GENETIC VARIABILITY IN THE SOLANUM NIGRUM L. COMPLEX AND RELATED SPECIES IN SOUTH AFRICA

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I wish to dedicate this work to my children,

Amy and Joshua.

Have a dream and know it can come true if you work hard for it

Love Ma.

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List of Abbreviations

ABB	abbreviation
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
bp	base pair(s)
BSA	bovine serum albumin
CTAB	cetyl triethyl ammonium bromide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DOC	sodium deoxycholate
EDTA	ethyl diamine tetra acetic acid
FAO	UN Food and Agriculture Organization
kb	kilobases (1kb=10^3 base-pairs)
LSD	least significant difference
M	molar
mM	millimolar
m/v	mass per volume
NCSS	number cruncher statistical systems
ng	nanogram
nm	nanometer
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PVP	polyvinylpurrolidone
r	correlation coefficient
RAPD	randomly amplified polymorphic DNA
RCBD	analysis for balanced randomized complete block design
RFLP	restriction fragment length polymorphism
rpm	rotation per minute
SD	standard deviation
SDS	sodium dodecyl sulfate
Sp.	species
SSR	simple sequence repeats

TAE	tris-acetate-EDTA buffer
T*aq*	*Thermus aquaticus*
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)-aminoethane
TE	tris-EDTA buffer
TSS	total soluble solids
U	units
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
µg	microgram
µl	microlitre

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Chapter 1
Introduction

1.1 General introduction

More than 4 million people in Southern Africa are threatened by serious food shortages, according to a report released by the UN Food and Agriculture Organization (FAO, 2002). This is largely due to declining food production caused by prolonged dry spells, floods and disruption of farming activities.

Poverty-wracked Africa should rally around making better use of its bio-diversity and by encouraging biotechnology. The African continent has an abundance of natural resources, which simply does not exist in the context of international economies (Kilama, 2002). There are numerous areas where the continent could cash in on its bio-diversity and Africans could benefit from their indigenous knowledge of among other things their medicinal plants. Kilama (2002) said that traditionally, Africa ignored the ingenuity of its local communities. They could provide ideas, which can lead to useful products for Africa and the world. He said Africa also rated its best food crops poorly. It will be sad if 10 years from now, inhabitants of Africa have done nothing positive.

Recent studies on subsistence farming societies in Africa, indicate that *Solanum nigrum* L. and it's related species play a significant role in nutrition and food supply, as well as generating an income (Edmonds and Chweya, 1997). In Sub-Saharan Africa, the nightshade species form an important part of farming and consumption systems. Several wild species are occasionally collected but there are only four species frequently cultivated as a vegetable (Schippers, 2000). It is, therefore worthwhile to note that the incorporation or maintenance of edible wild and noncultivated plant resources could be beneficial to nutritionally marginal populations, or to certain vulnerable groups within populations, in developing African countries (Edmonds and Chweya, 1997).

The Solanaceae, to which the genus *Solanum* belongs, is of great economic importance, containing many essential vegetables, fruits and tubers (aubergine, paprika, chilies and green, red and yellow peppers, tomatoes, Cape gooseberries and potatoes). Other species in this family are ornamentals such as *Petunia*, *Schizanthus* and *Lycium* species

(Bukenya and Hall, 1988; Edmonds and Chweya, 1997). Several species such as potato, tomato, peppers and tobacco have served well in pioneering biotechnology, genetic engineering and genetic analysis (Hadkins *et al.*, 1995). The genus also contains plants of medicinal value. The fruits of *S. anguivi* Lam. for example, contain alkaloids used in the treatment of a number of diseases, including chronic respiratory disease (Bector *et al.*, 1971). Unfortunately all solanums share the nightshade family's reputation for narcotic or toxic qualities (Henning, 1995).

The limited information available on many important and frequently basic aspects of neglected and under-utilised crops such as black nightshade hinders their development and their sustainable conservation. Edmonds and Chweya (1997), stated that one major factor hampering this development is that the information available on germplasm is scattered and not readily accessible. Existing knowledge on the genetic potential of these crops is limited.

