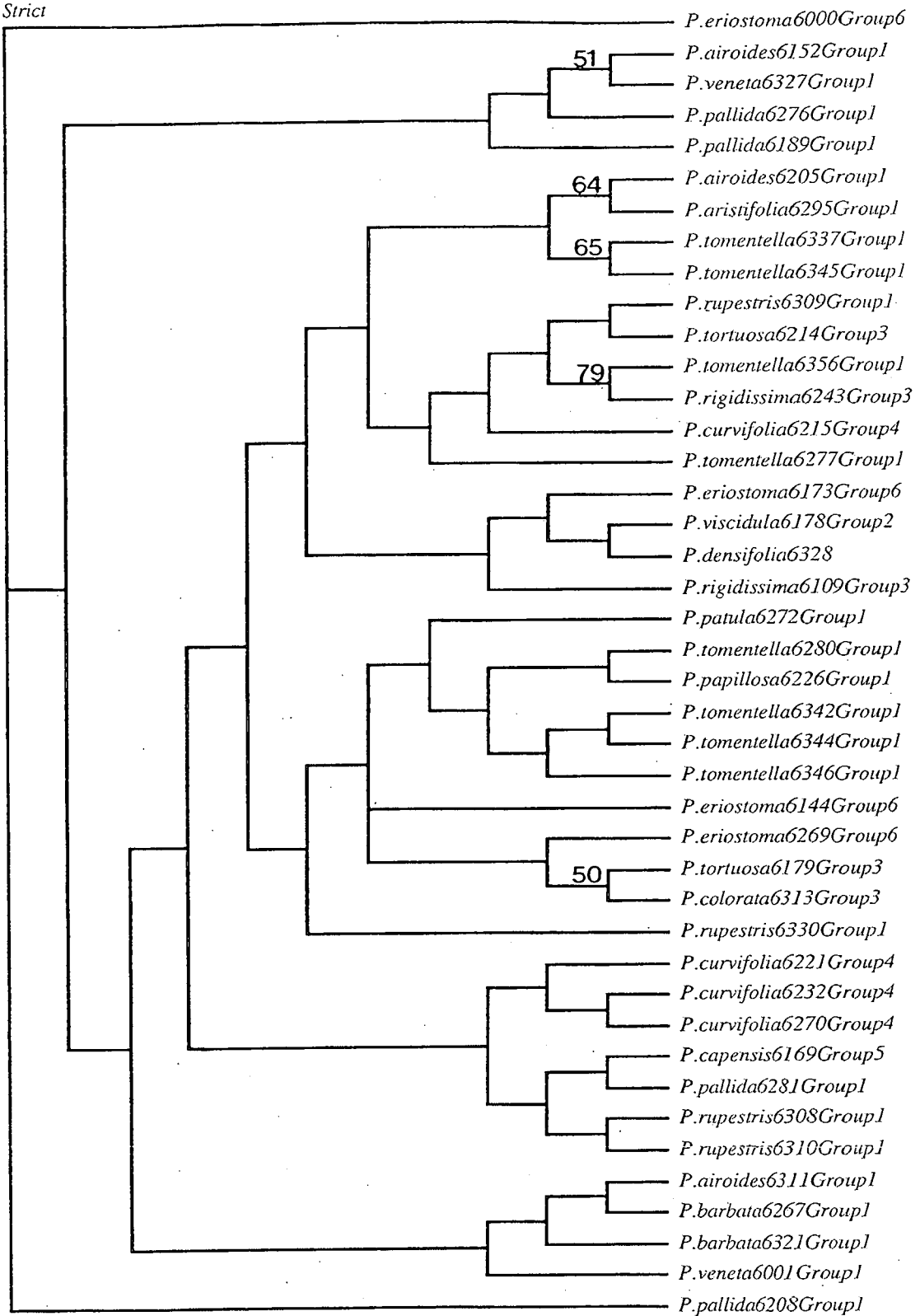


**Figure 5.3** Strict consensus of 12 most parsimonious trees (CI = 0.12, RI = 0.34), with length 2690, containing 42 *Pentaschistis* specimens, with *P. eriostoma* as outgroup. Numbers above branches are % bootstrap values of 200 replicates. Nodes without numbers have bootstrap values of <50%.



**Figure 5.4** Consensus of 42 most parsimonious trees (CI = 0.11, RI = 0.39), with length 1960, with some characters temporarily removed. Numbers above branches are Jackknife values.

Strict



**Figure 5.5** Strict consensus of two trees (CI = 0.12, RI = 0.42), with length 1621, with some characters temporarily removed. Numbers above branches are % bootstrap values of 200 replicates. Nodes without numbers have bootstrap values of <50%.

molecular biology. Over the last few years a single short primer based DNA amplification technique (DAF) was developed and applied to plant genomes. Complex relationships among reaction components are evident and these affect DNA amplification. The amplification of nucleic acids with arbitrary primers is mainly driven by the interaction between primer, template annealing sites and enzyme. Among all reagents MgCl<sub>2</sub> is the most important, its activity being modulated by the concentrations of template, primer and dNTPs (Weaver *et al.* 1995). Increased MgCl<sub>2</sub> levels allow the reaction to occur with more template and dNTPs. However, high magnesium levels should be avoided, since they generally decrease amplification stringency and increase primer template mismatching (Blanchard *et al.* 1993). In this study the lowest value (75mM) in the range tested was used.

**TABLE 5.3** The genetic distances within and between the species of the genus *Pentaschistis*. 1 = *P. airoides*, 2 = *P. aristifolia*, 3 = *P. barbata*, 4 = *P. densifolia*, 5 = *P. pallida*, 6 = *P. papillosa*, 7 = *P. patula*, 8 = *P. rupestris*, 9 = *P. tomentella*, 10 = *P. veneta*, 11 = *P. viscidula*, 12 = *P. colorata*, 13 = *P. rigidissima*, 14 = *P. tortuosa*, 15 = *P. curvifolia*, 16 = *P. capensis* and 17 = *P. eriostoma*.

	GROUP 1										GROUP 2		GROUP 3			GROUP 4	GROUP 5	GROUP 6	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
1	1.01	0.58	0.92	0.79	1.16	0.92	1.09	1.09	0.90	0.92	1.15	0.86	0.85	0.82	0.96	1.21	1.11		
2		0.90	0.61	0.83	0.71	0.68	0.76	0.71	0.75	1.22	0.69	0.70	0.76	0.75	1.01	0.80			
3			0.76	0.73	1.02	1.01	0.94	0.98	0.92	0.83	1.31	0.78	0.82	0.73	1.00	1.02	0.92		
4				0.80	0.69	0.56	0.87	0.62	0.74	1.19	0.50	0.57	0.57	0.85	0.91	0.67			
5					0.92	0.90	0.87	0.89	0.84	0.97	1.12	0.85	0.85	0.85	1.01	0.97	0.88		
6						0.49	0.74	0.61	0.85	1.03	0.63	0.65	0.77	0.80	0.93	0.75			
7							0.75	0.75	0.85	1.08	0.57	0.64	0.65	0.79	0.93	0.66			
8								0.68	0.70	0.91	0.84	0.73	0.76	0.87	0.76	0.74	0.84		
9									0.79	0.81	1.07	0.58	0.58	0.64	0.78	0.89	0.94		
10										1.00	1.32	0.76	0.85	0.80	0.92	1.20	0.92		
11												0.74	1.09	0.99	0.75	1.05	1.13		
12													0.64	0.50	0.73	0.96	0.64		
13														0.45	0.61	0.77	0.82		
14															0.61	0.89	0.79		
15																0.71	1.17		
16																		1.08	
17																			0.82

By varying the template DNA concentration, the reactions were affected, therefore the concentration was kept constant at 10 ng/μl. The resulting fragments were visualized by polyacrylamide gel electrophoresis, combined with silver staining and not by agarose gel electrophoresis and ethidium bromide staining, because the latter method only detects the few major fragments (Williams *et al.* 1990, Welsh & McClelland 1990). Thus, considerable loss of information can occur if suitable fragment separation and detection procedures are not employed. The silver staining technique is very sensitive, because of the formation of insoluble silver salts. These precipitates attach onto the gel surface and impair the contrast. This can be overcome by the pretreatment of fixed polyacrylamide gels in order to improve the sensitivity of the silver stain and the staining process (Heukeshoven 1985). The handicap of this is an increase of unspecific yellow or brown background staining, in parallel with increased sensitivity and velocity. This was experienced in our study and was reduced by using low amounts of thiosulfate (Blum *et al.* 1987) for the pretreatment of fixed gels.

These gels were scored via the schematic representation and a data matrix was obtained. The data matrix was used to determine the consistency and genetic distances within and between the morphological groups, as well as within, and between the different species (Table 5.2 & 5.3).

The genetic distance within the *Pentascistis* species has a high degree of variation. This was also the case with the RAPD analysis. High intra- and interspecific genetic distances are observed.

In this study three *P. airoides* specimens were examined, in comparison to the one specimen in the RAPD analysis. This species have a genetic distance of 1.01 (Table 5.3). With the DAF analysis two of the three specimens studied are morphologically identical. The only reason for the high variability can thus be the high polyploid levels in *P. airoides*. According to the genetic distances the *P. airoides* species is the closest to *P. aristifolia* (0.58), both belonging to morphological group 1. Morphologically these two species are very similar, the major difference being the absence of glands.

In *P. barbata* the genetic distance (0.76) (Table 5.3) supports the morphological variability and RAPD results (0.60). The two *P. barbata* specimens studied, differ morphologically. Earlier studies indicate high polyploid levels, contributing to the variability.

*Pentasthista pallida*'s genetic distance (0.92) also supports the observations of the RAPD analysis (0.82). The slight difference in genetic distances is contributed to the fact that more *P. pallida* specimens were used in the DAF study. Morphologically these specimens are identical, but cytogenetically polyploid levels of up to hexaploidy have been observed, indicating that this high genetic distance is likely to occur. Thus, again the subdivision (forms rather than subspecies) of *P. pallida*, by Linder & Ellis (1990a), is supported.

Only one *P. patula* specimen has been studied. According to Spies *et al.* (1994a), this species should be closely related to *P. airoides*, but a genetic distance of 1.09 is observed. Thus, again we would not recommend the grouping of these two species. The genetic distances indicate a very close relationship to *P. colorata* (0.57), with some shared morphological features. *P. colorata* can also be the tetraploid ancestor of *P. patula* (diploid).

Even though a high amount of variation is reported in *P. tomentella*, the DAF analysis also produced a low genetic distance within this species, which could be attributed to the fact that most of them comes from the same geographical territory.

All of the above-mentioned species belong to morphological group 1, but the genetic distance within this group is 0.82. Because of the morphological and cytogenetic similarities, no change in the grouping is recommended.

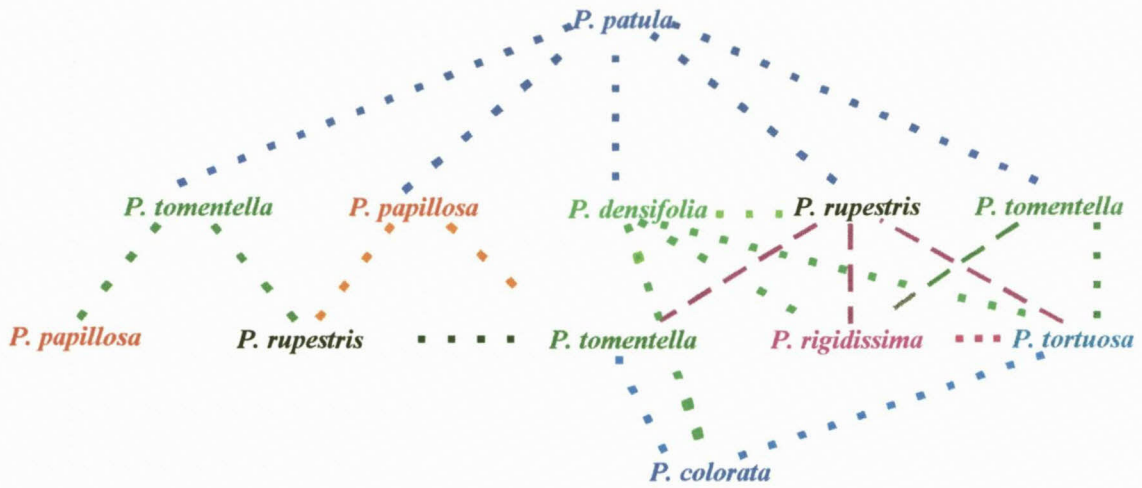
The low genetic distance (0.45) within *P. rigidissima* can be attributed to the fact that they come from the same geographical territory and the fact that they seem to be diploid. Morphologically they are also identical. This species is also closely related to *P. tortuosa*, both belonging to morphological group 3. *Pentasthista rigidissima* can also be the diploid ancestor of *P. tortuosa*, an octaploid.

*Pentasthista tortuosa* is sometimes difficult to separate from *P. colorata* and is thus morphologically similar, this is also seen in a genetic distance of 0.50. Again the genetic distance (0.71) within *P. curvifolia* is high, as was the case with RAPDs (0.93).

The morphological similar *P. capensis* specimens had a low genetic distance (0.52), whereas the *P. eriostoma* specimens had a high genetic distance, which differs from the RAPD analysis. The high polyploid levels observed in this species indicate that a high genetic distance is likely to occur.

Interesting observations according to the genetic distances are represented by Figure 5.6.





**Figure 5.6** Schematic representation of the genetic distances between some *Pentaschistis* species. - - - - represent genetic distances between 0.5 and 0.6 and — — — represent genetic distances smaller than 0.5.

The morphological and cytogenetic evidence corroborates most of these observations.

The genetic distances, within the morphological groups (Table 5.3), indicate the highest variation within morphological group 6 and the lowest variation in morphological group 3. This is contradictory to the RAPD analysis. The morphological group 1 seems to be the closest related to morphological group 3, sharing some morphological features and group 4 is the furthest from group 6. The average genetic distances between the morphological groups varied between 0.56 to 1.17, indicating a high degree of variation within the genus *Pentaschistis* and thus a big gene pool.

The genetic relationships within and between the species of the genus *Pentaschistis* were also determined using the parsimony methods of Hennig86 and PAUP.

In the first cladogram with *P. eriostoma* as outgroup (Figure 5.2), *P. airoides* (Spies 6152) & *P. pallida* (Spies 6189) is a sister group to *P. eriostoma* (Spies 6173), *P. viscidula* (Spies 6178) & *P. densifolia*. This relationship can be supported by the fact that all of these specimens are known to have glands.

The relationship between *P. eriostoma* & *P. pallida* is observed in both cladograms (Figure 5.2 & 5.4), and supported by their morphological similarities. This was also observed in the RAPD analysis. The cytogenetic data indicate polyploid levels of diploidy

up to hexaploidy for *P. eriostoma*, whereas *P. pallida* is diploid, therefore it is possible that *P. pallida* is the diploid ancestor of *P. eriostoma*. Thus, they may share a genome and this may lead to a close relationship.

The sister alliance of *P. tortuosa*, *P. rupestris* & *P. rigidissima* was also evident in the genetic distances, as well as their morphology. Thus supporting the groupings of *P. tortuosa* & *P. rigidissima* in group 3, with *P. rupestris* possibly sharing the same genome (evident from the high polyploid levels). The relationship between *P. rupestris* and *P. tomentella* is confirmed by both Hennig86 and PAUP as well as a genetic distance of 0.59. *Pentasthictis eriostoma* also appears to be related to *P. colorata* & *P. tortuosa*, with a genetic distance of 0.46 and 0.60 respectively, as well as tetraploidy present in all the species. Due to the morphological differences between these species, we would not recommend the groupings of *P. eriostoma* with *P. colorata* & *P. tortuosa*.

The *P. curvifolia* specimens formed a sister group to *P. capensis*, and not to *P. eriostoma*, as was hypothesised by Linder & Ellis (1990a). This is probably due to the fact that both species are diploid.

The two cladograms differ in their placements of the species, thus we excluded the characters with a lower retention index than consistency index (Lipscomb 1998). The cladogram obtained after character exclusion indicates the sister alliance between *P. airoides* & *P. veneta* again, both belonging to group 1, but in Figure 5.4, *P. curvifolia* & *P. capensis* seems to be sister groups to some of the species from the morphological group 1, however, this is not supported by Figure 5.5.

Few differences are observed between these cladograms, but because PAUP gives a lower tree length than Hennig86, the PAUP cladogram is considered to be more reliable. According to this cladogram the morphological groups within the genus *Pentasthictis* seem to be paraphyletic and once again the predicted relationship between *P. curvifolia* and *P. eriostoma* is not supported. The conclusion of Klopper (1996), that *P. eriostoma* is not closely related to any of the *Pentasthictis* species, is well supported by the genetic distances observed (Table 5.3), but the phylogenetic trees does indicate a relationship.

The high degree of variation between the morphological groups are supported by both RAPD and DAF analysis, it is thus safe to come to the conclusion that the genus *Pentasthictis* has a big gene pool with a high degree of hybridization.

Chromosome numbers are sometimes ineffective as a means of separating subspecies and morphologically similar cultivars of the same species, but DAF analysis is

able to detect the differences. DAF profiles are thus a more reliable method to determine the genetic relationships between the morphological groups. Our study provides an estimate of genetic diversity within *Pentascistis* and permits an assessment of genetic relationships between sample material.

## CHAPTER 6

### SEQUENCING

#### 6.1. Introduction.

Nuclear ribosomal DNA has proven to be a powerful phylogenetic tool, because it is ubiquitous in all organisms and is present, as repeated units, in high copy numbers (Hamby & Zimmer 1991). The nuclear rDNA units, separated by intergenic spacers, consist of the 18S, 5.8S and 26S coding regions in plants. The 5.8S coding region, flanked by internal transcribed spacer 1 and 2 (*ITS1* and *ITS2*), is located between the 18S and 26S coding regions. One of the advantages of rDNA as a phylogenetic tool is that the repeat unit consists of several regions that have different rates of nucleotide changes. Therefore, different regions of the molecule can be used to examine lineages with different levels of divergence. The 18S and 26S coding regions have been used to address phylogenetic questions on the family, or higher taxonomic levels in plants (Zimmer *et al.* 1989, Hamby & Zimmer 1991). On the other hand ITS sequences appear to be useful for assessing relationships at lower taxonomic levels, such as among genera or species, because the sequences of spacer regions evolve more rapidly than coding regions. Comparisons of DNA sequences of the internal transcribed spacers (ITS) of nuclear ribosomal RNA genes are useful for phylogenetic reconstructions of species at generic level (Baldwin 1992, 1993). For the purpose of this study we were interested in orthologous genes. ITS can be either orthologous or paralogous but, due to the concerted evolution of ITS, it became homogenised and therefore fewer variation is observed within the same specimen. Variation between ITS sequences is mostly attributable to point mutations, whereas a minor proportion of sites is affected by insertion/deletions (indels).

Only a few taxa of the family Poaceae, such as species of the genus *Zea* (Buckler & Holtsford 1996a, b), the tribe Triticeae (Hsiao *et al.* 1995b), the subfamily Arundinoideae (Hsiao *et al.* 1998), with one *Pentaschistis* species included and further members of the subfamily Pooideae (Hsiao *et al.* 1994, 1995a), have been investigated using this molecular marker.

The aim of this study is to determine the nucleotide sequences of the ITS regions of different species in the genus *Pentaschistis* and to use this data to analyse molecular

phylogenetic relationships among different species of *Pentaschistis*.

## **6.2 Results.**

The boundaries of the *ITS*<sub>1</sub> regions for all species sequenced were determined by comparison with published sequences for the subfamily Arundinoideae (Hsiao *et al.* 1998).

The *ITS*<sub>1</sub> region varied in length among the species from 187bp to 222bp. These lengths correspond with those reported for other taxa e.g. 214 for *P. aspera* (Hsiao *et al.* 1998). For comparative purposes *Merxmuellera dura*, of the subfamily Arundinoideae was used as outgroup. *Pentameris macrocalycina* and *Prionathium ecklonii* were also included in the analysis. Alignment of these sequences resulted in a consensus length of 233 base pairs, using Clustal W and 243 base pairs when the Malign program was used (Appendix E & F). Because of these differences in the alignment of the sequences with Clustal W and Malign, the data sets are used separately.

Estimated sequence divergence ranged from 0.86 % to 15.15 % within the genus *Pentaschistis* with Clustal W alignment and between 1.23 % and 15.63 % with Malign (Appendix G). Pairwise comparison of the taxa with the outgroup species reached 18.02 % with Clustal W alignment and 18.11 % with Malign. The G + C contents of *ITS*<sub>1</sub> averages 64.5 %, ranging from 60 % (*P. airoides*) to 68 % (*Merxmuellera dura*) (Table 6.1).

To find accurate cladogram topology, two cladogram reconstruction methods were used, i.e. PAUP and the Hennig86. Parsimony analysis of the *ITS*<sub>1</sub> region with PAUP yielded 36 equally parsimonious cladograms of 235 steps for the Clustal W data set (Figure 6.1.1) and 60 equally parsimonious cladograms of 211 steps for Malign (Figure 6.2.1). Some characters were temporarily excluded for both data sets and a consensus cladogram was obtained (Figure 6.1.3 & 6.2.3). Possible hybrid taxa were excluded and four equally parsimonious cladograms of 127 steps were obtained with the Clustal W data set and eight equally parsimonious cladograms of 129 steps with Malign.

The same set of data was also used with the Hennig86 computer program. In the first cladogram with all the data included, 18 equally parsimonious cladograms of 474 steps were obtained, from the Clustal W data set (Figure 6.1.2) and 60 equally parsimonious cladograms of 268 steps with the Malign data set (Figure 6.2.2). By excluding some characters, the Clustal W data set produced 16 equally parsimonious cladograms of 330 steps (Figure 6.1.4) and the Malign data set produced 30 equally

parsimonious cladograms of 121 steps (Figure 6.2.4).