African nightshades are amongst the most common and popular leafy vegetables found in the warmer and humid zones of Africa. Yet, this species group has received only minimal attention and virtually no research has taken place to further advance their potential as a valuable food crop. Schippers (2000) believes that the main reason for this is the misnomer "black nightshade" which is associated with the deadly nightshades, a poisonous plant species found in Europe.

In Australia, black nightshades are considered to be weeds but in the Pacific, Asia and to a lesser extent the Americas, they are minor food plants (Bassett and Munro, 1985). *S. retroflexum* is the most commonly occurring species of the *Solanum* group in Southern Africa. The leaves are cooked and eaten as a vegetable by the local people of Kwa-Zulu Natal and Lesotho. The leaves of *S. nigrum* are also used as a potherb. The indigenous people of Lesotho and the Free State eat the boiled leaves or the whole young plant as a relish with cereals. The ripe fruits are also eaten fresh or used in cooking and preserves. The fruit has a remarkable flavour, leaving an unusual taste in the mouth. The plants and fruit are also used in traditional medicine (Fox and Young, 1982).

To our knowledge, no cultivars have yet been developed through conventional plant breeding methods. Berries have not yet impacted largely on the South African fresh fruit market. It is hoped that this study will also serve to highlight the viability of improved cultivars of black nightshade berries as an alternate food crop in developing African countries and as a niche market crop in Southern Africa (yogurts, preserves, liqueurs etc.).

1.2 Aim of this study

The aim of this study was to study the *S. nigrum* complex by:

1. Conducting morphological trials on parental species and the progeny to assess diversity between the accessions, probable genetic variability and heterosis in the progeny as well as correlation and to identify desirable characters that can be used in further breeding studies.
2. Identifying the species correctly with the help of cytogenetics and investigate the crossability of the species.
3. Employing the Amplified Fragment Length Polymorphism (AFLP) fingerprinting technique and Simple Sequence Repeats (SSR) to identify and determine genetic relationships between the different species.
4. Performing biochemical analysis on the parental material to establish more precise information on differences between parental species and its nutritional value.

Chapter 2
Literature review

2.1 Introduction

African nightshades are the most important leaf crop in several places in Africa, surpassing cabbages and kale (Schippers, 2000). In 1969, Keller *et al.* already stated that edible wild leafy vegetables have an important role to play in African agricultural and nutritional systems. Owing to the lack of documentation of their total yield and sales, such traditional leafy vegetables have been regarded as minor crops and have been given low priority in most agronomic research and development programmes (Brown, 1983; Ruberté, 1984; Brush, 1986; Altieri and Merrick, 1987; Prescott-Allen and Prescott-Allen, 1990).

Little is known about the nightshades indigenous knowledge of the utilization, cultivation techniques, and structure of genetic variation and potential for crop improvement through domestication, selection and/ or breeding (Edmonds and Chweya, 1997). *Solanum nigrum* L. and related species occur world-wide as weeds, in moderately light and warm environments, particularly on soils rich in nitrogen, such as arable land, gardens, rubbish dumps and occur from sea to mountain levels. They are, however, also widely used as leafy herbs and vegetables, as a source of fruit and for various medicinal purposes. Unfortunately, there is also widespread confusion over the identification of the taxa involved, especially in those areas where the species are most commonly used as food source.

2.2 Origin

The family Solanaceae contains approximately 2300 species grouped into 96 genera and three subfamilies (D'Arcy, 1991). In Africa, Solanaceae is genus poor, with only 10 native genera in all, but in the genus *Solanum* there is ample diversity (D'Arcy, 1986). In this genus, there are about 1700 species world-wide (Heywood, 1978). One of the most widespread and variable species groups of the genus *Solanum*, is that contained in the section *Solanum*, centering around the type species *Solanum nigrum* L., the black nightshade (Edmonds, 1972).

The genus *Solanum* is widely distributed throughout the tropical and temperate regions of the world, with centres in Australia, Central and South America. Its great concentration in South America has led to the hypothesis that the family may have originated in that subcontinent (Heywood, 1978; Bassett and Munro, 1985). *Solanum nigrum* was probably introduced to America from Europe in the 1800's (Fuller and Mc Clintock, 1986). Thanks to human migration and simpler ways to travel, man has spread a lot of them, desirable or undesirable (as weeds), throughout the world. The modern distribution area of many *Solanum* species goes far beyond their original centre of origin (Daunay *et al.*, 1995). Majek (1981) stated that nightshades could be found in 73 countries of the world. Jaeger and Hepper (1986) estimated the number of *Solanum* species in Africa and its adjacent islands to be about 110.

Over 20 of the species of *Solanum* growing in Africa are not considered native to the continent (Jaeger and Hepper, 1986). Several of these are obviously intentional human introductions, such as those with nutritional or ornamental value. Food plants include the potato, *S. tuberosum* L., which is quite widely grown, having been known in Africa at least since the early nineteenth century (Correll, 1962). The pepino, *S. muricatum* Ait., is occasionally cultivated in Northeast Africa. Introduced ornamental species include *S. seaforthianum* Andr., *S. jasminoides* Paxton, *S. pseudocapsicum* L., *S. wendlandii* Hook.f., *S. capsicoides* Allioni, *S. mammosum* L. and *S. robustum* Wendl. (Jaeger and Hepper, 1986).

The National Botanical Institute in Pretoria (South Africa) stated that there are eight species in South Africa that belong to the *Solanum nigrum* complex, they are:

- *S. burbankii* Bitter
- *S. chenopodioides* Lam.
- *S. melanocerasum* All.
- *S. nigrum* L.
- *S. nodiflorum* Jacq.
- *S. retroflexum* Dun.
- *S. sarrachoides* Sendtn.
- *S. villosum* Mill. (Me. M. Welman-personnel comments).

Several wild species are occasionally collected in Africa but only four are frequently cultivated as a leafy vegetable i.e.

- *S. americanum* Mill.
- *S. eldoretii*
- *S. scabrum* Mill.
- *S. villosum* Mill. (Schippers, 2000).

2.3 Black nightshade, related and other species as food source and other uses

In parts of Africa, the ripe berries, especially the orange or red forms from *S. villosum* Mill, are frequently eaten raw as fruits. The black nightshade fruits are also widely used in pies and preserves and Simms (1997) reported that British-grown berries are particularly suitable for mixing with less colourful fruits such as apples. In North America, the fruits are occasionally used as a substitute for raisins in plum puddings. Fisher (1977) also reported that after the berries are washed and drained, they freeze well for winter pies. The fruits can also be used to make a jam (Palmer, 1985) eaten with bread and butter. People in Uganda occasionally use the dark-purple or black fruit as a source of ink (Schippers, 2000). Tribes of the American Southwest used the berries for tanning leather (Bassett and Munro, 1985).

In Australia, about 15 *Solanum* species are eaten by the Aborigines, such as *S. centrale* Black and *S. chippendalei* Symon (Peterson, 1979). There are several other minor *Solanum* species used for food, medicine, and rituals or for other uses, such as *S. torvum* Sw., a rootstock used for eggplant grafting (Jain and Borthakur, 1986; Bukenya, 1994; Daunay *et al.*, 1995).

S. nigrum L. has also been recorded as an ancient famine plant used by the Chinese (Henderson, 1974). In Lesotho and the Free State the Southern Sothos eat the boiled leaves or the whole young plant as a relish with cereals. This is also common among the Swazis and the Zulus. People in Zimbabwe collect the leaves and dry them on flat stones for later use as a boiled relish. The leaves and tender shoots are commonly used as a potherb (Fox and Young, 1982). In 1881, Bailey reported that the herbage of forms of *S. nigrum* was considered to be a very valuable culinary vegetable in Mauritius, and was imported to Australia by immigrants in the 1852 gold rush, for use as a vegetable. In Africa the leaves of *S. scabrum* Mill. are dried for use in soups and sauces during the dry season (Schippers, 2000).