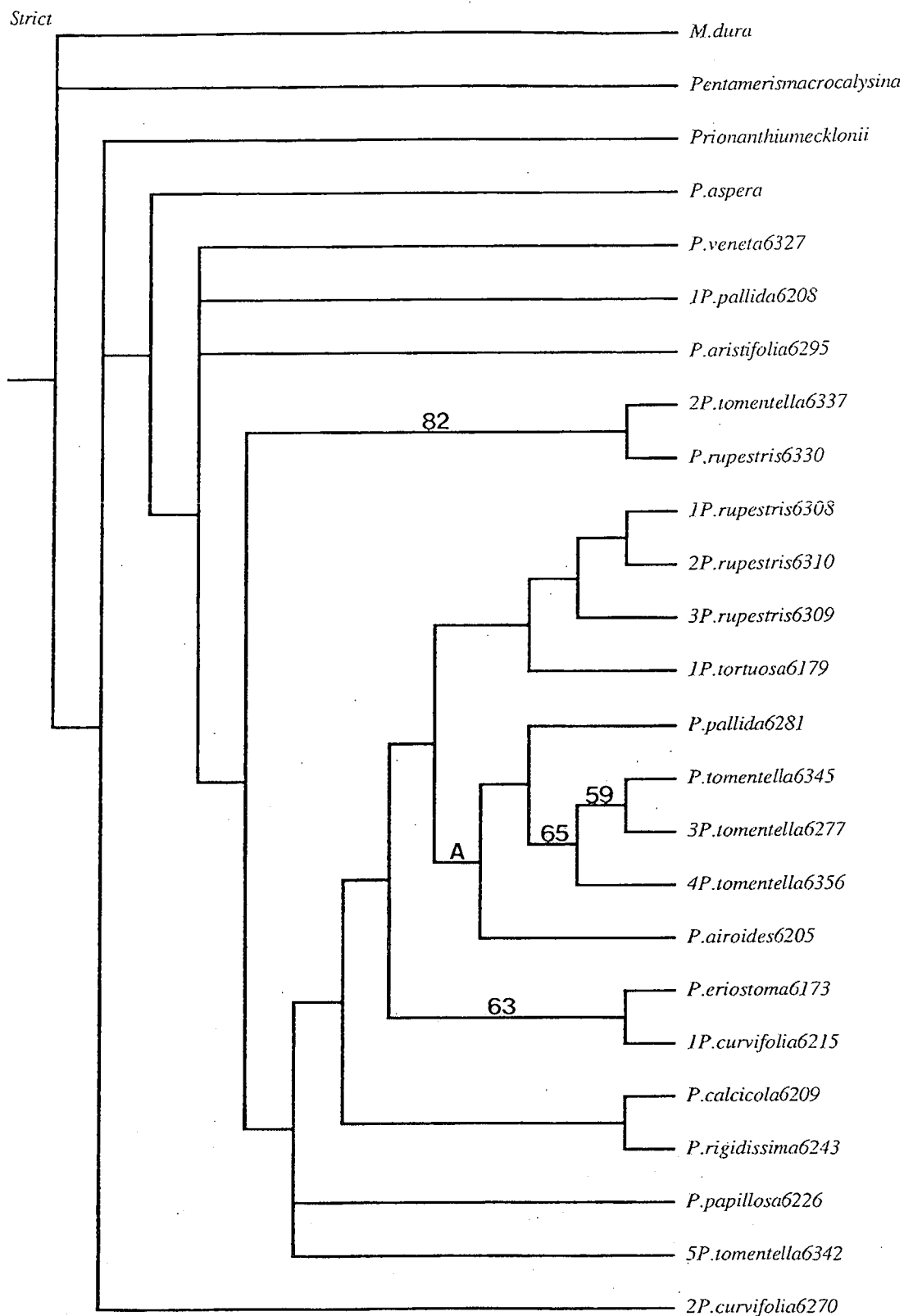
The topology of the cladograms obtained with both cladistic programs from sequences aligned with the Malign program corresponded. However, when Clustal W was used to align the sequences, the two cladistic methods produced cladograms with different topologies. Regardless of the differences in topology, cladograms obtained from PAUP gave fewer steps and consequently more parsimonious cladograms than those obtained after using Hennig86.

**TABLE 6.1** *ITS*<sub>1</sub> length and G + C content of the studied specimens.

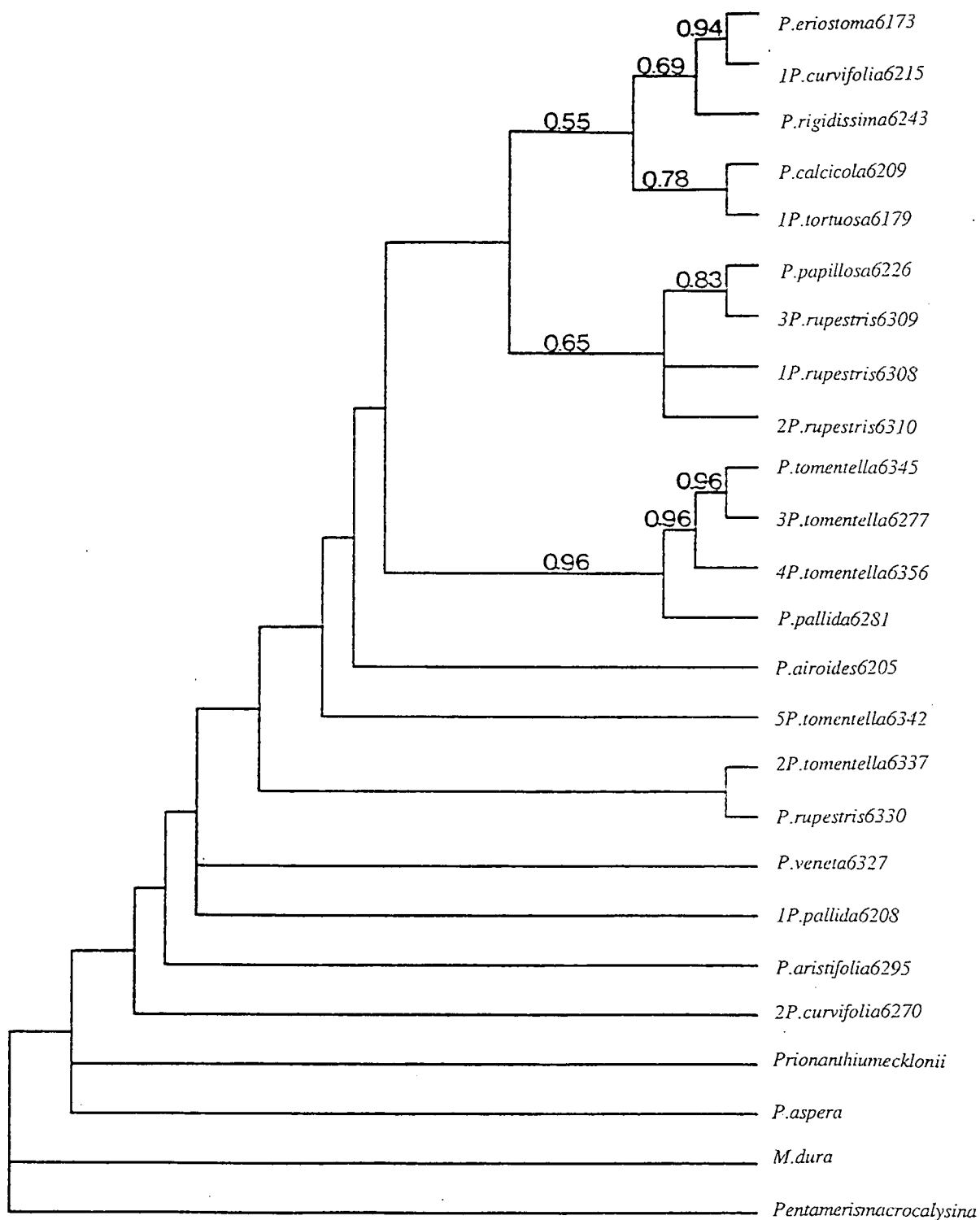
Species	Length	G + C content
<i>Merxmullera dura</i>	224	68 %
<i>Pentameris macrocalycina</i>	223	68 %
<i>Prionanthium ecklonii</i>	213	67 %
<i>Pentaschistis aspera</i>	212	66 %
<i>P. veneta</i> 6327	214	64 %
<i>P. pallida</i> 6208	218	64 %
<i>P. tomentella</i> 6337	218	64 %
<i>P. aristifolia</i> 6295	215	64 %
<i>P. airoides</i> 6205	215	60 %
<i>P. curvifolia</i> 6215	216	60 %
<i>P. curvifolia</i> 6270	214	64 %
<i>P. rupestris</i> 6330	215	65 %
<i>P. rupestris</i> 6308	214	64 %
<i>P. rupestris</i> 6310	217	65 %
<i>P. papillosa</i> 6226	187	61 %
<i>P. pallida</i> 6281	217	66 %
<i>P. rupestris</i> 6309	216	65 %
<i>P. calsicola</i> 6209	214	65 %
<i>P. tortuosa</i> 6179	218	66 %
<i>P. tomentella</i> 6345	215	64 %
<i>P. tomentella</i> 6277	222	61 %
<i>P. tomentella</i> 6356	217	66 %
<i>P. rigidissima</i> 6243	215	63 %
<i>P. eriostoma</i> 6173	213	66 %
<i>P. tomentella</i> 6342	214	66 %

### 6.3 Discussion.

The comparative analysis of DNA sequences provides information on three distinct evolutionary problems:

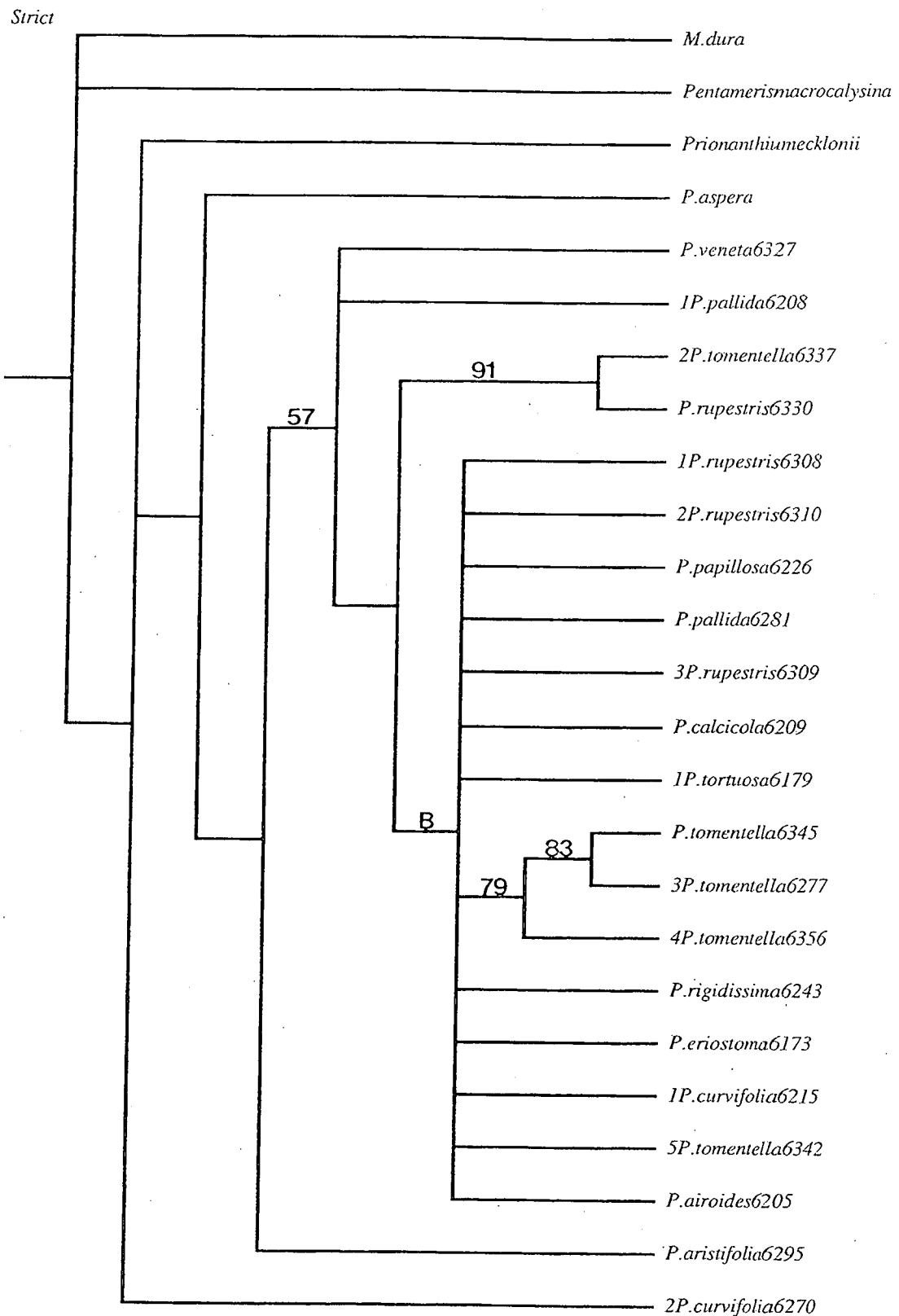


**Figure 6.1.1** Strict consensus of 36 most parsimonious trees (CI = 0.52, RI = 0.58), with length 235, containing 22 *Pentaschistis* species and three outgroups based on the Clustal W data set. Numbers above branches are % bootstrap values of 200 replicates. Nodes without numbers have bootstrap values of <50%.

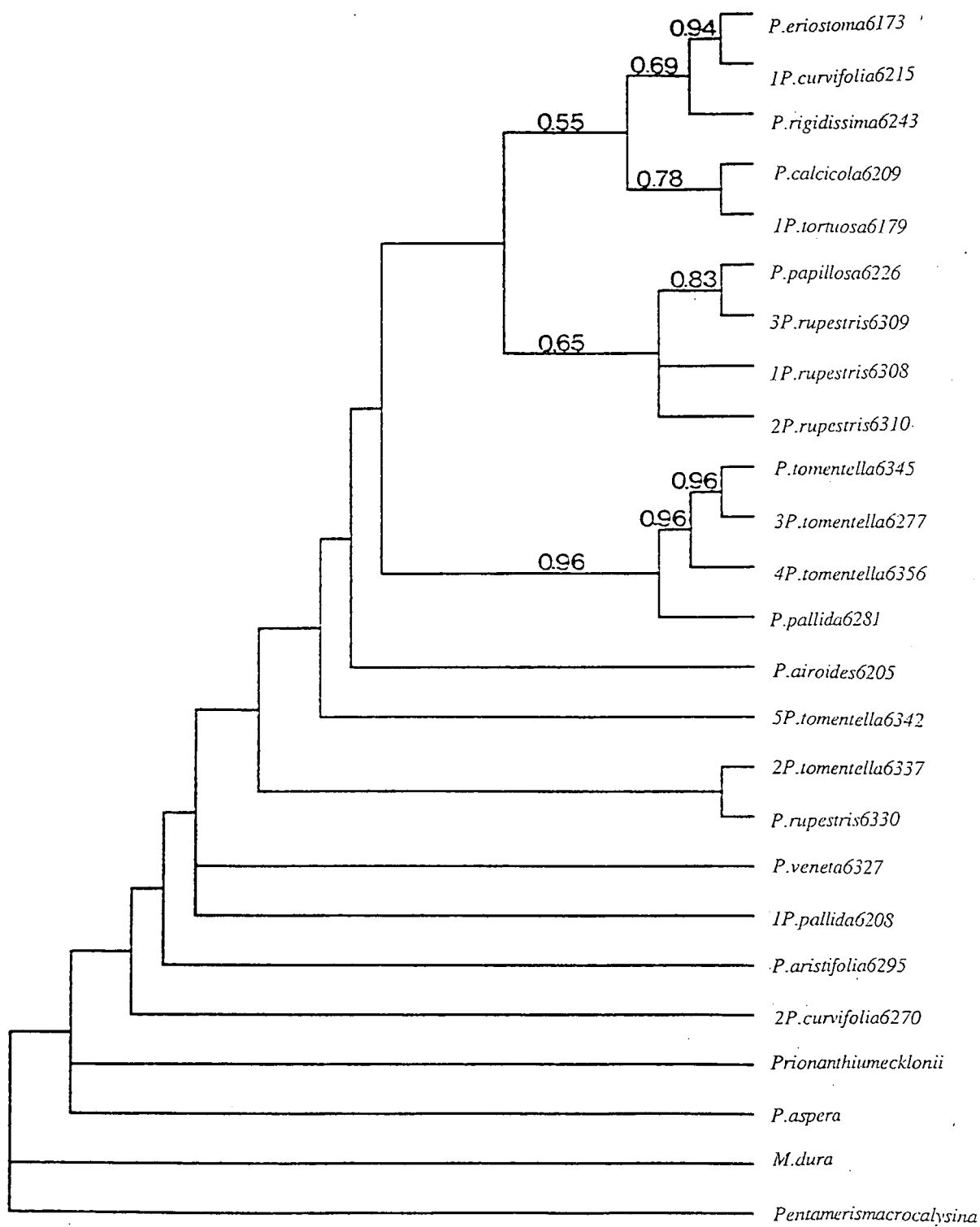


**Figure 6.1.2** Consensus (*nelsen*) of 18 trees (CI = 0.53, RI = 0.58), with length 474, containing 22 *Pentaschistis* specimens and three outgroup specimens, based on the Clustal W data set. Numbers above branches are % Jackknife values.



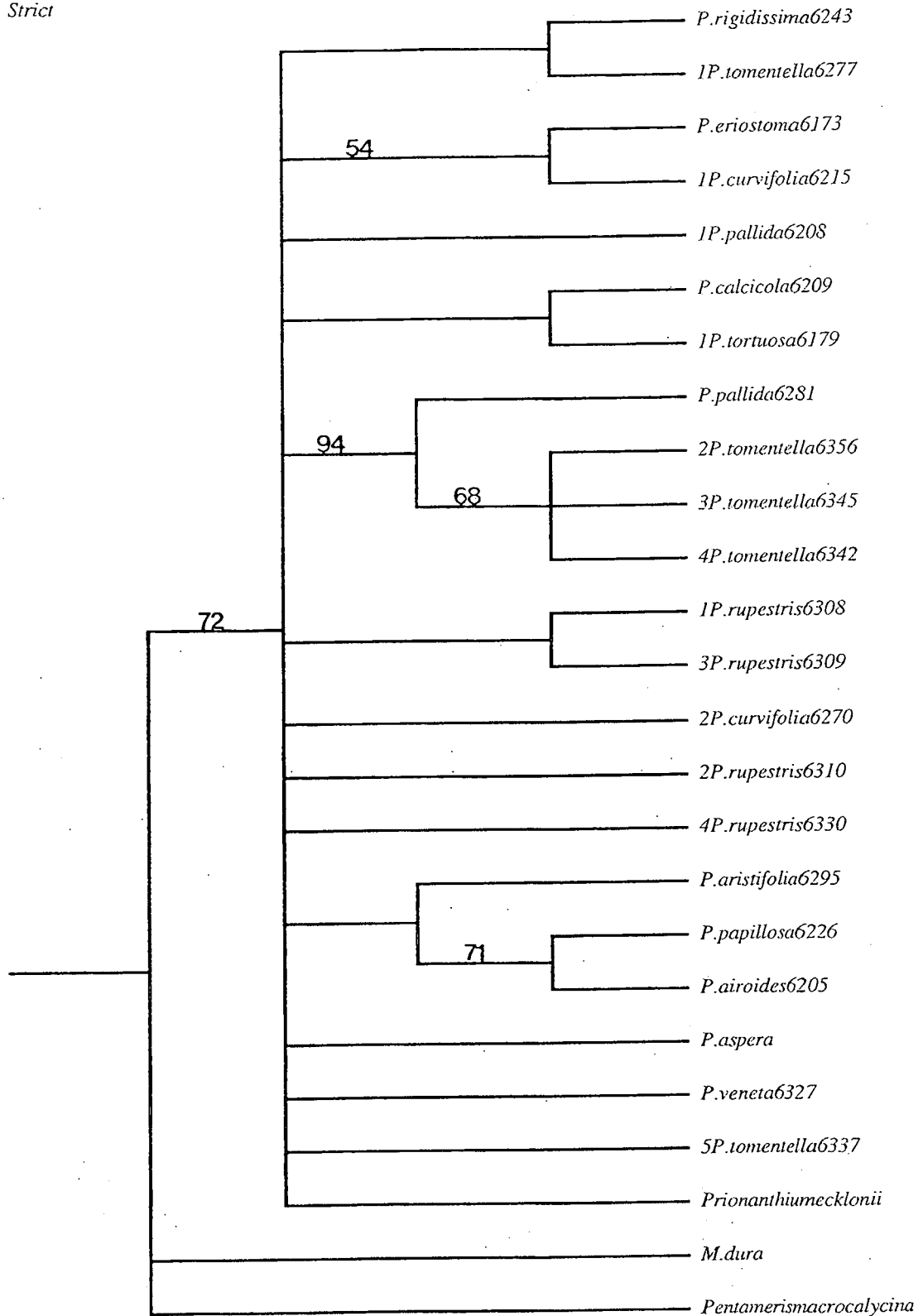


**Figure 6.1.3** Strict consensus of 16 most parsimonious trees (CI = 0.52, RI = 0.71), with length 131, based on the Clustal W data set, with some characters temporarily removed. Numbers above branches are % bootstrap values of 200 replicates. Nodes without numbers have bootstrap values of <50%.

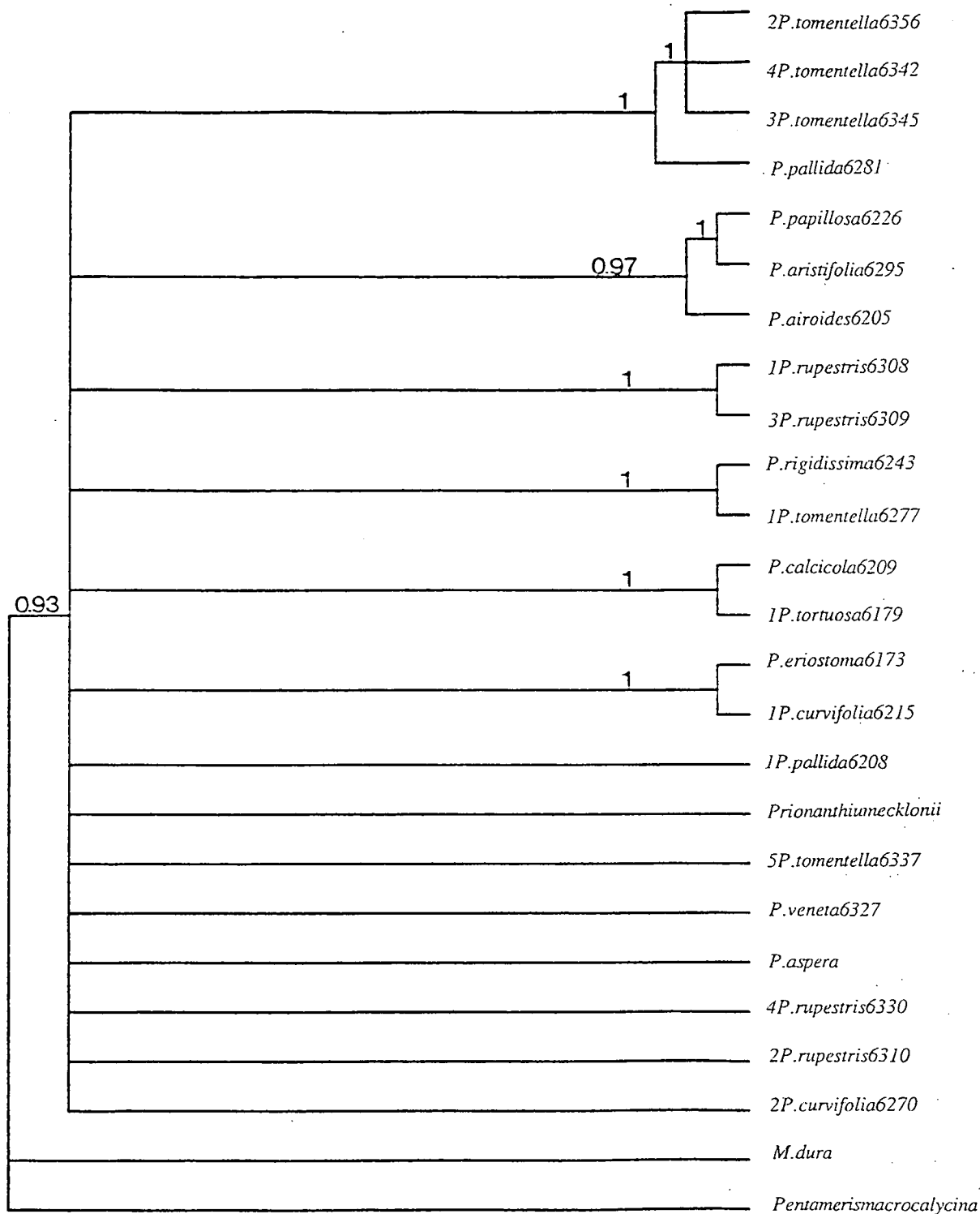


**Figure 6.1.4** Consensus (*nelsen*) of 16 parsimonious trees (CI = 0.57, RI = 0.68), with length 330, based on the Clustal W data set, with some characters temporarily removed. Numbers above branches are Jackknife values.