Edmonds and Chweya (1997) found that herbarium material records listed the fruit as being edible "when ripe", "red", "yellow" or "turning purple", suggesting that the local communities eating these fruits know how to avoid potentially dangerous forms. There are many ethnobotanical accounts of the water in which the vegetative parts have been boiled being discarded and replaced several times, or replaced with milk, to prevent the ingestion of toxins. Palmer (1985) stated that black nightshade is one of those extraordinary plants which is poisonous in some parts of the world and not in others. In South Africa, only the green fruits are toxic. Bromilow (1995) reported that the South African green berries are poisonous, and must be cooked before consumption.

Modern chemistry has borne out what the ancients suspected; many nightshades contain various combinations of powerful alkaloid chemicals, including steroidal alkaloids, commonly known as steroids. Overdoses of raw steroidal alkaloids can slow the heart, reduce body temperature, and cause delirium, convulsions and even death (Henning, 1995).

Species cultivated for their drug use include bittersweet (*S. dulcamara* L.) and *S. viarum* Dun, both used as sources of corticosteroids (Edmonds and Chweya, 1997). Researchers also observed that *Solanum* alkaloids have antifungal effects. It is, therefore possible that some of these alkaloids could be used as antibiotics. Beaman-Mbaya and Muhammed (1976) reported that alkaloids from fruit of *S. incanum* L. are used in Kenya in treatment of cutaneous mycotic infections and other pathological conditions.

Solanine is another toxic alkaloid contained by many Solanaceae. The black nightshades contain solanine and it is concentrated in the unripe berry. The sedative aerial parts have a paralysing effect on nerve ends and are used in painkilling ointments (Bremness, 1994). Hyoscine (scopolamine) with sedative properties which is used to control travel sickness, is another alkaloid found in Solanaceae (Hutchings, 1996).

Edmonds and Chweya (1997) refer to medicinal uses being recorded from the earliest times, and especially *S. nigrum* L. being mentioned and often illustrated in many of the early herbals. Among the great British herbals, Gerard's Herbal of 1636 (cited in Edmonds and Chweya, 1997) reported that the "Nightshade is used for those infirmities that need cooling and binding". The black nightshade was commonly used to cool hot inflammations. In Culpeper's Herbal of 1649 (cited in Edmonds and Chweya, 1997) among the soothing effects of the clarified juice of this plant, he mentioned inflamed throats, eye inflammations, shingles, and ringworm, as well as running ulcers, testicular swelling, gout and ear pains. In Asia a diuretic decoction treats fluid retention, eye disease, and infected sores. *S. lyratum* shows strong inhibiting action on cancer cells without affecting normal cells (Bremness, 1994). The whole plant of *S. nigrum* L. is used medicinally, the leaf juice or a leaf decoction for ulcers and skin troubles, the leaves as a dressing or poultice for wounds, parts (details lacking) for convulsions, malaria, dysentery, and the unripe berry as a paste for ringworm (Palmer, 1985). Uses ranging from snakebite remedies to cures for dysentery, toothache, venereal diseases, dandruff, rheumatism and so on is recorded by Jaeger and Hepper (1986).

Other uses of native *Solanum* species include the planting of the large and viciously prickled shrub *S. aculeastrum* Dun. as a hedge and the use of *S. coagulans* Forssk. fruits in tanning leather and coagulating milk (Jaeger and Hepper, 1986).

2.4 Taxonomic aspects

The traditional methods used for identifying different crop plants are based on conventional phenotypic expression of the plant in the field. Many morphological traits belonging to all development stages are required in order to assign an individual to a specific taxon. The *S. nigrum* complex is a group of plants that are difficult to distinguish because of their morphological similarity and a lot of confusion on the taxonomy of the plants exists. Various classical, experimental and numerical studies have demonstrated that the complexity is attributable to a number of causes one been that the boundaries between the species are generally ill-defined (Edmonds 1972, 1977, 1979a).

Most plants in the complex are erect or scrambling herbs with triangular stems, which bear oval, thin, textured, alternate leaves with wavy margins. The tiny white flowers are borne in drooping clusters on lateral stalks between the leaves. The flowers are bisexual, usually regular and composed of five sepals and five petals. The sepals are partly fused and the petals are variously fused, making the corolla round and flat. There are five stamens attached to the corolla tube and alternating with the petal lobes. The anthers are usually touching, but not fused. The anthers split longitudinally at anthesis. The ovary is superior, and consists of two fused carpels with a single style, and usually contains two locules. The style is elongated with a smooth flattened stigma. The fruit contain many seeds, which contain copious endosperm. The indehiscent berry fruit is green when immature, purplish-black when ripe. *S. villosum*'s fruit resemble black nightshade, except that the ripe fruit is an orange-yellow, yellow or red colour (Heywood, 1978).

2.5 Morphological characterization

Morphological characters have long been the means for the plant breeder of studying variability, genetic variation patterns and correlation in populations and accessions of plants. This method involves a lengthy survey of plant growth, which is costly, labour intensive and vulnerable to environmental conditions (Vega, 1993). Morphological data are affected by environmental interaction and descriptions must be made with sufficient replication and valid comparisons are only possible for descriptions taken at the same location during the same season (Smith and Smith, 1988). The management practices and human interpretation also have a strong influence on these phenotypic expressions.

Plants belonging to the *Solanum nigrum* complex display considerable genetic diversity between species, both florally and vegetatively. Among the more common features affected are: stems and leaves, varying from green to purple; stems are smooth to dentate; pubescence from sparse to dense and with glandular- or eglandular-headed trichomes. Leaf margins vary from entire to sinuate-dentate; flowers from white to purple; fruiting pedicels from erect to reflexed; berries from greenish-yellow to purple, or from yellow to orange or red. Features such as plant height and spread, the vegetative vigour, the number of berries per plant, and the number of seeds per berry, however, seem to be phenotypically plastic and dependent on the prevalent growing conditions (Edmonds and Chweya, 1997).

Specific references relating to characteristics like fruit ripening, colour, size and yield were not found. It was considered reasonable to compare black nightshades with statements for tomatoes, due to the fact that both belong in the same family (Solanaceae) and are morphologically similar.

2.5.1 *Fruit ripening*

Temperature has been found to have a profound effect on maturation of fruit (Ho, 1995). In tomato fruit, the onset of ripening is controlled by an increase in ethylene production. The ability to control ripening has interesting potential. To be able to ripen a field simultaneously would greatly reduce field losses and improve processing quality as all fruits could be harvested at optimum ripeness (Stevens, 1994).

2.5.2 *Fruit colour*

Colour is a significant factor in the consumer acceptability of foods. The colour is often directly related to nutritive value and can be correlated with general quality of some processed food products (Joslyn, 1970). For tomatoes it is found that fruit colour is an important quality parameter to the grower as it affects the grade of the fruits (Pomeranz and Meloan, 1994). Recessive genes have a major impact on the fruit colour of tomatoes (Stevens, 1994).

2.5.3 *Fruit size*

Cell number in the ovary determines the potential fruit size, but light, temperature and water relations in the plant can regulate the actual fruit size. Fruit size can also be manipulated by altering the fruit number per plant (Ho, 1995). The actual final size of a tomato fruit is determined by cultivar (i.e. genetic factor) and fruit position in the truss but may be modified by the growing conditions (Ho, 1992).

2.5.4 *Fruit yield*

High yield potential is one of the foremost objectives in many breeding programmes. Number of fruit and mean fruit mass of the fruits are the main components of total yield with the number of fruit being of greater importance than their mass (Yordanov, 1983). High productivity is one of the major goals of breeding, and yield is genetically complex and invariably influenced by environmental factors (Opeña, 1993).