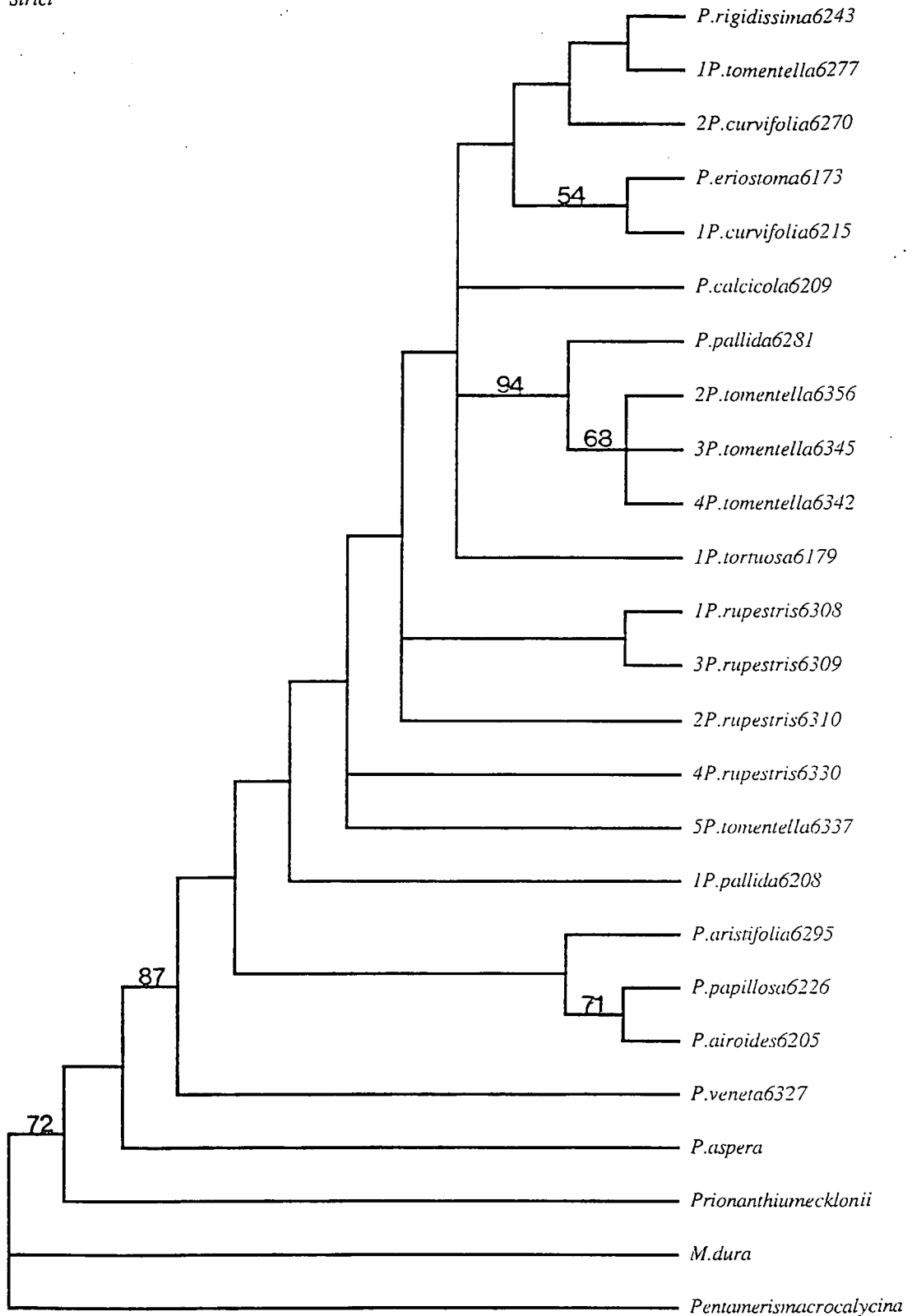
Strict



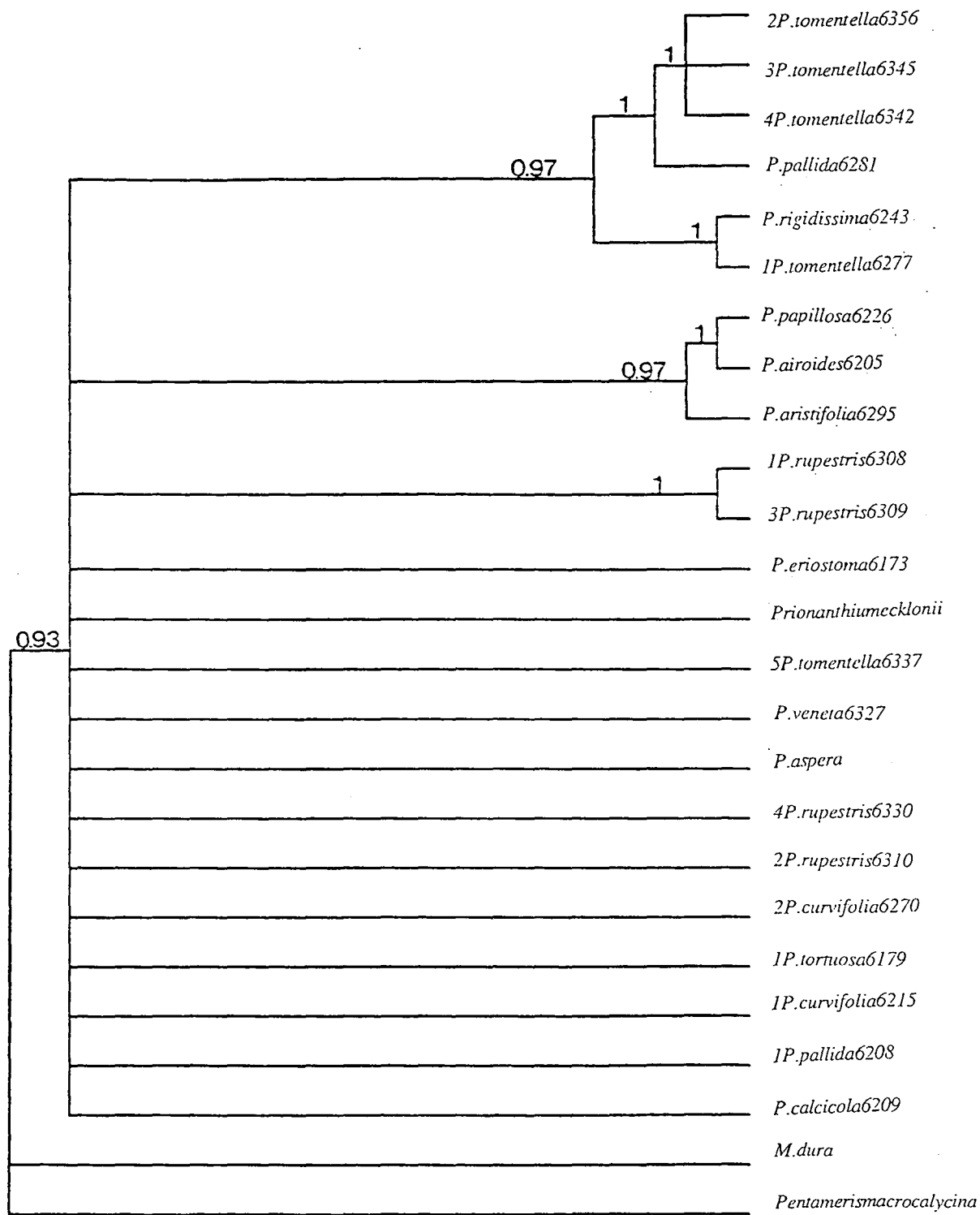
**Figure 6.2.1** Strict consensus of 60 most parsimonious trees (CI = 0.52, RI = 0.58), with length 211, containing 22 *Pentaschistis* species and three outgroups based on the Malign data set. Numbers above branches are % bootstrap values of 200 replicates. Nodes without numbers have bootstrap values of <50%.



**Figure 6.2.2** Consensus (*nelsen*) of 60 trees (CI = 0.62, RI = 0.58), with length 268, containing 22 *Pentaschistis* specimens and three outgroup specimens, based on the Malign data set. Numbers above branches are % Jackknife values.



**Figure 6.2.3** Strict consensus of 20 most parsimonious trees (CI = 0.53, RI = 0.69), with length 132, based on the Malign data set, with some characters temporarily removed. Numbers above branches are % bootstrap values of 200 replicates. Nodes without numbers have bootstrap values of <50%.



**Figure 6.2.4** Consensus (*nelsen*) of 30 parsimonious trees (CI = 0.79, RI = 0.76), with length 121, based on the Malign data set, with some characters temporarily removed. Numbers above branches are Jackknife values.

- The first is to infer the network of genetic relationships among homologous DNA sequences sampled from a set of taxa.
- The second concerns the processes that govern molecular evolutionary changes for the different classes of genes.
- The third, is the detection of differential rates of evolution for various regions within a gene or between separate genes (Ritland & Clegg 1987).

For this study we will concentrate on the first problem.

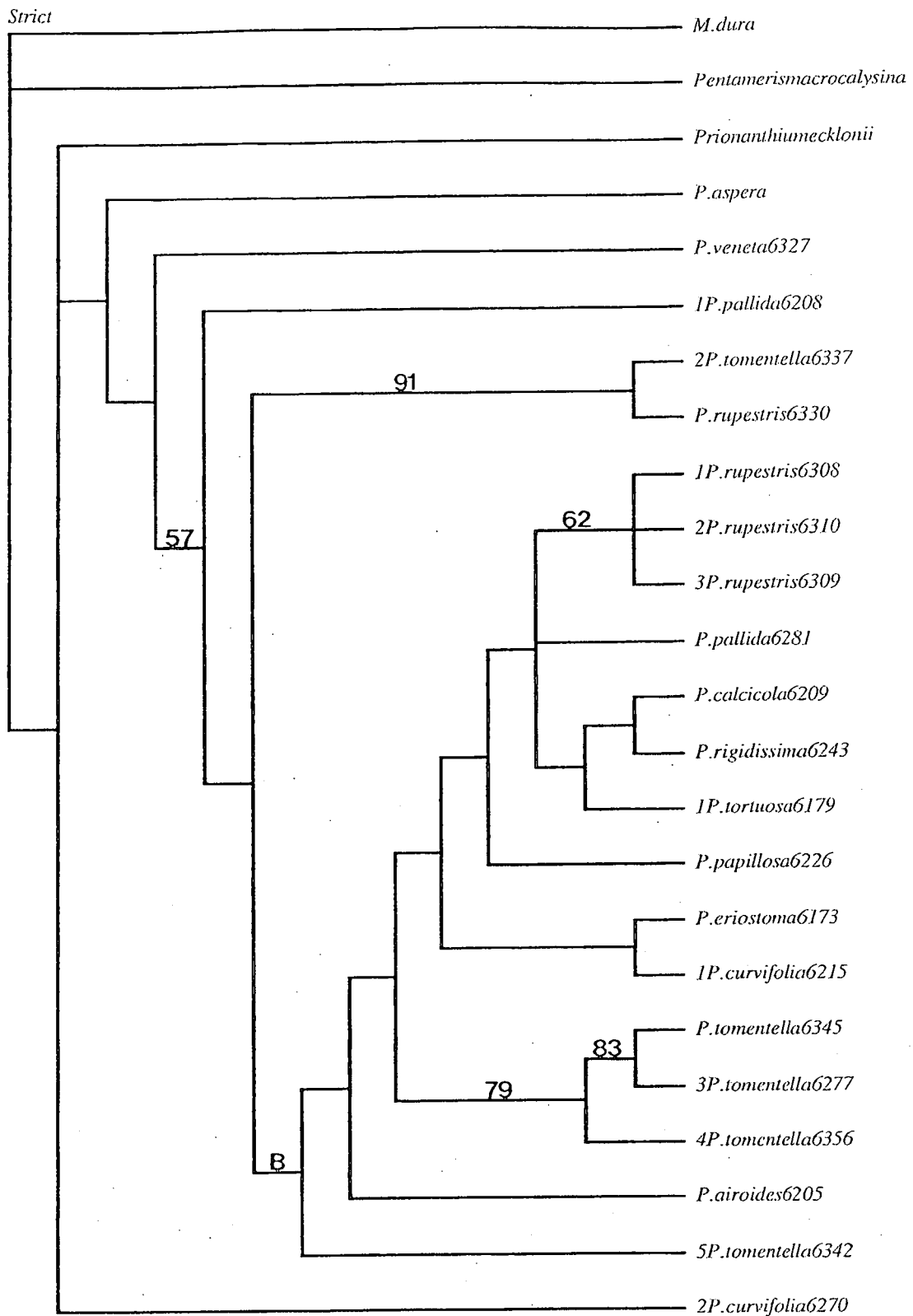
**G + C content and nucleotide site variation** — The high G + C contents observed in the *Pentaschistis* species may account for the difficulty experienced in the sequencing of some *Pentaschistis* specimens, because high G + C levels can cause stronger template secondary structure, which can confound sequencing reactions. Furthermore, grasses growing in arid regions, have on average a higher G + C content than plants from temperate areas (Salinas *et al.* 1988). However, our analysis of the G + C contents of the *ITS*<sub>1</sub> region of plants from temperate areas, indicates a high level of G + C content.

Of the variable sites observed in this study 70 are potentially phylogenetically informative. More transversions than transitions were observed among the nucleotide substitutions of the 25 grass species. The mean transition : transversion ratio across all sequences was 0.7 : 1 for Clustal W and 0.67 : 1 for Malign. Substitution patterns taken across all regions correspond with observations of Morton (1995), namely that positions flanked by A or T are more likely to undergo transversions. Although, upon inspection, this pattern is evident, insufficient data were obtained to test this phenomenon statistically.

**Phylogenetic analysis of the Clustal W data set with PAUP and Hennig86** — Detailed information obtained in species of the genus *Pentaschistis* and its close allies, *Pentameris* and *Prionanthium* are shown in the most parsimonious cladograms, with *Merxmuellera dura* as the outgroup (Figures 6.1.1-5).

A strict consensus cladogram (Figure 6.1.1), revealed a sister alliance between the *P. rupestris* specimen. This alliance was also observed with RAPD analysis.

*Pentaschistis tomentella* is a sister species to *P. rupestris*. A shared habitat and morphological similarities, corroborate this relationship. The genetic distances with the RAPD and DAF analysis (0.68) also indicate this relationship. *Pentaschistis tomentella*, a diploid specimen, may also contain a pivotal genome present in *P. rupestris*, a decaploid,



**Figure 6.1.5** Strict consensus of four most parsimonious trees (CI = 0.54, RI = 0.71), with length 127, based on the Clustal W data set, with possible hybrid *P. aristifolia* (Spies 6295) excluded. Numbers above branches are % bootstrap values of 200 replicates. Nodes without numbers have bootstrap values of <50%.



therefore the close relationship.

*P. tomentella* formed a sister group with *P. pallida*. Both belong to morphological group 1 and are diploid. Thus, this grouping is well supported.

*Pentastichis eriostoma* (Spies 6173) is a sister species to *P. curvifolia* (Spies 6215), and anatomically they are similar (Linder & Ellis).

The presence of basal leaves, contracted inflorescence and acuminate glumes in *P. calsicola* & *P. rigidissima* confirm the sister relationship between these species.

In the Hennig86 cladogram (Figure 6.1.2), the relationship between *P. eriostoma* & *P. curvifolia* is observed again, but in this case it forms a sister group to *P. rigidissima*, probably due to the diploid nature of these species. Jackknife analysis produced 17 cladograms with 22 groups. All groups had a value of between 0.4 and 1, and at least 8 of the groups had a value of 1 or ~1.

The topology of the two cladograms (Figures 6.1.1-2) is in general agreement, but because fewer steps are required for the PAUP cladogram, it is the most parsimonious cladogram and thus the preferred one.

Some characters were temporarily removed. This cladogram (Figure 6.1.3) also indicates that *P. tomentella* is a sister species to *P. rupestris* and the *P. tomentella* specimens again formed a sister alliance.

The Hennig86 cladogram (Figure 6.1.4) has a topological concord with Figure 6.1.2, but with this cladogram a shorter cladogram length was observed. The computer program PAUP again gave fewer steps (131) for the cladogram and is, therefore, the preferred cladogram.

Because of the fact that the genetic distances within the genus are so high and hybridisation is suspected, we excluded some of the taxa, one by one, to determine whether or not the removal of a taxa reduces the number of equally parsimonious cladograms and increases resolution in clade B. By excluding *P. aristifolia* (Spies 6295) only 4 parsimonious cladograms were obtained and clade B in Figure 6.1.5 had a better resolution than in Figure 6.1.3. This impact on cladogram topology may be explained by a hybrid origin of *P. aristifolia* (Spies 6295). Morphologically the *P. aristifolia* specimen deviate from the typical form and it can thus be a hybrid.

Gottlieb (1972) discussed several criteria for testing whether a particular diploid taxon originated through hybridisation. These features include a geographical distribution in the region of parental sympatry, morphological intermediary in several characters,

partial fertility and biochemical additivity. Phylogenetic analysis is not directly relevant to tests of hybrid origin hypothesis, as the placement of hybrids in phylogenetic cladograms does not appear to be predictable (McDade 1990,1992, Rieseberg & Morefield 1994). Although no single criterion can provide a clear means for testing a hybridisation hypothesis, each criterion that can be fulfilled provides a higher level of support for a hybrid origin (Gottlieb 1972). According to McDade (1992), the genes of species of hybrid origin will have their ancestry in one or the other of the lines that hybridised. For this reason there will not be a single phylogeny that explains the bulk of the genes.

All clades that are resolved in the consensus of all the data included (Figure 6.1.1) is preserved in the cladogram excluding *P. aristifolia* (Spies 6295) (Figure 6.1.5).

**Phylogenetic analysis of the Malign data set with PAUP as well as Hennig86** — The cladogram obtained with the Malign data set with *Merxmuellera dura* as the outgroup (Figure 6.2.1), indicated that *Pentastichis rigidissima* is a sister species of *P. tomentella*, which is supported by a morphological concord between these two species as well as the diploid nature of these species.

The sister alliance between *P. eriostoma* & *P. curvifolia* is once again observed, whereas *P. calicicola* emerges in a clade with *P. tortuosa*. This is corroborated by their morphological similarities, with the weak base type being one of them.

The morphological similar species, *P. patula* & *P. tomentella* forms sister species to one another. Both species are also diploid.

The topology between the PAUP cladogram (Figure 6.2.1) and the Hennig86 cladogram (Figure 6.2.2) is in concord, but the PAUP cladogram has fewer steps and is thus preferable. However, as soon as the characters with a low RI value are excluded (Lipscomb 1998), the Hennig86 cladogram is the more favorable because of its cladogram length.

This cladogram (Figure 6.2.4) preserved most of the clades observed in the previous cladograms, except for the sister relationships of *P. calicicola* & *P. tortuosa* and *P. eriostoma* and *P. curvifolia*.

The same test for a possible hybrid was done for the Malign data set, but this time *P. calicicola* was the possible hybrid (Figure 6.2.5). This is, however, not as well supported by impact on cladogram topology, as was the case with *P. aristifolia* (Spies 6295).

*Comparison between the phylogenetic cladograms of the two data sets* — Even though the Clustal W alignment is shorter than that of Malign, which is preferable, the Malign data set gave the cladogram with the fewest steps and is thus the most parsimonious cladogram.

In general, placement of the species within the genus *Pentaschistis* is in agreement between the two alignment methods as well as between the two parsimony methods. In cases of conflict, this is attributed to a lack of resolution rather than to incongruent topologies.

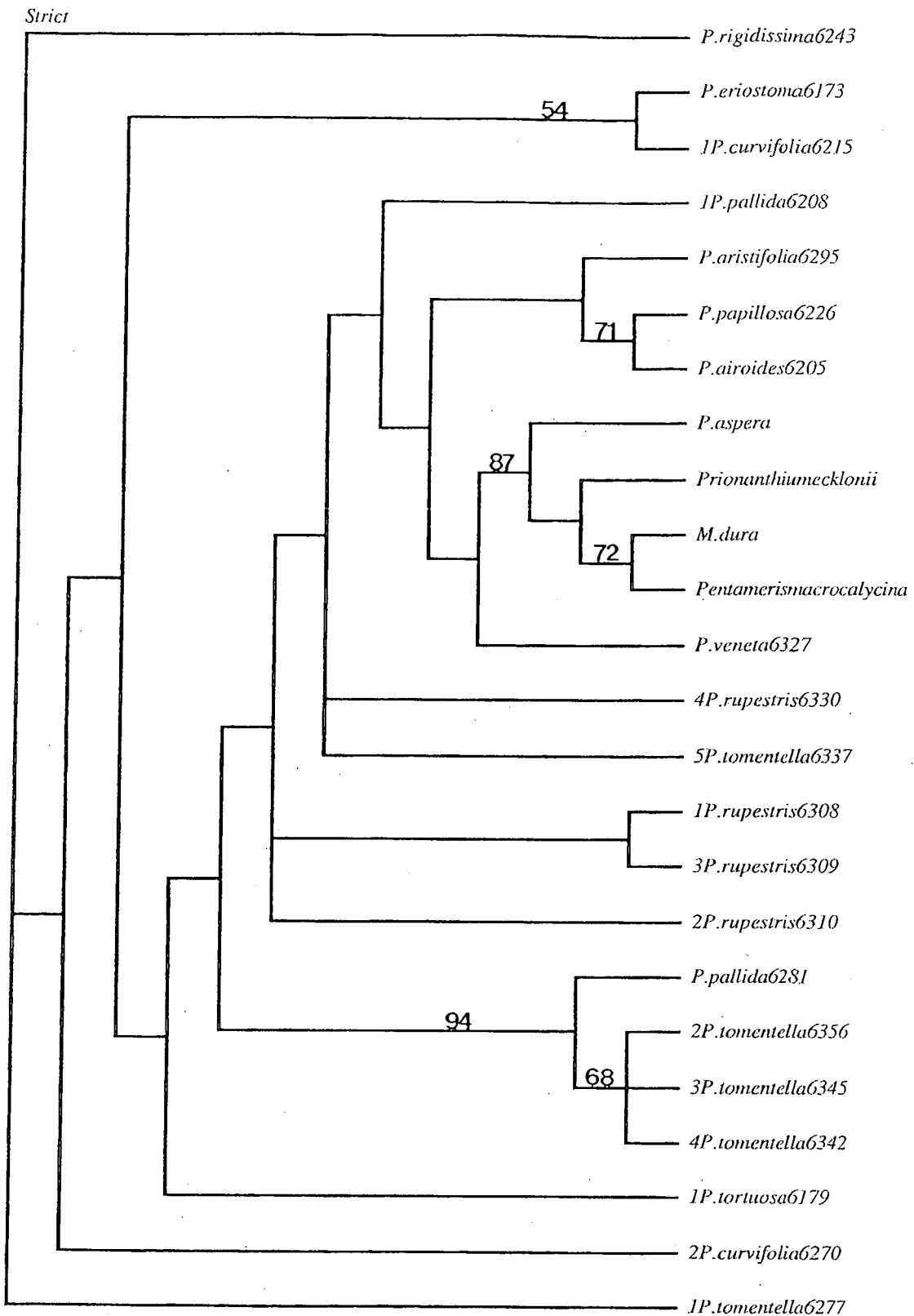
The relationship between the *P. tomentella* specimens is well supported by Jackknife values of 0.96. It is thus safe to come to the conclusion that they are monophyletic.