2.6 Agronomy

The characterising of berries in general as crops is:

- It is very labour intensive,
- It yields a high capital return and
- It is a high-value exotic product which can be sold into a growing export market or grown in a home garden for own use.

2.6.1 *Soil*

Generally most soils where cash crops are produced should be adequate. Good drainage is also very important, the crop does not perform well on clay (Mr. P. Mielmann, personal communication). The field should be prepared in advance by adding compost and working it well into the soil. The crop may occupy the field for six months or more, so well fertilised soil is required for optimum growth (Schippers, 2000).

2.6.2 *Cultivation*

The African nightshades produced for subsistence are mainly sown at the beginning of the rainy season. When sown directly, a few (3-10) seeds are sown together in the same location. The strongest plants are kept and the others removed as a first harvest or for planting in a different place. Flowering occurs earlier when the seeds are sown directly, than when seedlings are transplanted (Schippers, 2000).

Seeds can also be sown in seed boxes. Germination normally takes between five and 10 days. The plants stay in the seedbed for about five weeks after sowing. When the seedlings are 12 to 15cm high they are ready for transplanting (Schippers, 2000). Del Monte and Tarquis (1997) stated that the germination behaviour of species of the *S. nigrum* complex varies significantly. The seed population displays a dormancy effect, which is overcome with alternating temperatures. They found in their study that the optimum is 8h at 30°C and 16h at 15°C. Thullen and Keeley (1982) found that black nightshade germinated better in a 13h photoperiod followed by complete darkness and optimum temperatures of 26,7°C to 32,2°C.

One of the reasons farmers experience problems with the germination of seeds is the low vigour caused by inadequate removal of sugars and germination inhibitors present in the fruit. Such germination inhibitors include abscisic acid and ethylene, which normally prevent seeds from germinating within the fruit. To remove those inhibitors and thereby raise the seed vigour, it is recommended that the fermentation process be allowed to take place and that the seeds are properly washed prior to drying. Schippers (2000) also noticed that in Kenya and Nigeria the seeds require manure to germinate well. In Uganda the seeds were found to germinate very well on land where there had been recent fires (such land being rich in plant nutrients such as potash).

In Nigeria, *S. scabrum* Mill. is propagated through stem cuttings. The advantage of this method is that it only takes about three to four weeks before the first harvest of the leaves as vegetables can take place. The total yield is lower than transplanted or directly- sown seedlings and laboratory tests have revealed that the leaves contained more glycoalkaloids than leaves obtained from seedlings (Schippers, 2000).

Generally, berry plants tolerate some shade. Covering, however, is essential to protect crops from birds and to create a microclimate and that contribute to improve growth and crop production.

2.6.3 *Spacing*

The spacing between plants may differ, depending on the species. Most commercial farmers in West Africa grow their dry season crops in rows, facilitating easier irrigation. Spacing for vegetable crops is normally between 12 X 15cm or 30 X 50cm for the larger varieties. A wider spacing will be needed when the crop is to be kept for a long period, as well as for crops used for fruit or seed production. A wider spacing is said to reduce disease pressure, whereas branching is also stronger, compensating for the lower number of plants (Schippers, 2000).

2.6.4 *Irrigation*

Plant roots are sensitive to drought, therefore adequate irrigation is essential just before, and immediately after transplanting. Further irrigation is essential, and adequate soil moisture is needed to achieve optimum growth and yield. In the first week after transplanting, daily watering is needed, especially during the dry season. The irrigation interval can be reduced to three times per week depending on temperatures, cloud cover or possible rains. Overhead irrigation should be avoided because of the potential spread of diseases (Schippers, 2000).

2.6.5 *Fertilizers*

Fertilizers should be incorporated into the soil before sowing or transplanting. Supplementary fertilization is required when the crop remains in the field for a long time, but can be relatively inexpensive when considering the high value of the crop. It is found that nightshades require large amounts of nitrogen and other nutrients and do well in soils rich in organic matter (Schippers, 2000). With organic fertilization, the fruits have a good colour, while chemical fertilizers yield larger fruit with a dark colour, but a bland taste (Mr. P. Mielmann, personal communication). A common practice in Kenya is the use of a foliar spray containing a balanced mixture of both macro and micronutrients (Schippers, 2000).