The *P. eriostoma* & *P. curvifolia* clade is also preserved throughout the different cladograms, but these two species can not be classified together due to the paraphyletic nature of *P. curvifolia*.

On the cytogenetic level, the ITS cladogram was able to distinguish between the different polyploid levels of the species, for example the diploid clade (A) in Figure 6.1.1.

The fact that *Merxmuellera dura*, which was used as the outgroup due to its basic chromosome number of  $x = 6$ , which differs from the rest of the species included ( $x = 7$  &  $x = 13$ ), is closer to *Pentameris* than to the *P. eriostoma* species, indicates that the hypothesis of Du Plessis & Spies (1992), who suggested that the chromosome number of  $x = 13$  could have developed due to hybridisation with some *Merxmuellera* species ( $x = 6 + 7$ ), is not supported.

*Prionanthium* on the other hand does seem to be closely related to *Pentaschistis* as Hsiao *et al.* (1998) also observed with *ITS* data and Barker *et al.* (1995) with *rbcL* sequence data. This grouping is not surprising. These genera all share a basic chromosome number of seven. They are also very similar anatomically. *Prionanthium* and *Pentaschistis* are the only two genera, which possess glands. *Prionanthium* is unique in possessing two gland types. In *Pentaschistis* both these gland types also occur, but not simultaneously on the same plant. On the basis of this, *Prionanthium* appears to be intermediate between the *Pentaschistis* species group (Ellis 1989). Ellis (1985b, c, 1986a, Ellis & Linder 1992) also reported that on the basis of leaf anatomy, *Pentameris* is closely allied to *Pentaschistis*. There are three *Pentaschistis* species (*P. pallescens*, *P. silvatica* and *P. tortuosa*) that bear a very strong resemblance to *Pentameris thuarii* (Ellis 1985b, c).



**Figure 6.2.5** Strict consensus of four most parsimonious trees (CI = 0.54, RI = 0.70), with length 129, based on the Malign data set, with possible hybrid *P. calcicola* (Spies 6209) excluded. Numbers above branches are % bootstrap values of 200 replicates. Nodes without numbers have bootstrap values of <50%.

ITS data also support the postulation of Linder & Ellis (1990a) that the hybridisation of *P. curvifolia* could lead to the existence of *P. eriostoma*. The conclusion of Klopper *et al.* (1998), that *P. eriostoma* is not close to the other *Pentaschistis* species is however, not supported.

In conclusion it can thus be stated that ITS data do provide strong phylogenetic signal for some generic clades in *Pentaschistis*, but only 12 of the 68 species were sampled.

To resolve phylogenetic relationships within *Pentaschistis* additional sampling of species is necessary. Because gene cladograms may not represent species cladograms (Doyle 1992, Kellogg, Appels & Mason-Gamer 1996), more data are critical to enable confident determination of organismal relationships in *Pentaschistis*.

## CHAPTER 7

### PHYLOGENY

#### 7.1. Introduction.

Phylogenetic relationships among populations can be represented with tree-like structures. These are useful from a purely classification point of view, but can also be interpreted phylogenetically if certain assumptions are valid and especially when historical or fossil data are also available (Cavalli-Sforza & Edwards 1967). Renewed interest in phylogenies over the last few decades coincides with a growing sense that it will actually be possible to obtain an accurate picture of evolutionary history. Several methods have been developed to estimate these phylogenetic relationships from data on real populations.

There are two relevant kinds of phylogenetic studies:

- ❖ those that offer "reconstruction" by methodologically wrongly falling back on optimal criteria of, e.g. maximum parsimony, minimum falsifiability or maximum likelihood and maximum posterior probabilities, and
- ❖ those that treat their results not as optimisations but as contributions towards building a modern method for probabilistically estimating phylogenetic relationships for at least some groups and otherwise using explanations simply to guide classification.

Inference of phylogenetic relationships at and below the species level is relatively seldom carried out on plants. Morphological characters usually have a more or less continuous variation at this level, which makes unambiguous discrete character coding difficult (Stevens 1991). The rapidly increasing use of molecular techniques in phylogeny has focused attention on pros and cons of molecular versus morphological evidence.

In recent years there has been some debate on whether to combine the different data sets that are phylogenetically informative on the group of taxa studied, and whether to treat each data set separately (Linder & Crisp 1995). Sytsma *et al.* (1991) has attempted a general survey and comparison of molecular and morphological studies, regarding plant phylogeny in particular.

According to Sytsma *et al.* (1991), morphological evidence should be avoided, because it can often be phylogenetically uninformative or even misleading, because of the

operation of strong selection resulting in homoplasy, difficulty in ordering or even polarizing character states, the high number of autapomorphies, and the lack of well-defined synapomorphies. But, homoplasy does not by itself guarantee an inaccurate cladogram, particularly in large studies, which tend to have high levels of homoplasy by virtue of the number of taxa involved (Sanderson & Donoghue 1989). Because homoplasy is a function of the weight of multiple independent synapomorphies (Hennig 1966, Sanderson 1989), confidence can be low even if homoplasy is low or nonexistent, such as when only one synapomorphy supports each clade.

Molecular characters are however, less homoplastic than morphological characters. The beauty of molecular data is that there is potentially so much of it and increased numbers add evidential weight and statistical power to phylogenetic inferences. In general it appears that the more characters there are per taxon, the higher the level of confidence, at least as measured by the bootstrap (Sanderson 1989). And this relationship is unaffected by the amount of homoplasy present, which tends to vary independently of the number of characters (Sanderson & Donoghue 1989).

Molecular data, when gathered carefully and analyzed in an appropriate manner, are obviously very useful in understanding evolutionary history and the same can be said of morphological data. Both are extremely promising avenues to pursue and neither has come close to achieving its full potential.

To date most of what we know about phylogeny is based on morphology and this has been confirmed time and again by molecular studies (Donoghue & Sanderson 1992). It is thus a mistake to set morphological data aside and base phylogenetic relationships only on molecular evidence. According to Grant (1998) students of systematics and phylogeny need to make use of all available evidence. The prospect of combining molecular and morphological data sets raises a set of difficult issues, especially regarding character weighting and differences in the nature of the sampling of terminal taxa.

Independent data sets can be combined and analysed simultaneously. This 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). Donoghue & Sanderson (1990) also stated that it is better to combine data sets, rather than to use separate results for obtaining phylogenies in most cases.

In the previous chapters the data sets were analysed separately to determine the most parsimonious cladograms, to calculate genetic distances and to determine the phylogeny in each data set (Chapters 4, 5 & 6).

In this chapter the aim is to determine the phylogenetic relationships between the species by means of combining the data sets (RAPD, DAF & sequencing), and to determine whether or not the morphological data influence the phylogeny.

## **7.2 Results.**

Single specimens were used in the determination of the molecular data (RAPD, DAF & sequencing), but morphological data was obtained from previous studies by Ellis & Linder (1990), Linder & Ellis (1990a). To find accurate cladogram topology, the Wagner parsimony method (PAUP) was used. Hennig86 could unfortunately not be used, due to the fact that this program is only able to handle 500 characters and could thus not perform a search with 683 characters. Only taxa with at least two of the three molecular data sets available were included in this part of the study.

Parsimony analysis of the combined data sets yielded four equally parsimonious cladograms of 3249 steps (Figure 7.1). As soon as the outgroup specimen, *Merxmuellera dura* was excluded and some characters were removed, four equally parsimonious cladograms of 1715 steps were obtained (Figure 7.2).

The morphological data was included and a heuristic search with branch swapping was performed. This analysis yielded 49 equally parsimonious cladograms of 3420 steps (Figure 7.3). Again some characters and *M. dura* was temporarily excluded and 21 equally parsimonious cladograms of 2167 steps were obtained (Figure 7.4).

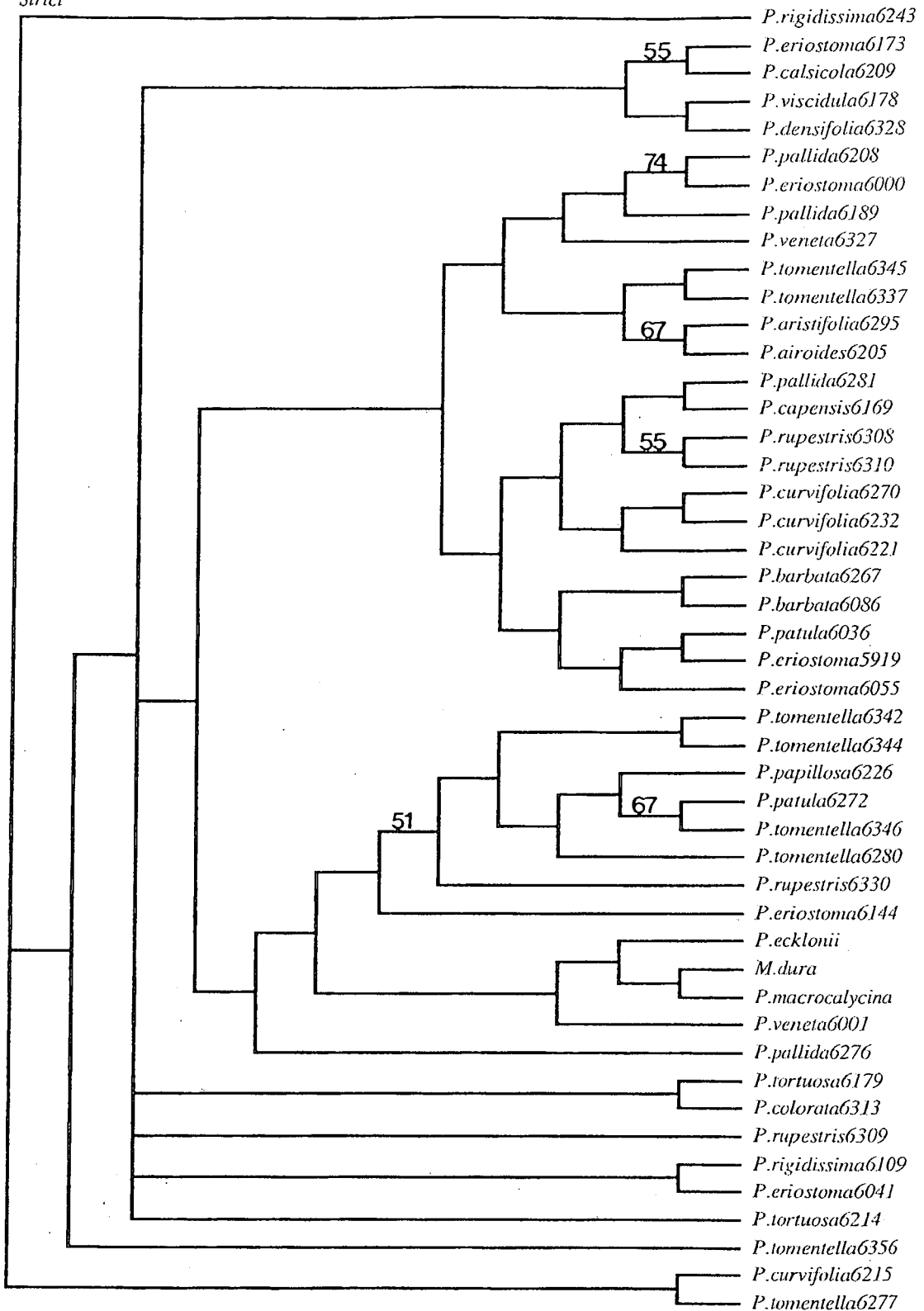
The topologies of the cladograms are in general agreement.

## **7.3 Discussion.**

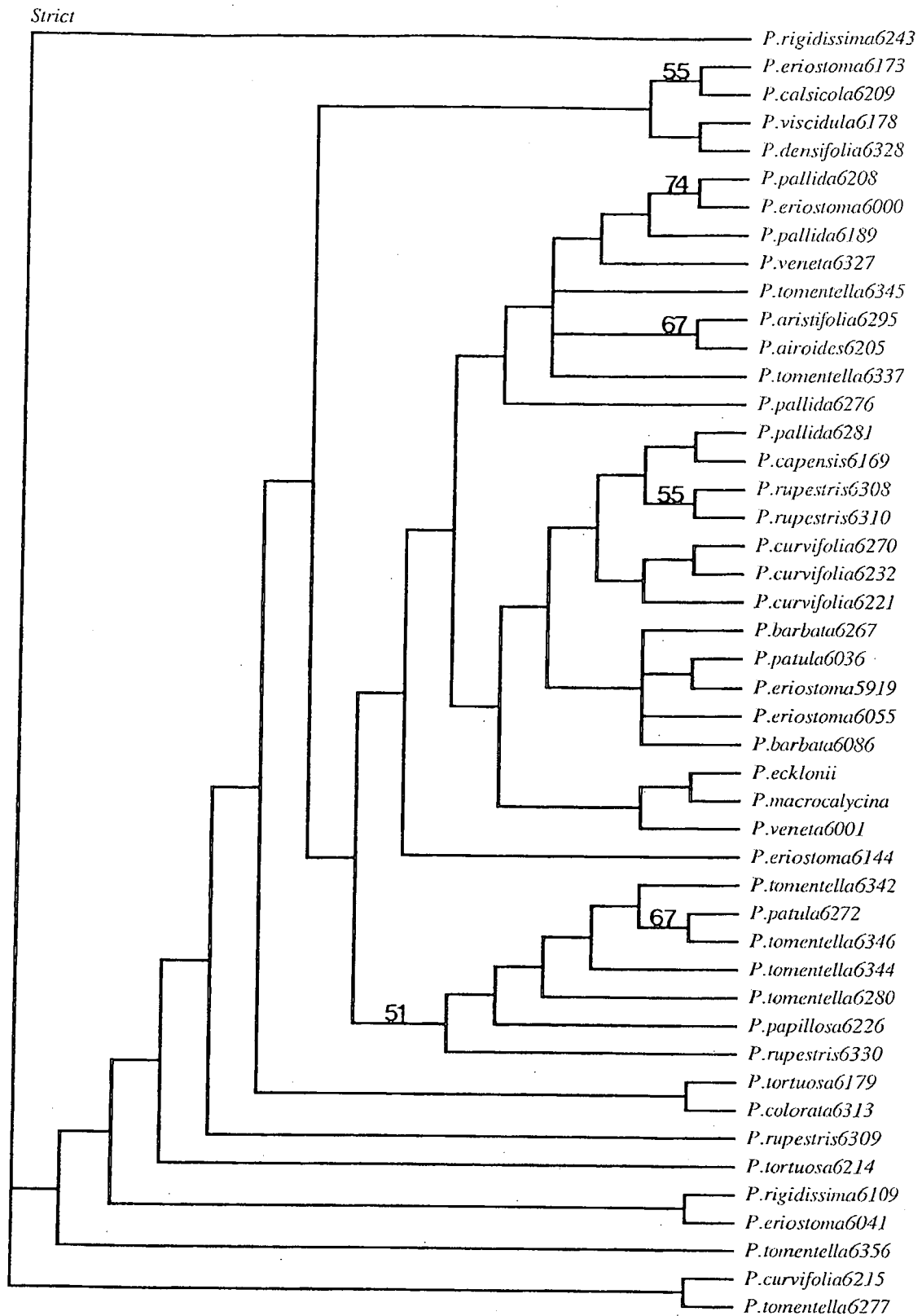
The cladogram obtained from the combined data set of RAPD, DAF & sequencing indicated that *P. curvifolia* (Spies 6215) and *P. tomentella* (Spies 6277) is a sister group to *P. rigidissima* (Spies 6243), whereas *P. rigidissima* (Spies 6109) forms a sister species to *P. eriostoma* (Spies 6041). This relationship is corroborated by absent glands, glabrous leaves with the leaf blades rolled, rigid and straight.



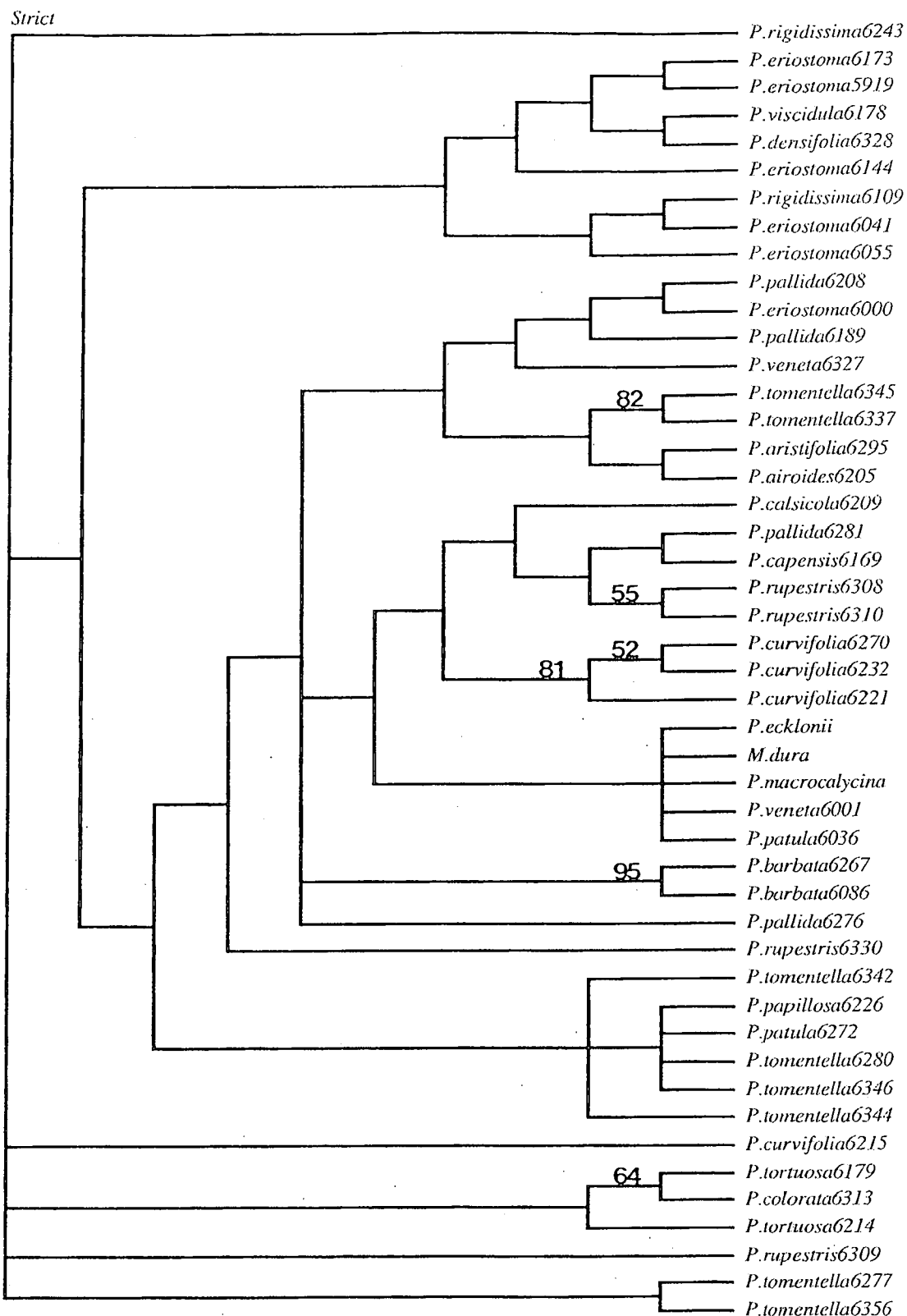
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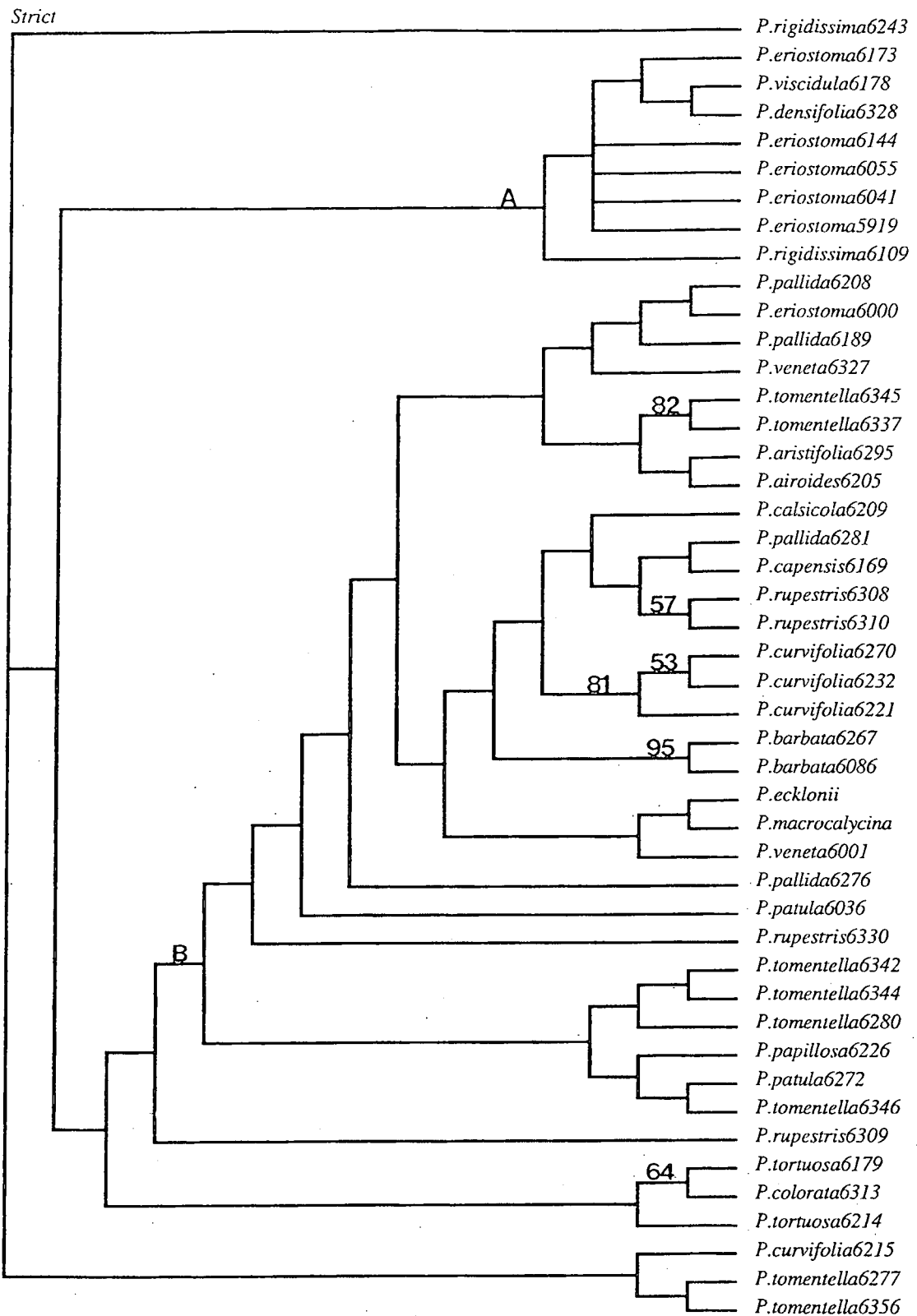
**Figure 7.1** Strict consensus of four most parsimonious trees (CI = 0.16, RI = 0.33), with length 3249, containing 44 *Pentaschistis* specimens and one outgroup, based on a combined data set of RAPD, DAF and ITS sequencing. Numbers above branches are % bootstrap values of 200 replicates. Nodes without numbers have bootstrap values of <50%.



**Figure 7.2** Strict consensus of four most parsimonious trees (CI = 0.17, RI = 0.43), with length 1715, based on a combined data set of RAPD, DAF and ITS sequencing, with some characters temporarily removed. Numbers above branches are % bootstrap values of 200 replicates. Nodes without numbers have bootstrap values of <50%.



**Figure 7.3** Strict consensus of 49 most parsimonious trees (CI = 0.16, RI = 0.34), with length 3420, containing 44 *Pentaschistis* specimens and one outgroup, based on a combined data set of RAPD, DAF and ITS sequencing and morphology. Numbers above branches are % bootstrap values of 200 replicates. Nodes without numbers have bootstrap values of <50%.



**Figure 7.4** Strict consensus of 21 most parsimonious trees (CI = 0.14, RI = 0.41), with length 2167, containing 44 *Pentaschistis* specimens and one outgroup, based on a combined data set of RAPD, DAF and ITS sequencing and morphology. Numbers above branches are % bootstrap values of 200 replicates. Nodes without numbers have bootstrap values of <50%.

*Pentaschistis eriostoma* (Spies 6173) forms a sister species to *P. calcicola* (Spies 6209) and *P. densifolia* to *P. viscidula*, with morphological similarities between these species. The morphological similarities between *P. patula* & *P. tomentella* and *P. papillosa* confirm the sister alliance between these three species. *P. pallida* forms a sister species to *P. eriostoma* & *P. veneta*. This relationship can be attributed to the tetraploid nature of these species. *P. pallida* also formed a sister species to *P. capensis*. In both these species diploidy have been observed, therefore it is safe to come to the conclusion that they share a genome. Another interesting observation is the sister relationship between *P. patula* & *P. eriostoma*, but *P. patula* is well known for its morphological variability.

Some characters with a CI : RI ratio of less than two and *M. dura* were temporarily removed. This cladogram had the same topology as the first cladogram; the only difference is the resolution of *P. eriostoma* (Spies 6055) & *P. barbata* (Spies 6086) and *P. patula* & *P. tomentella* and *P. papillosa*. The cladogram obtained by including morphology gave poor resolution with a high amount of homoplasy. By excluding some characters, better resolution was obtained.

The topology of this cladogram was in general agreement with Figure 7.2. Some differences were, however, observed. For instance, most of the *P. eriostoma* specimens are grouped together, whereas in Figure 7.2 they seemed to be polyphyletic. It is thus clear that the inclusion of morphology does have a minor influence on the cladogram. The cladogram does seem to agree with the convenient morphological grouping of Linder & Ellis (1990), as can be seen from the clades marked A & B, with only a few exceptions. What is interesting is that *P. curvifolia* are grouped with the specimens from group 1 and not with *P. eriostoma* as would be expected from the postulation of Linder & Ellis (1990). *P. tortuosa* which is very difficult to distinguish from *P. colorata* does form a sister species to *P. colorata*. Because Figure 7.2 has fewer steps and is the most parsimonious cladogram, this cladogram is the preferable cladogram.

When Figure 7.2 is compared with Figure 4.6 (RAPD), almost no similarity is observed. The only similarities are the sister alliance between *P. eriostoma* & *P. pallida* and *P. tomentella* (Spies 6277) & *P. curvifolia* (Spies 6215). A comparison between Figure 7.2 and Figure 5.5 indicated a few more similarities. For instance the sister relationship between *P. eriostoma* & *P. pallida* is again observed. The sister group relationships between *P. tomentella* & *P. rigidissima*, *P. viscidula* & *P. densifolia*, *P. tomentella* & *P.*

*papillosa* and *P. colorata* & *P. tortuosa* are observed in both cladograms, whereas a comparison with the *ITS1* cladogram (Figure 6.1.5), revealed almost no similarities.

From this observations it is thus safe to come to the conclusion that the combined analysis give a clearer indication of the phylogenetic relationships. According to this cladogram (Figure 7.2), most of the species are monophyletic, but a few exceptions were observed.

Linder & Ellis (1990) described *P. pallida* as a very variable species, with some specimens being very distinct and others are very similar to one another. It is thus not surprising that this species is paraphyletic. This is also the case with *P. patula* and the fact that this species is morphologically very similar to *P. airoides* and *P. pallida* is supported by their phylogenetic relationships.

Although Linder & Ellis (1990), describe *P. densifolia* to be morphologically difficult to distinguish from *P. pallida*, this species seems to be phylogenetically closer related to *P. viscidula*. This relationship is corroborated by cytogenetic evidence.

*Pentaschistis rupestris*, *P. barbata* and *P. veneta* are morphologically very similar, but they do not form sister species in the cladogram.

It is thus clear that even though the morphological groupings are supported, many morphological similar species do not form sister species to one another. The hypothesis of Linder & Ellis (1990), that there is a relationship between *P. curvifolia* and *P. eriostoma* (Spies 5919 & 6055) is supported by the combined analysis.

The statement that *P. eriostoma* should "most certainly" not be placed in *Pentaschistis* (Klopper 1996), is not supported; neither is the hypothesis that the hybridisation of some *Pentaschistis* species with *Merxmuellera* species (Du Plessis & Spies 1988), lead to *P. eriostoma*.

We can thus come to the conclusion that the morphological groupings of Linder & Ellis (1990) corresponds with the crude basis for the phylogeny of *Pentaschistis* and that the hybridisation of *P. curvifolia* could well have led to *P. eriostoma*. The difficulties in studying morphological and molecular based phylogenies must be a reflection of their complex evolutionary history. The incongruence of the morphological data set could be explained by difficulties in delimiting morphological states and stochastic effects due to the relatively small number of informative characters.

In conclusion, the combination of sequence data from the *ITS1* region, DNA amplification fingerprinting and random amplified polymorphic DNA, have sufficient

variation to resolve intraspecific relationships within *Pentaschistis*. Thus our study provides reason for both encouragement and caution in the continuing quest for additional and more informative tools for phylogenetic analysis in plants.

## CHAPTER 8.

### SUMMARY.

The genus *Pentasthitis* (Nees) Spach consists of 68 species and is endemic to Africa, with 57 species being indigenous to South Africa and 40 species endemic (Gibbs Russell *et al.* 1990).

To date, the chromosome number of 30 species have been reported, as well as the sequences of the *rpoC<sub>2</sub>* gene of two species and the ITS region of one species.

In this study, seventeen specimens were cytogenetically examined. The polyploid levels ranged from diploid ( $n = x = 7$ ) to 14-ploid ( $n = 7x = 49$ ). Two species were examined for the first time, namely: *P. capensis* (diploid) and *P. veneta* (tetraploid). New polyploid levels were also observed for *P. viscidula* (tetraploidy), *P. densifolia* (octaploidy), *P. rupestris* (decaploidy & 14-ploidy) and *P. tortuosa* (octaploidy).

Due to the fact that no, or very few, multivalents were observed, we concluded that the species are allopolyploids or segmental allopolyploids tending towards allopolyploidy. The morphological groupings (Linder & Ellis 1990a) could unfortunately neither be supported nor rejected by cytogenetic evidence alone, therefore cytogenetics was used in conjunction with molecular data to determine the phylogeny.

The fragment patterns obtained from RAPDs were used to calculate the genetic distances. A high degree of variation was observed within and between the morphological groups. Cladograms were obtained with the computer programs PAUP and Hennig86, and PAUP gave the most parsimonious cladogram. The resolutions of these cladograms were, however, not good, therefore DAFs was performed.

Again PAUP and Hennig86 were used and again PAUP proved to give the most parsimonious cladogram. These cladograms gave a clearer indication of the phylogeny of *Pentasthitis*, but the genetic distances within and between the species again proved to be high.

The *ITS<sub>1</sub>* region was sequenced and aligned separately with Clustal W and Malign. These cladograms indicated a close alliance between *P. eriostoma* and *P. curvifolia*.



The three data sets were combined and a cladogram with much better resolution was obtained. The morphological data was included and had a minor influence on the phylogeny. This cladogram also indicated a sister relationship between *P. eriostoma* and *P. curvifolia*.

Current data suggest that *P. eriostoma* could well have developed through the hybridisation of *P. curvifolia* and that both *P. eriostoma* are correctly grouped with *Pentasthitis*. The combined analysis also indicate that the morphological groupings of Linder & Ellis (1990a) is somewhat supported by phylogeny.

**Keywords:** Arundineae, Arundinoideae, cytogenetics, DNA amplification fingerprinting (DAFs), internal transcribed spacers (ITS), *Pentasthitis*, Poaceae, polyploidy, random amplified polymorphic DNA (RAPDs), sequencing.

## CHAPTER 9

### OPSOMMING

Die genus *Pentaschistis* (Nees) Spach bestaan uit 68 spesies, waarvan 57 inheems en 40 endemies tot Suid-Afrika is (Gibbs Russell *et al.* 1990).

Die chromosoomgetalle van 30 spesies is tot dusver gepubliseer, sowel as die nukleotiedvolgordes van die *rpoC<sub>2</sub>* geen van twee spesies en die nukleotiedvolgorde van die ITS gebied van een spesie.

In hierdie studie is 17 eksemplare sitogeneties ondersoek. Die poliploïede vlakke het gevarieer van 'n diploïd ( $n = x = 7$ ) tot 'n 14-ploïed ( $n = 7x = 49$ ). Twee spesies is vir die eerste keer sitogeneties ondersoek, naamlik *P. capensis* (diploïed) en *P. veneta* (tetraploïed). Nuwe poliploïede vlakke is ook waargeneem vir *P. viscidula* (tetraploïed), *P. densifolia* (oktaploïed), *P. rupestris* (dekaploïed & 14-ploïed) en *P. tortuosa* (oktaploïed).

Omdat min of geen multivalente waargeneem is nie, kan aanvaar word dat die spesies allopoloïede of segmentele allopoloïede is, wat neig tot allopoloïede. Die sitogenetiese data alleen kon nie die morfologiese groeperings van Linder & Ellis (1990a) ondersteun nie. Daarom word sitogenetika in samewerking met molekulêre data gebruik om die filogenie te bepaal.

Die fragmentpatrone verkry vanaf RAPD data is gebruik om die genetiese afstande te bereken. 'n Hoë mate van variasie is binne en tussen die morfologiese groepe waargeneem. Beide PAUP and Hennig86 is gebruik om kladogramme te verkry en PAUP het die mees parsinomiese kladogram verskaf. Die resolusie van hierdie bome was egter nie baie goed nie, daarom is daar ook van DAFs gebruik gemaak.

Beide PAUP en Hennig86 is gebruik en weereens het PAUP die mees parsinomiese kladogramme verskaf. Hierdie kladogramme het 'n beter indikasie van die filogenie van *Pentaschistis* gegee, maar die genetiese afstande binne en tussen die spesies was weereens hoog.

Die nukleotiedvolgorde van die *ITS<sub>1</sub>* gebied is bepaal en inlyn gestel met onderskeidelik Clustal W en Malign. Kladogramme verkry vanaf hierdie data het 'n noue verwantskap tussen *P. eriostroma* en *P. curvifolia* aangedui.

Al drie datastelle is gekombineer en 'n kladogram met 'n beter resolusie is verkry. Morfologiese data is ingesluit en het 'n geringe invloed op die filogenie gehad. Hierdie kladogram het aangetoon dat *P. eriostoma* en *P. curvifolia* naverwant is.

Data verkry tydens hierdie studie stel voor dat die verbastering van *P. curvifolia* wel tot die ontwikkeling van *P. eriostoma* kon gelei het, en dat *P. eriostoma* korrek binne *Pentasthitis* gegroepeer is. Volgens die gekombineerde data vorm die morfologiese groeperings van Linder & Ellis (1990a) wel 'n basis vir filogenetiese studies.

**Sleutelwoorde:** Arundineae, Arundinoideae, DNA amplifiseringsvingerafdrukke (DAFs), intern getranskribeerde streke (ITS), lukraak geamplifiseerde polimorfiese DNA (RAPDs), nukleotiedvolgordebepaling, *Pentasthitis*, Poaceae, poliploëdie, sitogenetika.

## CHAPTER 10

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**APPENDIX A:** RAPD data matrix of the *Pentaschistis* species used for the cladistic analysis in PAUP and Hennig86, for all fragments obtained after amplification with primers OPA11, OPA16, OPA7, OPA20, OPC4.

<i>P. aristifolia</i> (Spies 6295) Group 1
000011001011000000 00101111000011000000 0010100010001010100 001110000110100000 ????????????????????
<i>P. airoides</i> (Spies 6205) Group 1
000110001000111000 00010110110000000000 0000000000001110000 000000100100000000 0001100000110001000
<i>P. barbata</i> (Spies 6267) Group 1
000010011001010100 ??????????????????? 0000000000000100100 000000000000010001 ???????????????????
<i>P. veneta</i> (Spies 6327) Group 1
000000001000100000 00111010110000000000 0000001001000100100 000010010100000100 0001100000010101000
<i>P. pallida</i> (Spies 6208) Group 1
000011001001010000 ??????????????????? 0000001000100100100 000010010000000001 0001101000100000000
<i>P. tomentella</i> (Spies 6345) Group 1
000011001010000000 00110101001000000000 0000000000000100100 100010001111101101 0010100010101010000
<i>P. tomentella</i> (Spies 6342) Group 1
000010111010000000 00011111011100000000 0000000000000100100 101000100001000000 1011101100010100000
<i>P. rupestris</i> (Spies 6330) Group 2
000010111000100000 ??????????????????? 0000000000000100100 001000100011010000 0011110010111100000
<i>P. eriostoma</i> (Spies 6173) Group 6
000010111100100000 11011010100100100100 0110001001011100000 001110000101000100 0001000010101100000
<i>P. veneta</i> (Spies 6001) Group 1
110000011010001000 00010001011001010000 0000000010101101010 010010000001100010 0010101010000000000
<i>P. lima</i> (Spies 5961) Group 1
010001100000110000 00011101010011010000 0110011100000110110 000000000100010010 0010011010100000000
<i>P. pallida</i> (Spies 5922) Group 1
110111011010101000 00011001010010011000 0000000000100110100 001000100100111001 0000110010000000000
<i>P. pallida</i> (Spies 5924) Group 1
111010010001000000 ??????????????????? 0000000001000110100 ???????????????????? ???????????????????
<i>P. patula</i> (Spies 6076) Group 1
010010100100100000 00000010100010010111 ??????????????????? ???????????????????? 0010100100000000000

<i>P. tomentella</i> (Spies 5960) Group 1
100010000100000000 00011010110010001000 0000000010000110100 ?????????????????? 0010101110000100000
<i>P. tomentella</i> (Spies 5898) Group 1
110001001011000000 00000101000100000010 ??????????????????? ?????????????????? 0000101100000000000
<i>P. viscidula</i> (Spies 6059) Group 2
010011011000000000 00001001100110010101 0000000001000110100 000001000011000110 0000100110000000000
<i>P. patula</i> (Spies 6036) Group 1
011011001000000000 10011001011110001001 0000000010000100100 ?????????????????? 0000100110000000000
<i>P. elegans</i> (Spies 5927) Group 3
010011001000000000 11100111001001110011 0100001001000100100 000010110011001001 0000010100000110000
<i>P. eriostoma</i> (Spies 6055) Group 3
110110010110000000 00111011000010000000 0000000010000110100 000000100011000101 0010010100000000000
<i>P. rigidissima</i> (Spies 6109) Group 3
111110011000000000 00000101110101010010 0000000000000110100 000010110010101001 0000001010000000000
<i>P. eriostoma</i> (Spies 6041) Group 3
010011010010000000 00111001101101010011 0000000001000110100 ?????????????????? ???????????????????
<i>P. tortuosa</i> (Spies 6102) Group 3
000001101000001000 00100101001000110101 0000000001100110110 000010100101100000 000000111111100000
<i>P. barbata</i> (Spies 6086) Group 1
001000010001011111 10110010110011000000 0000000011100110100 000000000000010001 0000100101000000000
<i>P. eriostoma</i> (Spies 5919) Group 4
010010011000101110 0100001011110110000 0000010000000100111 001100101000000101 1000111110000000000
<i>P. densifolia</i> (Spies 6328) Group 1
000010110111101000 00001011010000000000 ??????????????????? 011001000001010000 0011010100110110001
<i>P. calcicola</i> (Spies 6210) Group 3
000010100001000000 00000000000100100100 ??????????????????? ?????????????????? ???????????????????
<i>P. tortuosa</i> (Spies 6179) Group 3
000010011010101000 ??????????????????? ??????????????????? 101011010100000100 0001100110110100100
<i>P. colorata</i> (Spies 6313) Group 3
001111101111101000 0001101101100010010 0001110011010000000 000000000100001100 1100000011011000000

<i>P. rupestris</i> (Spies 6309) Group 3
000011010010000000 10011101000100010010 0010000000010000000 011000101000100000 0001010010110100000
<i>P. rupestris</i> (Spies 6356) Group 3
000111010000000000 01001010110100000000 0000000000110010100 011111000101100100 1101100001111001000
<i>P. rigidissima</i> (Spies 6243) Group 3
010100000010000000 00111010010000010011 0011000000011000000 000100100010100000 1010100000010100000
<i>P. tomentella</i> (Spies 6277) Group 1
000110000001000000 10110001001000010010 0110110100011011000 110010000100100100 0110000010111110010
<i>P. curvifolia</i> (Spies 6215) Group 4
010110110010000000 01111011011101010000 0000100000011010000 010011000000100000 1010010000101101000
<i>P. pallida</i> (Spies 6281) Group 5
000010010000000000 01001101010000000000 0001000000010101100 100010000000000000 0010100000010000000
<i>P. rupestris</i> (Spies 6308) Group 6
000010010000000000 01000011010110000000 0001000000010100100 100110000111011000 0000101011100000000
<i>P. rupestris</i> (Spies 6310) Group 6
000010010000000000 10100110111010010001 0000000000000100100 000110010101010000 0100101001000000000
<i>P. curvifolia</i> (Spies 6026) Group 4
000110111000000000 00010011111010000000 0000110100000100101 101101100011101000 0000000110000000000
<i>P. curvifolia</i> (Spies 6073) Group 4
000011111000100000 00000111011010010110 0000000100011100101 000000100001100011 0000000010100001000













Average of all the primers.

1 - P. aristifolia (Spies 6295), 2 - P. airoides (Spies 6205), 3 - P. barbata (Spies 6267), 4 - P. veneta (Spies 6327), 5 - P. pallida (Spies 6208), 6 - P. tomentella (Spies 6345), 7 - P. tomentella (Spies 6342), 8 - P. rupestris (Spies 6330), 9 - P. eriostoma (Spies 6173), 10 - P. veneta (Spies 6001), 11 - P. lima (Spies 5961), 12 - P. pallida (Spies 5922), 13 - P. pallida (Spies 5924), 14 - P. patula (Spies 5924), 15 - P. tomentella (Spies 5960), 16 - P. tomentella (Spies 5898), 17 - P. viscidula (Spies 6059), 18 - P. patula (Spies 6036), 19 - P. elegans (Spies 5927), 20 - P. eriostoma (Spies 6055), 21 - P. rigidissima (Spies 6109), 22 - P. eriostoma (Spies 6041), 23 - P. tortuosa (Spies 6102), 24 - P. barbata (Spies 6086), 25 - P. eriostoma (Spies 5919), 256- P. densifolia (Spies 6329), 27 - P. calcicola (Spies 6210), 28 - P. tortuosa (Spies 6179), 29 - P. colorata (Spies 6313), 30 - P. rupestris (Spies 6309), 31 - P. tomentella (Spies 6356), 32 - P. rigidissima (Spies 6243), 33 - P. tomentella (Spies 6277), 34 - P. curvifolia (Spies 6215), 35 - P. pallida (Spies 6281), 36 - P. rupestris (Spies 6308), 37 - P. rupestris (Spies 6310), 38 - P. curvifolia (Spies 6026), 39 - P. curvifolia (Spies 6073).

Below diagonal: genetic distances.

Above diagonal: coefficients of similarities

Large triangular matrix table containing genetic distances (below diagonal) and coefficients of similarities (above diagonal) for 39 species of Psidium. The table is organized with species numbers 1 through 39 along both the top and left sides of the matrix.

**APPENDIX C:** DAF data matrix of the *Pentasthitis* species used for the cladistic analysis in PAUP and Hennig86, for all fragments of primers DAF<sub>1</sub>, DAF<sub>2</sub>, DAF<sub>3</sub>, DAF<sub>5</sub>, DAF<sub>6</sub>, DAF<sub>7</sub>, DAF<sub>8</sub>, DAF<sub>9</sub>, DAF<sub>10</sub>, DAF<sub>11</sub>, DAF<sub>12</sub>.

<i>P. airoides</i> (Spies 6205) Group1
001011011110111011111100000000 1000010111111110001101000000 11111111111011011111101100 1110011011111111000000 111111111000101000111001100 101110100011000000 101000011110111011000000000 000111101101110110011100100 0000101110101010110111110000000 10111011111101110011101000000000 000011010111111110110010100000000
<i>P. aristifolia</i> (Spies 6295) Group1
01111011011010010010111100100100 110100001011010010001000000000 1111111110100110111010101000 100111111010111110101000 ???????????????????????????????? 000101100110000000 01111001111001111011100100000 00011110101111110000010100 00100001110010100101101000000000 101010011011011100010101000000000 1001011001101110010010111110100000
<i>P. airoides</i> (Spies 6311) Group1
???????????????????????????????? 001000001000000000000000000000 11110111110110010101101110000 ????????????????????????????? ???????????????????????????????? 010000101101000000 01011100100000100011000000000 000111001000000000000000000000 01101001000000000100100000000000 ????????????????????????????? 0001011001101011010101100110110100
<i>P. veneta</i> (Spies 6001) Group1
00010000010100010010000000010000 10000000100100011000100001000 00010011010100010000100010000 00010101001100011100000000 ???????????????????????????????? 000011100010000000 01100010010000111000010000000 001111001001100000000111000 00110100010000101000100101000101 ????????????????????????????? 0010101001011011000100010000000000
<i>P. barbata</i> (Spies 6267) Group1
00011100001101111110110101000000 01000000000001010010001110000 11010111010111001000100111000 10111010111101011000000000 1111100010001000111101111100 0100001001001000000 11101010100000111111100000000 0011110001001110100100000000 0111100110011010101000000000000 101111010010011100000110000000000 100110110111011110010000000000000
<i>P. barbata</i> (Spies 6321) Group1
???????????????????????????????? 01000100010101000000000001000 00011011000000000001100010000 1100000000000000000001000 1111111000000001000100000110 100000000101001000 01100011110100111011110000000 ????????????????????????????? 11111001001000101000000010000100 01101101001010001000000000000000 000010000100100001000000000000000

<i>P. veneta</i> (Spies 6327) Group1
01101000000100000100111010000011 1101001100010011111110000000 01011011000010110101001011000 001010111111011110000000 100101111100010101010110110 110100111010110010 00001111010001011011110000000 01111000011111100011000110 01111001010110011010011101010010 011110110110111110011000000110100 0000101101001001100111011011111000
<i>P. eriostoma</i> (Spies 6000) Group1
01001110000010011000110000001001 01001111101101111110000000000 ???????????????????????????????? 0000001100111101110101110 0101101101010111110100110100 110101100110111000 1000111011111111001110011100 100100101111010110100000100 11111001011010011010100101100100 001010110000000001010100100101000 ????????????????????????????????
<i>P. pallida</i> (Spies 6189) Group1
01111010101110001001111110000101 10001111011111011011000100100 00000001000001100000101100000 1000010101010110010100000 ???????????????????????????????? ???? ??????????1????? 01010000000001001000000000000 101111100111110100000001100 11011000111010010101011001001000 110110010010010101000010100011100 ????????????????????????????????
<i>P. pallida</i> (Spies 6208) Group1
01000110000000000000000000000111 01011111101101001010000000000 0110100000000000011100110100 1100001000001000000000010 ???????????????????????????????? ???? ?????????????????? 11101111111000111000000000000 100011100101010000000001110 01011001110010011010010101000100 101011111101000110010000001000101 ????????????????????????????????
<i>P. pallida</i> (Spies 6276) Group1
01001110010001001010011111010011 11001001101100001111010001001 00011111111101111101001111000 01010011111111111111010100 1110110000111011010110010010 ???? ?????????????????? 00100110000000011000000000000 001100100110001000010010000 10101010110110010000100101101100 101010110010011100001000000110000 0010001001111010101110001001000000
<i>P. tomentella</i> (Spies 6337) Group1
01001100001011001010111101100101 11000110111011010001110001010 0000000111111011111100110000 1111110111111111111110111 110011000001111111111011110 111111010111000010 10111111001111111111011000000 001111011111100100000000000 11010110111010011010011001000000 000110000111001110110000001000010 0000101001111011110111011111100000



<i>P. tomentella</i> (Spies 6345) Group1
01001100000101001010110100100011 100000000111111110101010010 0111101101001111110101010000 101111111111001110001111 101101111111111110101111010 110100101011000100 11011000101101010101001010000 0111111111111110100000000 11110001011100101100010010110010 001110011001001111110100000010110 0000011001111010110100011101100100
<i>P. patula</i> (Spies 6272) Group1
000100011110111110111110111101 11111010000100010011111000000 0100100000010101110000000011 1111001011110100110000000 0001100000010111011110101100 ?????????????????? 11101011000000010111011010000 010010110011110010001111110 001000010000101011111111111100 01001011111111110111101011001000 0011110001111010101000000010000000
<i>P. tomentella</i> (Spies 6280) Group1
00101000110110011111110100011101 10111110101100111011000000000 01010001110111110111000000010 1100001011011100000000000 0000100001101111100110010000 ?????????????????? 11111110011100111000000010000 000001011100110110111111110 00100111110010111001001101100100 000101011011011110110001011010000 0000100000001000000110000000000000
<i>P. tomentella</i> (Spies 6342) Group1
00111111110111011111001100011010 11001010110101010010100001110 10111001110110100100000000000 1111101011110000000000000 0010011110010111000110000000 100100110011001111 01100110000111111010110010000 000111111101111010100010000 01101000010011000100100101010000 00011010111101111111101111110000 0010010000110010010101001011011010
<i>P. tomentella</i> (Spies 6346) Group1
00000111111011001110111100001001 11011111011101100110110000000 00000000010000000000000000010 111111110110000001100000 0001111101010111010111100000 0000000000000001110 00101010110100111111010010000 001100011100100100000111000 000010011001110010111111110000 00011011101110111111111110000000 0011010000010000000110000010101101
<i>P. tomentella</i> (Spies 6344) Group1
00001111101110011111110101111010 11001010101111010010101001000 01001001011011000101000000011 1011010111110101011010010 0011010111111101000110000000 000000100000000101 01010101000100110011000010000 000101110111110011100110100 00000000110010010001101111110000 0001101001101011101111110110000000 1110000000010010001100000010110101

<i>P. papillosa</i> (Spies 6226) Group1
00000001111011101010011100111010 11001110111100111111111010000 01010110011011110101110000000 0100011110111111111111000 100100111110111110110110000 ?????????????????? 11101010000100010011000000000 000001101001011010001011110 00000001100001001011111100001000 000100101001011110111111101000000 0010100000101001010110110010010000
<i>P. rupestris</i> (Spies 6330) Group2
00000100000010010010001000000110 11001110111011111011110011000 01001001010101111111101111010 1110110010100001000010000 0100011100110111000010000000 001000010010001101 01110000010100011111000010000 001001111111111111000010100 00001001010000010001101100100000 00100101010100111111100000000000 0010100001011101110100000001010000
<i>P. eriostoma</i> (Spies 6144) Group6
00111001010010011001110111100001 01010010111101011011110000010 00000100000100110100100011010 01001011111010001000000000 1000000000010011011100000000 ?????????????????? 00000010000000010001010000000 010000101100010010010100000 01011001100000001000101111010100 0111111011011111011110000000000 0001100101111000110000010000000000
<i>P. eriostoma</i> (Spies 6173) Group6
01111100010100001001110101110001 10011110111101110111100000000 01000001010100011100000000110 1000101111110000010100000 0100100010001001011000110001 ?????????????????? 00100001100000111001100000000 000101000111000100000110000 01011001101000001101000101011000 000010111011010110110010111000100 0101110001011001100111111111100000
<i>P. eriostoma</i> (Spies 6269) Group6
01111000000101001101111001011101 11001001111101100100101011100 00000000000000110100011101000 1101110101100000000000000 1011001111110111000011010011 ?????????????????? 10110010010010110001010000000 001011001011111001001110000 01011100010011001101101101111000 00101101001110111111100110010000 0010110101101000100100000000000000
<i>P. viscidula</i> (Spies 6178) Group2
11100000000100001001000000001001 1111110111110110111111100000 11110111100110001110000110100 1????????????????????????? 0111000000110100111101111101 ?????????????????? ???????????????????????????????? 11010101111101110000000000 ???????????????????????????????? ????????????????????????????? ???????????????????????????????? ?????????????????????????????

<i>P. densifolia</i> (Spies 6328) Group 1
11111000011100101001110101100010 11001001101100000101110001001 11110101100110001101110110100 0101001101110000010000000 1011101000000001110101101101 ??????????????????? 00100001000000011001100000000 001111010111110010000110000 0111100011001101110110111100000 1110101111101111011110110000000 001011100001011110111101111111100
<i>P. tortuosa</i> (Spies 6179) Group3
01101010000000101001101011001001 11100111011101111101111001011 0101001111011111111011111010 ?????????????????????? 1111100000000000000000011011 ??????????????????? 1011001010001111111000000000 001111010111100001100110100 0111101010111010111010111101110 010100010010101111011110111110000 101000000111100101110101011110000
<i>P. colorata</i> (Spies 6313) Group3
00111010000010010100101100001001 11001010111111111011111001011 10110011100100111101001011111 1101111100100100000000000 1111010111101111010111111111 ??????????????????? 01100001100110110011010000000 111111011110001111010010000 0101010101011001101110110111110 011010100000101111011111011000000 001110000011011101011100100010000
<i>P. rupestris</i> (Spies 6309) Group3
01110010000100101100010100000100 010111111111111101111100001000 00000000101101011111011111010 1011111101110000100000000 0100010111111111100100001000 ??????????????????? 01100111111011110111010010010 001100111000001010001011011 001011111111011111010000000000 111110111101011110111100011100000 0010010101111011000111001000101100
<i>P. tomentella</i> (Spies 6356) Group3
1110000111111101010010110010000 01001110101110111111111001110 01011100100101011101101100010 0101010100000000000000000 011010111011111101111111000 ??????????????????? 10110110011011010010000000010 001010101100100110100001100 001011111111010111101111100000 101111011110111110100000100000000 1011010011111111110100010010001000
<i>P. rigidissima</i> (Spies 6243) Group3
01101000110011110110001100111000 01000100111101110011000111110 10010001000000011101100110010 0101001101110000010001000 0011101000101111001111101000 ??????????????????? 11111111110100110011100000010 001001001000100100000010111 001110111111111111111111000000 100111011011011111100100100000000 11111111111111111101110100100010000

<i>P. rigidissima</i> (Spies 6109) Group3
01110000010000101010001001111100 00010100010000010010100111100 01000101110101011001011010010 1????????????????????????? 0011010110100101001100010000 ?????????????????? 01010001001000111010000000010 100100100111111010001000000 01111101100110101010110101100000 100110110101101110110010101000000 000011000001001110011101111011000
<i>P. tortuosa</i> (Spies 6214) Group3
01011000000100101111101100001100 1000111010111111100000001001 01010111000010111101000010010 ???????1????????????????? 0010010101100011101110101100 ???????????????1???? 01110101001100010000000000010 01100011111010000000000000 0011101111001010111110111010100 11111111110111010010111000010000 110101111111011000101101010101111
<i>P. tomentella</i> (Spies 6277) Group1
11111010000000011100011000100000 00000110111111111000001000001 10000101001111011100000010010 11?????????????1????????? 111000000110111101111101000 ?????????????????? 10101111010111011000010110010 100110101101011010110100000 00111110101011101001101010000000 00111111111111110111101101000010 0011111101111111110110011110100010
<i>P. curvifolia</i> (Spies 6215) Group4
01000000010000000100011110010010 01111111110101111100000001000 00001000101101011111101011010 010010000011010100000000 1001100111010111001111011000 ?????????????????? 10111111110100111010000000100 011100110111111010001111100 00011110110111001111011010000000 00111111011101111101110010000000 100111111111111111111111100000110
<i>P. curvifolia</i> (Spies 6221) Group4
00000101110010110010000100010100 10000110000011010100100000000 00011011000010101011001011111 1001001000000001110000010 1100100110100111010110010000 ?????????????????? 0000101100100111101111110010 100101110101111111010001000 ????????????????????????????????? ?????????????????????????????? 1010101011011111100101111011001000
<i>P. curvifolia</i> (Spies 6232) Group4
01011011001111111011100100111001 00001110001010010010000110100 00001101001110111100001011111 0100101010010001100000010 1000000101001111001000100000 ?????????????????? 11000000001100101011110100011 001011011110101110111000100 ????????????????????????????????? 00100001000000001000000000000000 111111110111101111011111111111110

<i>P. curvifolia</i> (Spies 6270) Group4
00000001000010000000000000010000 1000000000100000000000000000001000 11101011101010001100001111101 1001000100010111110011010 0101110011110111001000000000 ???? ?????????????? 11000011011101101011110100010 01101101111111111111110110 ???????????????????????????????????? 1100001001000100000000000000000000 ????????????????????????????????????
<i>P. capensis</i> (Spies 6169) Group5
00000101100101111000110100100000 100011101111111100000000000000 11101011000000001000000000010 ???????????????????????11?? 1011011101000111000011000000 ?????????????????????? 11111000101001010100110010010 000110010011111010011010100 00101010101010001000000000000000 ????????????????????????????????? ????????????????????????????????????
<i>P. pallida</i> (Spies 6281) Group5
00000011011100011001000101001000 110111010001011100000000000000 10101010110010001001000110010 ???????????????????????1111? 0001111101100011011110100000 ?????????????????????? 11111011110101111110110011010 000110101101010010010011000 00011011001010010000000000000000 1001001001000100000000000000000000 ????????????????????????????????????
<i>P. rupestris</i> (Spies 6308) Group6
01001100110011011111111111011000 000000000100111101110000000000 10010101000100011010000011010 1111101100010011100011110 1100111010000000100000000000 ?????????????????????? 1111111111001110111110010010 001101111111110010001101000 01100000100000000000000000000000 ????????????????????????????????? ????????????????????????????????????
<i>P. rupestris</i> (Spies 6310) Group6
00000001001101011010011101100000 10011010011101111110110011101 ???????????????????????????????????? 111110100101101110011111 1001110100000000110111100000 ?????????????????????? 01010111011011110011101111010 100011011111101101010100110 ???????????????????????????????????? ????????????????????????????????? ????????????????????????????????????;

APPENDIX D: The coefficients of similarities and the genetic distances between the different specimens studied for DAFs.

Primer DAF<sub>1</sub>.

- 1 - *P. airoides* (Spies 6152), 2 - *P. airoides* (Spies 6205), 3 - *P. aristifolia* (Spies 6295), 4 - *P. airoides* (Spies 6311), 5 - *P. veneta* (Spies 6001),
- 6 - *P. barbata* (Spies 6267), 7 - *P. barbata* (Spies 6321), 8 - *P. veneta* (Spies 6327), 9 - *P. eriostoma* (Spies 6000), 10 - *P. pallida* (Spies 6189),
- 11 - *P. pallida* (Spies 6208), 12 - *P. pallida* (Spies 6276), 13 - *P. tomentella* (Spies 6337), 14 - *P. tomentella* (Spies 6345), 15 - *P. patula* (Spies 6272),
- 16 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 17 - *P. tomentella* (Spies 6344), 18 - *P. tomentella* (Spies 6346),
- 19 = *P. tomentella* (Spies 6344), 20 = *P. papillosa* (Spies 6226), 21 - *P. rupestris* (Spies 6330), 22 - *P. eriostoma* (Spies 6144),
- 23 - *P. eriostoma* (Spies 6173), 24 - *P. eriostoma* (Spies 6269), 25 - *P. viscidula* (Spies 6178), 26 - *P. densifolia* (Spies 6328), 27 - *P. tortuosa* (Spies 6179),
- 28 - *P. colorata* (Spies 6313), 29 - *P. rupestris* (Spies 6309), 30 - *P. tomentella* (Spies 6356), 31 - *P. rigidissima* (Spies 6243),
- 32 - *P. rigidissima* (Spies 6109), 33 - *P. tortuosa* (Spies 6214), 34 - *P. tomentella* (Spies 6277), 35 - *P. curvifolia* (Spies 6215), 36 - *P. curvifolia* (Spies 6221),
- 37 - *P. curvifolia* (Spies 6232), 38 - *P. curvifolia* (Spies 6270), 39 - *P. capensis* (Spies 6169), 40 - *P. pallida* (Spies 6281),
- 41 - *P. rupestris* (Spies 6308), 42 - *P. rupestris* (Spies 6310).

Below diagonal: genetic distances.

Above diagonal: coefficients of similarities

Table with 42 rows and 42 columns representing genetic distances and coefficients of similarities between specimens 1-42.

Primer DAF<sub>2</sub>.

1 - *P. airoides* (Spies 6152), 2 - *P. airoides* (Spies 6205), 3 - *P. aristifolia* (Spies 6295), 4 - *P. airoides* (Spies 6311), 5 - *P. veneta* (Spies 6001), 6 - *P. barbata* (Spies 6267), 7 - *P. barbata* (Spies 6321), 8 - *P. veneta* (Spies 6327), 9 - *P. eriostoma* (Spies 6000), 10 - *P. pallida* (Spies 6189), 11 - *P. pallida* (Spies 6208), 12 - *P. pallida* (Spies 6276), 13 - *P. tomentella* (Spies 6337), 14 - *P. tomentella* (Spies 6345), 15 - *P. patula* (Spies 6272), 16 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 17 - *P. tomentella* (Spies 6344), 18 - *P. tomentella* (Spies 6346), 19 = *P. tomentella* (Spies 6344), 20 = *P. papillosa* (Spies 6226), 21 - *P. rupestris* (Spies 6330), 22 - *P. eriostoma* (Spies 6144), 23 - *P. eriostoma* (Spies 6173), 24 - *P. eriostoma* (Spies 6269), 25 - *P. viscidula* (Spies 6178), 26 - *P. densifolia* (Spies 6328), 27 - *P. tortuosa* (Spies 6179), 28 - *P. colorata* (Spies 6313), 29 - *P. rupestris* (Spies 6309), 30 - *P. tomentella* (Spies 6356), 31 - *P. rigidissima* (Spies 6243), 32 - *P. rigidissima* (Spies 6109), 33 - *P. tortuosa* (Spies 6214), 34 - *P. tomentella* (Spies 6277), 35 - *P. curvifolia* (Spies 6215), 36 - *P. curvifolia* (Spies 6221), 37 - *P. curvifolia* (Spies 6232), 38 - *P. curvifolia* (Spies 6270), 39 - *P. capensis* (Spies 6169), 40 - *P. pallida* (Spies 6281), 41 - *P. rupestris* (Spies 6308), 42 - *P. rupestris* (Spies 6310).

Below diagonal: genetic distances.

Above diagonal: coefficients of similarities

42x42 similarity matrix table with genetic distances below the diagonal and similarity coefficients above the diagonal.









Primer DAF<sub>7</sub>.

1 - *P. airoides* (Spies 6152), 2 - *P. airoides* (Spies 6205), 3 - *P. aristifolia* (Spies 6295), 4 - *P. airoides* (Spies 6311), 5 - *P. veneta* (Spies 6001), 6 - *P. barbata* (Spies 6267), 7 - *P. barbata* (Spies 6321), 8 - *P. veneta* (Spies 6327), 9 - *P. eriostoma* (Spies 6000), 10 - *P. pallida* (Spies 6189), 11 - *P. pallida* (Spies 6208), 12 - *P. pallida* (Spies 6276), 13 - *P. tomentella* (Spies 6337), 14 - *P. tomentella* (Spies 6345), 15 - *P. patula* (Spies 6272), 16 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 17 - *P. tomentella* (Spies 6344), 18 - *P. tomentella* (Spies 6346), 19 = *P. tomentella* (Spies 6344), 20 = *P. papillosa* (Spies 6226), 21 - *P. rupestris* (Spies 6330), 22 - *P. eriostoma* (Spies 6144), 23 - *P. eriostoma* (Spies 6173), 24 - *P. eriostoma* (Spies 6269), 25 - *P. viscidula* (Spies 6178), 26 - *P. densifolia* (Spies 6328), 27 - *P. tortuosa* (Spies 6179), 28 - *P. colorata* (Spies 6313), 29 - *P. rupestris* (Spies 6309), 30 - *P. tomentella* (Spies 6356), 31 - *P. rigidissima* (Spies 6243), 32 - *P. rigidissima* (Spies 6109), 33 - *P. tortuosa* (Spies 6214), 34 - *P. tomentella* (Spies 6277), 35 - *P. curvifolia* (Spies 6215), 36 - *P. curvifolia* (Spies 6221), 37 - *P. curvifolia* (Spies 6232), 38 - *P. curvifolia* (Spies 6270), 39 - *P. capensis* (Spies 6169), 40 - *P. pallida* (Spies 6281), 41 - *P. rupestris* (Spies 6308), 42 - *P. rupestris* (Spies 6310).

Below diagonal: genetic distances.

Above diagonal: coefficients of similarities

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	35	36	37	38	39	40	41	42											
1																																																				
2	0.58																																																			
3	0.89	0.51																																																		
4	1.10	0.97	0.49																																																	
5	0.91	0.41	0.41	1.20																																																
6	0.91	1.01	0.91	0.41	1.55																																															
7	1.10	1.01	1.01	0.81	1.31	1.31																																														
8	1.10	0.91	0.87	0.97	1.23	0.61	1.91																																													
9	1.70	0.71	0.41	0.57	0.81	0.51	0.91	1.70																																												
10					0.51		1.70	1.70																																												
11																																																				
12																																																				
13																																																				
14	0.41	0.59	0.55	0.97	0.97	0.97	0.36	0.61																																												
15	0.31	0.71	0.49	0.41	1.10	1.10	0.41	0.59																																												
16																																																				
17	0.59	0.91	1.27	1.27	1.27	0.51	0.59																																													
18																																																				
19	1.41	1.39	1.39	1.25	1.25	1.25	1.57	1.57																																												
20																																																				
21	0.55	0.91			0.81	0.59	0.91	0.91																																												
22																																																				
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**Primer DAF<sub>8</sub>.**

1 - *P. airoides* (Spies 6152), 2 - *P. airoides* (Spies 6205), 3 - *P. aristifolia* (Spies 6295), 4 - *P. airoides* (Spies 6311), 5 - *P. veneta* (Spies 6001), 6 - *P. barbata* (Spies 6267), 7 - *P. barbata* (Spies 6321), 8 - *P. veneta* (Spies 6327), 9 - *P. eriostoma* (Spies 6000), 10 - *P. pallida* (Spies 6189), 11 - *P. pallida* (Spies 6208), 12 - *P. pallida* (Spies 6276), 13 - *P. tomentella* (Spies 6337), 14 - *P. tomentella* (Spies 6345), 15 - *P. patula* (Spies 6272), 16 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 17 - *P. tomentella* (Spies 6344), 18 - *P. tomentella* (Spies 6346), 19 = *P. tomentella* (Spies 6344), 20 = *P. papillosa* (Spies 6226), 21 - *P. rupestris* (Spies 6330), 22 - *P. eriostoma* (Spies 6144), 23 - *P. eriostoma* (Spies 6173), 24 - *P. eriostoma* (Spies 6269), 25 - *P. viscidula* (Spies 6178), 26 - *P. densifolia* (Spies 6328), 27 - *P. tortuosa* (Spies 6179), 28 - *P. colorata* (Spies 6313), 29 - *P. rupestris* (Spies 6309), 30 - *P. tomentella* (Spies 6356), 31 - *P. rigidissima* (Spies 6243), 32 - *P. rigidissima* (Spies 6109), 33 - *P. tortuosa* (Spies 6214), 34 - *P. tomentella* (Spies 6277), 35 - *P. curvifolia* (Spies 6215), 36 - *P. curvifolia* (Spies 6221), 37 - *P. curvifolia* (Spies 6232), 38 - *P. curvifolia* (Spies 6270), 39 - *P. capensis* (Spies 6169), 40 - *P. pallida* (Spies 6281), 41 - *P. rupestris* (Spies 6308), 42 - *P. rupestris* (Spies 6310).

Below diagonal: genetic distances.

Above diagonal: coefficients of similarities

	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	35	36	37	38	39	40	41	42				
1																																														
2	0.781																																													
3	0.391	0.581																																												
4	0.721	0.511	0.591																																											
5	0.311	0.591	1.391	0.711																																										
6	0.491	0.371	0.551	0.751	0.711																																									
7	0.591	0.311	0.791	0.811	0.701	0.911																																								
8	0.591	0.591	0.591	0.611	0.591	0.591	0.611																																							
9	0.591	0.591	0.591	0.611	0.591	0.591	0.611	0.591																																						
10	1.521	0.721	1.191	1.101	1.251	1.191	1.161	1.451	1.451																																					
11	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451																																				
12	1.101	1.251	1.151	1.091	1.101	1.251	1.101	1.251	1.451	1.451	1.451																																			
13	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451																																		
14	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451																																	
15	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451																																
16	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451																															
17	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																														
18	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																													
19	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																												
20	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																											
21	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																										
22	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																									
23	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																								
24	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																							
25	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																						
26	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																					
27	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																				
28	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																			
29	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																		
30	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																	
31	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451																
32	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451															
33	0.721	0.721	0.811	0.811	0.711	0.811	0.811	0.811	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451	1.451														
34	0.721	0.721	0.811	0.811	0.711																																									

**Primer DAF<sub>9</sub>.**

1 - *P. airoides* (Spies 6152), 2 - *P. airoides* (Spies 6205), 3 - *P. aristifolia* (Spies 6295), 4 - *P. airoides* (Spies 6311), 5 - *P. veneta* (Spies 6001), 6 - *P. barbata* (Spies 6267), 7 - *P. barbata* (Spies 6321), 8 - *P. veneta* (Spies 6327), 9 - *P. eriostoma* (Spies 6000), 10 - *P. pallida* (Spies 6189), 11 - *P. pallida* (Spies 6208), 12 - *P. pallida* (Spies 6276), 13 - *P. tomentella* (Spies 6337), 14 - *P. tomentella* (Spies 6345), 15 - *P. patula* (Spies 6272), 16 - *P. tomentella* (Spies 6280), 19 - *P. tomentella* (Spies 6342), 20 - *P. tomentella* (Spies 6346), 17 - *P. tomentella* (Spies 6344), 18 - *P. tomentella* (Spies 6346), 19 = *P. tomentella* (Spies 6344), 20 = *P. papillosa* (Spies 6226), 21 - *P. rupestris* (Spies 6330), 22 - *P. eriostoma* (Spies 6144), 23 - *P. eriostoma* (Spies 6173), 24 - *P. eriostoma* (Spies 6269), 25 - *P. viscidula* (Spies 6178), 26 - *P. densifolia* (Spies 6328), 27 - *P. tortuosa* (Spies 6179), 28 - *P. colorata* (Spies 6313), 29 - *P. rupestris* (Spies 6309), 30 - *P. tomentella* (Spies 6356), 31 - *P. rigidissima* (Spies 6243), 32 - *P. rigidissima* (Spies 6109), 33 - *P. tortuosa* (Spies 6214), 34 - *P. tomentella* (Spies 6277), 35 - *P. curvifolia* (Spies 6215), 36 - *P. curvifolia* (Spies 6221), 37 - *P. curvifolia* (Spies 6232), 38 - *P. curvifolia* (Spies 6270), 39 - *P. capensis* (Spies 6169), 40 - *P. pallida* (Spies 6281), 41 - *P. rupestris* (Spies 6308), 42 - *P. rupestris* (Spies 6310).

Below diagonal: genetic distances.

Above diagonal: coefficients of similarities

	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	35	36	37	38	39	40	41	42							
1																																																	
2	0.72																																																











**APPENDIX E: CLUSTAL W (1.74) multiple sequence alignment data of the *Pentaschistis* species used for the cladistic analysis in PAUP and Hennig86.**

<i>Merxmullera dura</i>	TCGTGACCCGAAACCAAAA-CCGACCGCGAA--TGCGTCA-CCCTGTCCGGCCGCGCGC
<i>Pentameris macrocalycina</i>	TCGTGACCCGAAACCAAAA--CTGACCGCGAA--CGCGTCA-CCTTGTCCGGCCGACGG
<i>Prionanthium ecklonii</i>	TCGTGACCCGAAACCAAAA--CTGACCGCGAA--CAAGTCA-CCTTGTCCGGTCCGACGG
<i>Pentaschistis aspera</i> (Spies 6001)	TCGTGACC--GAACCAAAA--CTGACCGCGAA--CAAGTCA-CCTTGTCCGGCCACGCGG
<i>P. veneta</i> (Spies 6327)	TCGTGACTCGCAACCAATG--CTGACCGTGAA--CAAGTCA-CATTGTCCGGCCACGCGG
<i>P. pallida</i> (Spies 6208)	TCGTGACTCGCAACCAATG--CTGACCGTGCAACTAAGTCATCATTGTCCGGCCACGCGA
<i>P. aristifolia</i> (Spies 6295)	TCGTGACC-GAAACCAATG--CAGACCGTGCAA-CAAGTCA-CATTGTCCGGCCACACGA
<i>P. curvifolia</i> (Spies 6270)	TCGTGACC---GAGGACGAT-CTGACCGTGCAATCAAGTCA-CCTTGTCCGGTCCGATGG
<i>P. tomentella</i> (Spies 6337)	TGCTGAATC--ATCGACGATGCTGACCGTGCAAGTAAACGCATCATTGTCCGGCCACGCGA
<i>P. rupestris</i> (Spies 6330)	TCGTGACTC--AG-GACGATGCTGACCGTGCAACCAA-GTCTCATTGTCCGGCCACGCG-
<i>P. rupestris</i> (Spies 6308)	TCGTGA-GT--CA-GTAGGTGCTGACCATG-AACCAAGTCATCATTGTCCGGCCA-CGC-
<i>P. rupestris</i> (Spies 6310)	TCGTGACGT--CACGTCCGTGCTGACCCTGCAACCAAGTCATCATTGTCCGGCCA-CGC-
<i>P. papillosa</i> (Spies 6226)	TCGTGACGCTCCACNT-GGTGCTGACCT--CTAACAAGTC-TCATTGTCC-----
<i>P. pallida</i> (Spies 6281)	TCGTGACGT--CACGT-GGTGCGTACCCTGCAACCAAGTC-TCATTGTCCGGCCAGCXC-
<i>P. rupestris</i> (Spies 6309)	TCGTGACGT--CACGTCCGTGCTGACCCTTC-AACAAGTCATCATTGTCCGGCCA-CGC-
<i>P. calcicola</i> (Spies 6209)	TCGTGACCC---AAGTCNGTGTGACCG--CAACCAAGTC-ACATTGTCCGGCCACGTC-
<i>P. tortuosa</i> (Spies 6179)	TCGTGACGT--GCAGTCCGTGCTGACCGTGCAACCAAGTCATCATTGTCCGGCCAGCGC-
<i>P. tomentella</i> (Spies 6345)	TGCTGAGT---CATGTAGATGCCTACCCTGCAACCAAGTCATCATTGTCCGGCCACCTC-
<i>P. tomentella</i> (Spies 6277)	TCGTGAGTG--CACGTAGGTGCGTACCCTGCAACCAAGTCATCATTGTCCGGCCACATG-
<i>P. tomentella</i> (Spies 6356)	TCGTGACTG--CACGTAGGTGCGT-CCGTGCAACCAAGTCATCATTGTCCGGCCAGCTC-
<i>P. rigidissima</i> (Spies 6243)	TCGTGAGCT--TACGTNGGTGCGTACCCTGCAACCAAGTCATCATTGTCTCCGGCACT---
<i>P. eriostoma</i> (Spies 6173)	TCGTGACTC---GCATCGATGCTGACC--GCAACCAAGTC-ACATTGTCCGGTC-GCAC-
<i>P. curvifolia</i> (Spies 6215)	TCGTGAGTA---TCATCGGTGTGACCCCTGCAACCAAGTC-ACATTGTCCGGTCAGCAC-
<i>P. tomentella</i> (Spies 6342)	TCGTGAGT----CATCAGTGCCGACCGTGCAA-CAAGTC-TCATTGTCCGGCCGCTCG-
<i>P. airoides</i> (Spies 6205)	TCGTGAGTCTCAA--TCAATGCAGACCGTGCAA-CAAG----CATTATCACGCCCGCAGC
<i>Merxmullera dura</i>	CGGGGCTTGTCCCTGTGCGGTGGCCCAAGGC-CGCCGACCTCCGCTAGGG---GGGAGC
<i>Pentameris macrocalycina</i>	CGTGGCTCGCTCACGCCGCTGGCCTA-GGC-CGCCGACCTCCGCCAGGAT--GGGGAGC
<i>Prionanthium ecklonii</i>	C-----TCACGCCGCGCGGCTA-GGC-CGCCGACCTCCGCAAGGAC--GGGGAGC
<i>P. veneta</i> (Spies 6001)	C-----TCACGCCGCGCGGCTA-GGC-CGCCGACCTCCGCGAGGAA--GGGGAGC
<i>P. veneta</i> (Spies 6327)	C-----TCACGCCGCGCGGCTA-GGC-CGCCGACCTCCGCAAGGAA--GGGGAGC
<i>P. pallida</i> (Spies 6208)	G-----CTCACGCCGCGCGGCTA-GGC-CGCCGACCTCCGCAAGGAA--GGGGAGC
<i>P. aristifolia</i> (Spies 6295)	G-----CTCACGCCGCTGTGGCCTA-GGC-CGCCGACCTCCGCAAGGAC--GGGGAGC
<i>P. curvifolia</i> (Spies 6270)	C-----TCACGCCGCGCGGCTA-GGC-CGCCGACCTCCGCAAGGAT--GGGGAGC
<i>P. tomentella</i> (Spies 6337)	G-----CTCACGCCGCGCGGCTA-GGC-CGCCGACCTCCGCAAGGAA--GGGGAGC
<i>P. rupestris</i> (Spies 6330)	G-----CTCACGCCGCGCGGCTA-GGC-CGCCGACCTCCGCAAGGAA--GGGGAGC
<i>P. rupestris</i> (Spies 6308)	---GGCT--CACGC--CGCGCGGCTA-GGC-CGCCGACCTCCGCAAGGAA--GGGGAGC
<i>P. rupestris</i> (Spies 6310)	---GGCT--CACGC--CGCGCGGCTA-CGC-CGCCGACCTCCGCAAGGAA--GGGGAGC
<i>P. papillosa</i> (Spies 6226)	-----A-GGC-CGCCGACCTCCGCAAGGAT--GGGGAGC
<i>P. pallida</i> (Spies 6281)	---GGCT--CACGC--CGCGCGGCTA-GGC-CGCCGACCTGAGCGCTGCTC-GGGGAGC
<i>P. rupestris</i> (Spies 6309)	---GGCT--CACGC--CGCGCGGCTA-GGC-CGCCGACCTCCGCGAGGAA--GGGGAGC
<i>P. calcicola</i> (Spies 6209)	---GGCT--CACGC--CGCGCGGCTA-GGC-CGCCGACCTCCGCAAGGAC--GGGGAGC
<i>P. tortuosa</i> (Spies 6179)	---GGCT--CACGC--CGCGTGGCCTA-GGC-CGCCGACCTCCGCGAGGAC--GGGGAGC
<i>P. tomentella</i> (Spies 6345)	---GGCT--C-TCC--CGCGCGGCCAC-GGC-CACT-ACCGAGGCTCCTCX-GGGGAGC
<i>P. tomentella</i> (Spies 6277)	---AGCT--CATGCTACACGCGCATA-GAT-TGCT-ACCTCCGGCTCGGAGTGGGGAGC
<i>P. tomentella</i> (Spies 6356)	---GGCT--CACGC--CGCGCGGCTA-GGC-CGCCGACCGAGGCTCGTCG-GGGAGC

*P. rigidissima* (Spies 6243) ---GGCT--C-TGC--TGC GTGGCCTA-GGC-CGCCGACCTCCGCAAGGACG--GGGAGC  
*P. eriostoma* (Spies 6173) ---GGCT--CACGC--CGCGCGCCTA-GGC-CGCCGACCTCCGCAAGGAT--GGGGAGC  
*P. curvififolia* (Spies 6215) ---GGCT--CCGCC--TGC GCGCXTA-GGC-CGCCGACCTCCGCAAGGAT--GGGGAGC  
*P. tomentella* (Spies 6342) ---G-----CTCACGCCGCGCGCCTA-GGC-CGCCGACCGACGCGTGGTC--GGGGAGC  
*P. airoides* (Spies 6205) AGCGG-----TCGCCGCGTGGCCTAGTCTCTCGACGACCTCTCTCTCGGAGCGGGC

*Merxmuellera dura* G-GCCGCAAAGAACCACGGCGCCGACGGCGTCAAGGAACTTATATTGCCTTGCGC  
*Pentameris macrocalycina* G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*Prionanthium ecklonii* G-GCCGCAAAGAACCACGGCGCCGTACGGC-TCAAGGAAACTGTTATTGCCTTGCGC  
*P. veneta* (Spies 6001) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. veneta* (Spies 6327) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. pallida* (Spies 6208) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. aristifolia* (Spies 6295) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. curvififolia* (Spies 6270) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. tomentella* (Spies 6337) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. rupestris* (Spies 6330) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. rupestris* (Spies 6308) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. rupestris* (Spies 6310) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. papillosa* (Spies 6226) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. pallida* (Spies 6281) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. rupestris* (Spies 6309) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. calcicola* (Spies 6209) A-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. tortuosa* (Spies 6179) G-GCCGCAAAGAACCACGGCGCCGTATGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. tomentella* (Spies 6345) G-GCCGCAAAG-AACCCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. tomentella* (Spies 6277) GCGCTGCAAATAACCCACGGCGCCGTATGGCGTCCAGGAACTGTTATTGCCTTGCGC  
*P. tomentella* (Spies 6356) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. rigidissima* (Spies 6243) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. eriostoma* (Spies 6173) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. curvififolia* (Spies 6215) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. tomentella* (Spies 6342) G-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAACTGTTATTGCCTTGCGC  
*P. airoides* (Spies 6205) TCGGGTAAAGAACCACGGCGCCGTAAGGCGTCAAGGAACTGTGCTAGCTTAGG-

*Merxmuellera dura* GCGGCGGTGGCTGGCCTGCCGGCCGCTCCGTGCGCAGCGATTGTATGCTAATC  
*Pentameris macrocalycina* GCGGTGGCGGCTGGCCTGCCGGTCCGCCGCGCGCAGCGATTCTATACTAATC  
*Prionanthium ecklonii* GCGGCCGCGGCTGGCCCGCAGCCGACCGCGCGCAGCGATTCTATACTAATC  
*P. veneta* (Spies 6001) GTGGCCGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTAATC  
*P. veneta* (Spies 6327) GTGGACGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTAATC  
*P. pallida* (Spies 6208) GTGGACGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTAATC  
*P. aristifolia* (Spies 6295) GTGGCCGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTAATC  
*P. curvififolia* (Spies 6270) GTGGCCGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTAATC  
*P. tomentella* (Spies 6337) GTGGACGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTAATC  
*P. rupestris* (Spies 6330) GTGGACGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTAATC  
*P. rupestris* (Spies 6308) GTGGTCGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTAATC  
*P. rupestris* (Spies 6310) GTGGTCGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTAATC  
*P. papillosa* (Spies 6226) GTGGACTG-CTGGCCTGCCAGCCGCGCCCGCGCAGCGATTCTATACTAATC  
*P. pallida* (Spies 6281) GTGGCCGTGGCTGGCCTGCCAGCCGCGCGCGCGCAGCGATTCTATACTAATC  
*P. rupestris* (Spies 6309) GTGGTCGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTAATC  
*P. calcicola* (Spies 6209) GTGGCCGCGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTAATC  
*P. tortuosa* (Spies 6179) GTGGCCGCGGCTGGCCTGCCAGTGCACCGCGCGCAGCGATTCTATACTAATC  
*P. tomentella* (Spies 6345) GCGGCCGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTAATC  
*P. tomentella* (Spies 6277) GCGGCCGTGGCTGGCCTGCCAGCCGCGCGCGCAGCGATTCTATACTAATC

<i>P. tomentella</i> (Spies 6356)	GCGGCCGTGGCTGGCCTGCCAGCCGCGACGCGCGCAGCGATTCTATACTAATC
<i>P. rigidissima</i> (Spies 6243)	GTGGCCGTGGCTGGCCTGCCAGCCGCACCGCGCGCAGCGATTCTATACTAATC
<i>P. eriostoma</i> (Spies 6173)	GCGGCCGTGGCTGGCCTGCCAGCCGCACCGCGCGCAGCGATTCTATACTAATC
<i>P. curvififolia</i> (Spies 6215)	GCGGCCGTGGCTGGCCTGCCACCCGCACCGCGCGCAGCGATTCTTTACTAATC
<i>P. tomentella</i> (Spies 6342)	GCGGACGTGGCTGGCCTGCCACCCGCGCCGCGCGCAGCGATTCTATACTAATC
<i>P. airoides</i> (Spies 6205)	--GGATGCGGCTGGCTTGCTGGCCGCACCCTTTGCTGC-ATTCTATACTAATC

**APPENDIX F: MALIGN (1.93) multiple sequence alignment data of the *Pentasthisis* species used for the cladistic analysis in PAUP and Hennig86.**

<i>P. rigidissima</i> (Spies 6243)	TCGTGAGCT-T-ACGTNGG-TG-CGTACCCTGCAACCAAGTC-ATCATTGTCTCGGC-AC
<i>P. eriostoma</i> (Spies 6173)	TCGTGA-CT-C-GCATCGA-TG-CTGACC--GCAACCAAGTC-A-CATTGTCCGGTC-GC
<i>P. pallida</i> (Spies 6208)	TCGTGA-CT-C-GCAACCAATG-CTGACCGTGCAACTAAGTC-ATCATTGTCCGGCC-AC
<i>P. calcicola</i> (Spies 6209)	TCGTGA-CC-C-AAGTCNG-TG-CTGACC--GCAACCAAGTC-A-CATTGTCCGGCC-AC
<i>P. curvifolia</i> (Spies 6215)	TCGTGA-GT-A-TCATCGG-TG-CTGACCCTGCAACCAAGTC-A-CCTTGTCCGGTCAGC
<i>P. pallida</i> (Spies 6281)	TCGTGACGT-C-ACG-TGG-TG-CGTACCCTGCAACCAAGTC-T-CATTGTCCGGCCAGC
<i>P. rupestris</i> (Spies 6308)	TCGTGA-GT-C-AGT-AGG-TG-CTGACCA-TGAACCAAGTC-ATCATTGTCCGGCC-AC
<i>P. tortuosa</i> (Spies 6179)	TCGTGACGT-G-CAGTCCG-TG-CTGACCGTGCAACCAAGTC-ATCATTGTCCGGCCAGC
<i>P. rupestris</i> (Spies 6309)	TCGTGACGT-C-ACGTCCG-TG-CTGACCCTCAA-CAAGTC-ATCATTGTCCGGCC-AC
<i>P. curvifolia</i> (Spies 6270)	TCGTGA-CC-G-AGG-ACG-AT-CTGACCGTGCAATCAAGTC-ACC-TTGTCCGGTC-GC
<i>P. tomentella</i> (Spies 6277)	TCGTGA-GT-GCAGTAGG-TG-CGTACCCTGCAACCAAGTC-ATCATTGTCCGGCC-AC
<i>P. rupestris</i> (Spies 6310)	TCGTGACGT-C-ACGTCCG-TG-CTGACCCTGCAACCAAGTC-ATCATTGTCCGGCC-AC
<i>P. tomentella</i> (Spies 6356)	TCGTGA-CTGC-ACGTAGG-TG-CGT-CCGTGCAACCAAGTCAT-CATTGGTCCGGCCAGC
<i>P. tomentella</i> (Spies 6345)	TGCTGA-GT-C-ATGTAGA-TG-CCTACCCTGCAACCAAGTCAT-CATTGTCCGGCCACC
<i>P. tomentella</i> (Spies 6342)	TCGTGA-GT-C-ATC-AG--TG-CCGACCGTGCAAC-AAGTC-T-CATTGTCCGGCC-GC
<i>P. rupestris</i> (Spies 6330)	TCGTGA-CT-C-AGGACGA-TG-CTGACCGTGCAACCAAGTC-T-CATTGTCCGGCC-AC
<i>P. aristifolia</i> (Spies 6295)	TCGTGA-C-CG---AACCAATG-CAGACCGTGCAAC-AAGTC-A-CATTGTCCGGCC-AC
<i>P. papillosa</i> (Spies 6226)	TCGTGA-C-G-----C---TC-CA--CN-TG-----GTG---C-T-GACCT-CT-A-
<i>P. veneta</i> (Spies 6001)	TCGTGA-C-CG---AACCAAAA-CTGACCGCG-AAC-AAGTC-A-CCTTGTCCGGCC-AC
<i>P. veneta</i> (Spies 6327)	TCGTGA-CTCG--CAACCAATG-CTGACCGTG-AAC-AAGTC-A-CATTGTCCGGCC-AC
<i>P. tomentella</i> (Spies 6337)	TGCTGA-ATCA-TGCAC-GATG-CTGACCGTGCAAGTAACGC-ATCATTGTCCGGCC-AC
<i>P. airoides</i> (Spies 6205)	TCGTGA-GTCT--CAATCAATG-CAGACCGTGCAAC-A-AGC-ATTAT-CACGC-CC-GC
<i>Prionanthium ecklonii</i>	TCGTGA-CCCG-A-AACCAAAA-CTGACCGCG-AAC-AAGTC-A-CCTTGTCCGGTC-GC
<i>Merxmuellera dura</i>	TCGTGA-CCCG-A-AACCAAAAACCGACCGCG-AATGC-GTC-A-CCCTGTCCGGCC-GC
<i>Pentameris macrocalycina</i>	TCGTGA-CCCG-A-AACCAAAA-CTGACCGCG-AACGC-GTC-A-CCTTGTCCGGCC-GC
<i>P. rigidissima</i> (Spies 6243)	--TG-GCT--C-TG-CTGCGT-G-G---CCTA-GGCCGC--CGACCTCCG-C---AAG-G
<i>P. eriostoma</i> (Spies 6173)	A-CG-GCT--CACG-CCGCGC-G-G---CCTA-GGCCGC--CGACCTCCG-C---AAG-G
<i>P. pallida</i> (Spies 6208)	G-CGAGCT--CACG-CCGCGC-G-G---CCTA-GGCCGC--CGACCTCCG-C---AAG-G
<i>P. calcicola</i> (Spies 6209)	GTCG-GCT--CACG-CCGCGC-G-G---CCTA-GGCCGC--CGACCTCCG-C---AAG-G
<i>P. curvifolia</i> (Spies 6215)	A-CG-GCT--C-CGCTGCGC-G-G---CXTA-GGCCGC--CGACCTCCG-C---AAG-G
<i>P. pallida</i> (Spies 6281)	XXCG-GCT--CACG-CCGCGC-G-G---CCTA-GGCCGC--CGACCTGAG-CG--CTG-C
<i>P. rupestris</i> (Spies 6308)	G-CG-GCT--CACG-CCGCGC-G-G---TCTA-GGCCGC--CGACCTCCG-C---AAG-G
<i>P. tortuosa</i> (Spies 6179)	G-CG-GCT--CACG-CCGCGT-G-G---CCTA-GGCCGC--CGACCTCCG-C---GAG-G
<i>P. rupestris</i> (Spies 6309)	G-CG-GCT--CACG-CCGCGC-G-G---CCTA-GGCCGC--CGACCTCCG-C---GAG-G
<i>P. curvifolia</i> (Spies 6270)	A-TG-GCT--CACG-CCGCGC-G-G---CCTA-GGCCGC--CGACCTCCG-C---AAG-G
<i>P. tomentella</i> (Spies 6277)	A-TGAGCT--CATG-CTACAC-GCG---CATA-CGATTG--CTACCTCCGGC---TCG-G
<i>P. rupestris</i> (Spies 6310)	G-CG-GCT--CACG-CCGCGC-G-G---CCTA-CGCCGC--CGACCTCCG-C---AAG-G
<i>P. tomentella</i> (Spies 6356)	T-CG-GCT--CACG-CCGCGC-G-G---CCTA-GGCCGC--CGACCGAGG-C---CTC-G
<i>P. tomentella</i> (Spies 6345)	T-CG-GCT--CTC--CCGCGC-G-G---CCAC-GGCCAC--T-ACCGAGG-C---CTC-C
<i>P. tomentella</i> (Spies 6342)	T-CG-GCT--CACG-CCGCGC-G-G---CCTA-GGCCGC--CGACCGAGC-C---GTCGG
<i>P. rupestris</i> (Spies 6330)	G-CG-GCT--CACG-CCGCGC-G-G---CCTA-GGCCGC--CGACCTCCG-C---AAG-G
<i>P. aristifolia</i> (Spies 6295)	A-CGAGCT--CACG-CCGTGT-G-G---CCTA-GGCCGC--CGACCTCCG-C---AAG-G
<i>P. papillosa</i> (Spies 6226)	A-CAAG-T--CTCA-T--TGT-----CC-A-GGCCGC--CGACCTCCG-C---AXG-G
<i>P. veneta</i> (Spies 6001)	G-CGGC-T--CACG-CCGCGC-G-G---CCTA-GGCCGC--CGACCTCCG-C---GAG-G
<i>P. veneta</i> (Spies 6327)	G-CGGC-T--CACG-CCGCGC-G-G---CCTA-GGCCGC--CGACCTCCG-C---AAG-G

<i>P. tomentella</i> (Spies 6337)	G-CGAGCT--CACG-CCGCGC-G-G---CCTA-GGCCGC--CGACCTCCG-C---AAG-G
<i>P. airoides</i> (Spies 6205)	A-CGAGCG--GTCG-CCGCGT-G-G---CCTA-GTCCTCGACGACCTCCT-CTCCTCG-G
<i>Prionanthium ecklonii</i>	A-CGGC-T--CACG-CCGCGC-G-G---CCTA-GGCCGC--CGACCTCCG-C---AAG-G
<i>Merxmuellera dura</i>	G-CGCCGGGGCTTGTCCTGTGCGGTGGCCCAAGGCCGC--CGACCTCCG-C---TAG-G
<i>Pentameris macrocalycina</i>	A-CGGCGTGGCTCGCTCACGCCGCGTGGCCTA-GGCCGC--CGACCTCCG-C---CAG-G
<i>P. rigidissima</i> (Spies 6243)	A-CGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. eriostoma</i> (Spies 6173)	A-TGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. pallida</i> (Spies 6208)	A-AGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. calcicola</i> (Spies 6209)	A-CGGGGAGCA-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. curvifolia</i> (Spies 6215)	A-TGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. pallida</i> (Spies 6281)	T-CGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. rupestris</i> (Spies 6308)	A-AGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. tortuosa</i> (Spies 6179)	A-CGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTATGGCGTCAAGGAAAACGTGTAT
<i>P. rupestris</i> (Spies 6309)	A-AGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. curvifolia</i> (Spies 6270)	A-TGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. tomentella</i> (Spies 6277)	AGTGGGGAGCGCGCTGCAAAATAACCCACGGCGCCGTATGGCGTCCAGGAACACTGTAT
<i>P. rupestris</i> (Spies 6310)	A-AGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. tomentella</i> (Spies 6356)	T-CGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. tomentella</i> (Spies 6345)	TCXGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. tomentella</i> (Spies 6342)	T-CGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. rupestris</i> (Spies 6330)	A-AGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. aristifolia</i> (Spies 6295)	A-CGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. papillosa</i> (Spies 6226)	A-TGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. veneta</i> (Spies 6001)	A-AGGGGAHCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. veneta</i> (Spies 6327)	A-AGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. tomentella</i> (Spies 6337)	A-AGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. airoides</i> (Spies 6205)	AGCGGGCTCG-G-GTAAAAGAACCACGGCGCCGTAAAGCGTCAAGGAACACTGTGCC
<i>Prionanthium ecklonii</i>	A-CGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGC-TCAAGGAAAACGTGTAT
<i>Merxmuellera dura</i>	--GGGGCAGCG-GCCGCAAAGAACCACGGCGCCGACGGCGTCAAGGAACACTTATAT
<i>Pentameris macrocalycina</i>	A-TGGGGAGCG-GCCGCAAAGAACCACGGCGCCGTACGGCGTCAAGGAAAACGTGTAT
<i>P. rigidissima</i> (Spies 6243)	TGCCTTGC GCGTGGCCGTGGCTGGCCTGCCAGCCGACCGCGCGCAGCGATTCTATACTA
<i>P. eriostoma</i> (Spies 6173)	TGCCTTGC GCGCGGCCGTGGCTGGCCTGCCAGCCGACCGCGCGCAGCGATTCTATACTA
<i>P. pallida</i> (Spies 6208)	TGCCTTGC GCGTGGACGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTA
<i>P. calcicola</i> (Spies 6209)	TGCCTTGC GCGTGGCCGCGGCTGGCCTGCCAXGCGCACCGCGCGCAGCGATTCTATACTA
<i>P. curvifolia</i> (Spies 6215)	TGCCTTGC GCGCGGCCGTGGCTGGCCTGCCACCCGACCGCGCGCAGCGATTCTATACTA
<i>P. pallida</i> (Spies 6281)	TGCCTTGC GCGTGGCCGTGGCTGGCCTGCCAGCCGCGCAGCGCGCAGCGATTCTATACTA
<i>P. rupestris</i> (Spies 6308)	TGCCTTGC GCGTGGTCTGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTA
<i>P. tortuosa</i> (Spies 6179)	TGCCTTGC GCGTGGCCGCGGCTGGCCTGCCAGTCCGACCGCGCGCAGCGATTCTATACTA
<i>P. rupestris</i> (Spies 6309)	TGCCTTGC GCGTGGTCTGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTA
<i>P. curvifolia</i> (Spies 6270)	TGCCTTGC GCGTGGCCGTGGCTGGCCTGCCAGCCGCGCAGCGCGCAGCGATTCTATACTA
<i>P. tomentella</i> (Spies 6277)	TGCCTTGC GCGCGGCCGTGGCTGGCCTGCCAGCCGCGCAGCGCGCAGCGATTCTATACTA
<i>P. rupestris</i> (Spies 6310)	TGCCTTGC GCGTGGTCTGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTA
<i>P. tomentella</i> (Spies 6356)	TGCCTTGC GCGCGGCCGTGGCTGGCCTGCCAGCCGCGCAGCGCGCAGCGATTCTATACTA
<i>P. tomentella</i> (Spies 6345)	TGCCTTGC GCGCGGCCGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTA
<i>P. tomentella</i> (Spies 6342)	TGCCTTGC GCGCGGACGTGGCTGGCCTGCCACCCGCGCCGCGCGCAGCGATTCTATACTA
<i>P. rupestris</i> (Spies 6330)	TGCCTTGC GCGTGGACGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTA
<i>P. aristifolia</i> (Spies 6295)	TGCCTTGC GCGTGGCCGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTA
<i>P. papillosa</i> (Spies 6226)	TGCCTTGC GCGTGGACCTG-CTGGCCTGCCAGCCGCGCCCGCGCAGCGATTCTATACTA
<i>P. veneta</i> (Spies 6001)	TGCCTTGC GCGTGGCCGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTA

<i>P. veneta</i> (Spies 6327)	TGCCTTGCGCGTGGACGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTA
<i>P. tomentella</i> (Spies 6337)	TGCCTTGCGCGTGGACGTGGCTGGCCTGCCAGCCGCGCCGCGCGCAGCGATTCTATACTA
<i>P. airoides</i> (Spies 6205)	TAGCTTA-G-G-GGATGCGGCTGGCTTGCTGGCCGCACCCTTTGCTGC-ATTCTATACTA
<i>Prionanthium ecklonii</i>	TGCCTCGCGCGCGCCGCGGCTGGCCCGCCAGCCGCACCAGCGCGCAGCGATTCTATACTA
<i>Merxmullera dura</i>	TGCCTTGCGCGCGGCGGTGGCTGGCCTGCCGCGCGCTCCGTGCGCAGCGATTGTATGCTA
<i>Pentameris macrocalycina</i>	TGCCTTGCGCGCGGTGGCGGCTGGCCTGCCGCGTCCCGCCGCGCGCAGCGATTCTATACTA

<i>P. rigidissima</i> (Spies 6243)	ATC
<i>P. eriostoma</i> (Spies 6173)	ATC
<i>P. pallida</i> (Spies 6208)	ATC
<i>P. calcicola</i> (Spies 6209)	ATC
<i>P. curvifolia</i> (Spies 6215)	ATC
<i>P. pallida</i> (Spies 6281)	ATC
<i>P. rupestris</i> (Spies 6308)	ATC
<i>P. tortuosa</i> (Spies 6179)	ATC
<i>P. rupestris</i> (Spies 6309)	ATC
<i>P. curvifolia</i> (Spies 6270)	ATC
<i>P. tomentella</i> (Spies 6277)	ATC
<i>P. rupestris</i> (Spies 6310)	ATC
<i>P. tomentella</i> (Spies 6356)	ATC
<i>P. tomentella</i> (Spies 6345)	ATC
<i>P. tomentella</i> (Spies 6342)	ATC
<i>P. rupestris</i> (Spies 6330)	ATC
<i>P. aristifolia</i> (Spies 6295)	ATC
<i>P. papillosa</i> (Spies 6226)	ATC
<i>P. veneta</i> (Spies 6001)	ATC
<i>P. veneta</i> (Spies 6327)	ATC
<i>P. tomentella</i> (Spies 6337)	ATC
<i>P. airoides</i> (Spies 6205)	ATC
<i>Prionanthium ecklonii</i>	ATC
<i>Merxmullera dura</i>	ATC
<i>Pentameris macrocalycina</i>	ATC

**APPENDIX G: The percentage mean divergence between the ITS sequences of the studied specimens.**

1 = *Merxmuellera dura*, 2 = *Pentameris macrocalycina*, 3 = *Prionanthium ecklonii*, 4 = *P. veneta* (Spies 6001), 5 = *P. veneta* (Spies 6327), 6 = *P. pallida* (Spies 6208), 7 = *P. aristifolia* (Spies 6295), 8 = *P. curvifolia* (Spies 6270), 9 = *P. tomentella* (Spies 6337), 10 = *P. rupestris* (Spies 6330), 11 = *P. rupestris* (Spies 6308), 12 = *P. rupestris* (Spies 6310), 13 = *P. currhilosa* (Spies 6226), 14 = *P. pallida* (Spies 6281), 15 = *P. rupestris* (Spies 6309), 16 = *P. calscicola* (Spies 6209), 17 = *P. tortuosa* (Spies 6179), 18 = *P. tomentella* (Spies 6345), 19 = *P. tomentella* (Spies 6277), 20 = *P. tomentella* (Spies 6356), 21 = *P. rigidissima* (Spies 6243), 22 = *P. eriostoma* (Spies 6173), 23 = *P. curvifolia* (Spies 6215), 24 = *P. tomentella* (Spies 6342) and 25 = *P. airoides* (Spies 6205).

Below diagonal: mean divergence % between the species with the Clustal w alignment.

Above diagonal: mean divergence % between the species with Malign.

	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
1		6.58	8.23	5.76		8.23	7.41	7.41	7	7.82	7	6.17	9.47	11.11	10.7	7.41	6.58	6.17	9.88	7.82	9.05	9.47	11.93	14.81	14.81
2	6.86		4.12	4.94	2.88	7.82	5.76	6.18	5.35	5.78	9.05	4.94	8.84	10.7	7.82	4.94	4.53	6.17	6.17	4.53	7.41	4.94	10.7	12.35	9.47
3	9.01	5.15		4.94	7.41	8.2	4.53	7.41	4.12	7.82	11.11	4.12	10.28	12.35	7.82	2.47	2.88	4.94	4.12	1.23	3.7	7.14	10.28	12.76	10.7
4	8.58	6.44	4.29		6.17	7.41	4.53	3.7	4.12	5.76	9.88	3.7	9.05	11.52	8.23	4.12	4.53	6.58	5.76	4.94	6.58	5.76	11.93	12.76	9.88
5	11.15	8.58	6.44	3.43		9.05	7	7	6.58	7	8.84	5.75	10.7	11.93	8.64	7.82	7	6.58	8.23	7	7.82	7	11.93	13.99	11.11
6	11.59	10.3	8.15	5.15	1.72		7.41	7.82	6.17	9.88	8.64	6.58	4.12	7.41	6.17	6.58	7.82	8.64	9.88	8.23	9.05	11.11	12.76	16.05	14.4
7	10.73	8.58	6.44	5.58	4.29	3.43		6.58	2.06	7.41	9.05	2.47	9.47	10.7	7.41	3.29	5.75	6.58	6.17	4.53	5.76	9.05	12.76	14.05	11.93
8	12.02	9.44	6	6.87	7.73	9.01	7.73		4.94	7.82	9.88	5.76	10.29	12.76	8.64	6.58	5.76	7.41	7.41	6.58	7.82	8.23	10.7	13.99	11.59
9	13.73	12.44	10.3	7.29	5.58	4.29	6.87	9.44		7.41	8.64	1.85	8.64	10.7	7	3.29	5.76	6.58	5.76	4.12	4.53	9.47	12.35	13.99	11.93
10	12.45	10.3	8.15	5.15	3.43	3	5.15	6.87	2.58		10.29	7.82	11.11	13.99	10.7	7	6.17	7	6.58	7	8.64	5.35	13.58	11.11	9.88
11	13.73	11.59	9.87	6.87	5.58	6.01	6.44	8.58	8.15	6.44		7.14	9.88	11.93	11.11	10.29	9.88	8.64	12.35	10.7	11.52	13.58	13.17	18.46	15.84
12	14.59	12.45	10.73	7.73	6.44	6.44	6.87	8.58	8.15	6.01	0.88		9.05	10.29	7.82	3.29	5.76	6.17	6.58	4.12	4.94	9.47	12.76	14.81	12.35
13	10.73	9.01	7.29	5.58	3.66	4.29	4.72	5.58	6.01	4.29	2.58	2.58		5.76	4.94	8.64	9.88	9.88	11.93	10.29	11.52	12.35	15.23	17.28	15.64
14	15.88	15.02	13.3	9.87	9.87	9.87	9.01	11.16	12.01	9.87	5.15	5.15	5.58		6.17	10.29	11.93	10.7	13.58	12.35	14.81	15.64	18.11	16.87	
15	14.16	12.02	10.73	6.87	6.44	6.87	6.87	8.58	8.15	6.44	1.72	1.29	2.58	4.72		6.58	8.23	9.05	9.88	8.23	9.47	10.7	12.76	14.81	13.58
16	12.45	10.3	7.29	6.87	8.44	6.87	6.01	6.67	9.01	8.44	5.15	5.15	4.72	8.15	5.58		4.12	4.94	4.94	2.47	4.53	7.82	11.93	13.16	11.11
17	15.88	12.88	11.57	10.3	10.3	10.3	8.58	9.87	11.59	9.01	5.15	5.15	6.01	7.3	4.72	4.72		3.29	5.35	3.29	5.76	6.17	9.47	10.29	9.88
18	17.76	18.03	15.88	14.59	14.16	14.16	13.73	15.45	13.73	13.3	10.73	11.59	9.87	11.16	12.45	12.88	14.59		7.41	5.35	6.58	8.64	8.64	9.88	8.23
19	15.86	16.31	14.59	12.45	11.59	11.59	11.16	13.73	13.73	12.01	9.01	9.44	8.15	9.44	10.3	11.59	12.88	7.72		2.88	7	8.12	12.76	8.64	6.58
20	16.74	15.88	13.73	12.02	11.16	11.16	10.3	13.3	13.73	11.59	7.72	8.15	7.72	5.58	8.15	9.44	10.3	6.44	7.3		4.12	5.76	11.11	10.7	8.23
21	14.59	13.73	9.87	9.87	9.44	9.44	6.87	9.44	11.16	9.44	6.44	6.44	5.58	9.44	7.72	6.87	8.15	11.16	9.44	10.3		10.29	11.93	15.23	13.58
22	12.44	10.73	7.29	6.15	7.73	8.15	8.15	6.87	9.01	6.44	5.58	5.15	3.86	7.72	6.01	5.58	6.87	12.45	11.59	9.01	8.15		12.35	9.05	5.35
23	15.02	14.16	10.73	10.73	10.3	10.73	10.3	9.44	11.59	10.3	8.15	7.72	5.58	10.73	9.01	8.58	9.87	13.3	12.88	12.01	8.15	4.29		13.17	13.58
24	12.88	11.59	10.3	9.01	7.73	0.7	8.58	10.3	9.01	7.72	9.44	9.44	5.58	9.44	8.58	10.3	11.59	11.16	10.73	9.44	11.16	9.01	9.87		7.82
25	14.59	14.59	14.59	14.16	13.3	13.73	13.73	13.3	13.73	12.01	12.01	12.01	8.15	12.45	11.59	10.73	11.16	14.59	14.16	13.73	12.45	9.44	12.01	11.59	



**APPENDIX H:** Cytological, morphological and anatomical data matrix for phylogenetic analysis with PAUP (X = undecided, ? = unknown data).

SPECIES	CYTOGENETICAL	MORPHOLOGICAL	ANATOMICAL
<i>P. airoides</i>	71110000S	X??00101X00?X0111X0X??0	00X00XX1
<i>P. aristifolia</i>	70100000?	1??00101101?20111100??0	00000XX1
<i>P. barbata</i>	70100011?	00?001X1X01221111101??0	00X00XX1
<i>P. colorata</i>	70100000?	0X111001001010211000??X	11X00110
<i>P. curvifolia</i>	71000000S	00100101001011200010001	11X11101
<i>P. densifolia</i>	71000000?	011000X1?01220X11001??1	00000XX1
<i>P. eriostoma</i>	11111000U	00110001001000211011??0	11X11101
<i>P. lima</i>	70000010?	00110000000000201010010	2XX01101
<i>P. pallida</i>	71110000S	00?00101X010X1211X01010	XXXXXXXX1
<i>P. papillosa</i>	71000000?	00?00011X012X121110X??1	00100X01
<i>P. patula</i>	71000000?	1??00101X01?20X11001??0	XXXXXXXXX
<i>P. rigidissima</i>	71010000?	01110000001010211012??0	21X01101
<i>P. rupestris</i>	70000100?	00?00101101020211001??0	00000XX1
<i>P. tomentella</i>	71100000S	0?X00101101000101010??0	00000XX1
<i>P. tortuosa</i>	71000000?	001X1201001010201010??0	11X00110
<i>P. viscidula</i>	70010000S	0??10121X1010121110X??0	11X01011

**APPENDIX I:** List of chromosomal, cytogenetical (A), morphological (B), anatomical (C) characters used during the cladistic analysis and their values.

CHARACTER	ALLOCATED VALUE
<b>A:</b>	
Basic chromosome number	7 or 1=13
Diploidy (2x)	0 = absent, 1 = present
Tetraploidy (4x)	0 = absent, 1 = present
Hexaploidy (6x)	0 = absent, 1 = present
Heptaploidy (7x)	0 = absent, 1 = present
Octaploidy (8x)	0 = absent, 1 = present
12-ploid (12x)	0 = absent, 1 = present
13-ploid (13x)	0 = absent, 1 = present
Reproduction	U = asexual, S = sexual
<b>B:</b>	
<b>Habitat:</b>	
Perennial or annual	0,1
Caespitose or cushion forming	0,1
Roots	1 = rhizome, 1 = stolon
<b>Leaves:</b>	
Expanded or rolled	0,1
Linear or lorate	0,1
Rigid or flacid	0,1
Basal, cauline or radical	0,1,2
Pungent or not	0,1
Glabrous or villous	0,1
<b>Lemmas:</b>	
Awned or not	0,1
Glabrous, villous or puberulous	0,1,2
<b>Leave sheath:</b>	
White, brown and burnt or decaying	0,1,2
Sheath mouth	0 = villous, 1 = glabrous, 2 = bristles
<b>Pedicels:</b>	
Length in relation to spikelet	0 = longer, 1 = shorter
<b>Glumes:</b>	
Obstuse, acute or acuminate	0,1,2
White-Yellow or Green-Purple	0,1
Villous or glabrous	0,1
<b>Lateral awns:</b>	
Included or extended from glumes	0,1
<b>Inflorescence:</b>	
Open or contracted	0,1
Nodes glabrous, villous or puberulous	0,1,2
<b>Awns:</b>	
Geniculate or straight	0,1
Spreading or erect	0,1

Culms:	
Simple or branching	0,1
C:	
Anatomical type	0 = mesic, 1 = sclerophyllous, 2 = intermediate
Glands	0 = present, 1 = absent
Foliar gland type	0 = stalked, 1 = sessile
Epidermal zonation	0 = differentiated, 1 = undeveloped
Shape of abaxial silica bodies	0 = dumbbell, 1 = elliptical
Abaxial stomata	0 = present, 1 = absent
Adaxial prickles	0 = present, 1 = absent
Adaxial papillae	0 = present, 1 = absent

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