2.6.6 *Harvesting*

The leafy vegetables are harvested for the first time five weeks after transplanting. *S. scabrum* Mill. stems are cut down to about 15cm from the ground, allowing new side shoots to develop. Further harvests take place at seven to 14 day intervals, on average three to four times per plant, if no additional manure or fertilizers are given. The smaller plant types have a shorter harvest season, because when the plants grow older, their shoots become thinner. Harvesting takes place very early in the morning and the produce is sold the same day (Schippers, 2000).

Fruits can generally be harvested about 35 to 65 days from sowing. They require intensive labour and management during picking and packing for the market.

2.7 Pests and diseases

Insect pests are common to nightshades and the plants are frequently eaten. Ants and/ or flea beetles cause small holes in the leaves. Black aphids are a serious problem, not only sucking the plant's sap and reducing its growth, but also causing the leaves to curl. It affects further growth and makes the leaves less attractive for sale. Caterpillars and occasionally grasshoppers, can be a nuisance. Millipedes and snails have also been reported on plants (Edmonds and Chweya, 1997; Schippers, 2000).

A traditional cure for pests is wood ash spread onto the leaves, but consumers are deterred by the resulting grey colour of the leaves. Insecticides are used, and spraying is carried out once or twice a month depending on the gravity of the infection. Many farmers, however, do not follow the instructions given on the label of the insecticide container, which may require that one week should pass before harvesting can take place. Instead they believe that the effect of chemicals lasts only 24h and will harvest one day after spraying. This lack of knowledge and lack of proper advice have resulted in consumers experiencing stomach complaints or worse, and have given leafy vegetables a bad name (Schippers, 2000).

During heavy rains, diseases can be troublesome, especially when plant spacing is close. A major fungal disease, which is found particularly in *S. scabrum* Mill, is late blight (*Phytophthora infestans).* This causes a greyish rot of leaves and stems and subsequent leaf drop. Early blight, caused by *Altemaria solani* also causes significant problems. Bacterial wilt, caused by *Ralstonia solanacearum* is also a problem, especially when the humidity is high. A 5% plant loss due to this infestation was recorded in Kenya (Schippers, 2000).

Karschon and Horowitz (1985) stated that species related to the black nightshade are frequently associated with a broad spectrum of potentially destructive nematodes and micro-organisms. They serve as altemative hosts and potential disease vectors. Henderson (1974) reported that plants of both *S. americanum* Mill. and *S. nigrum* L. persisted as weeds of cultivation in Australia. They were known to be alternative hosts for insects attacking crops such as tobacco, for plant viruses transmitted by insects, and for pathogenic bacteria attacking commercial strains of ginger.

2.8 Quality and nutritional value

The quality of fresh fruit is complex. It involves not only physical appearance and firmness, but flavour and nutritive value as well (Jones and Scott, 1983). Several studies have been conducted to investigate the nutritive value of the vegetable black nightshade. The leaves provide appreciable amounts of protein and amino acids and minerals including calcium, iron and phosphorus. Vitamins A and C, fat and fibre as well as appreciable amounts of methionine, an amino acid scarce in other vegetables, are found in the leaves (Fortuin and Omta, 1980; FAO, 1988). They also stated that the berries can yield high amounts of iron, calcium and vitamin B. Appreciable amounts of vitamin C and carotene are found in the fruits and seeds (Watt and Breyer-Brandwijk, 1962). The nutrient values depend on many factors, including crop species, whether it was grown during the rainy or dry season, soil fertility and plant age (Chweya, 1997; Schippers, 2000).

2.8.1 *Proteins*

Good nutrition is the cornerstone for survival, health and development of current and succeeding generations. Chweya (1997) found the crude protein nutrient per 100g edible portion of vegetable black nightshade to be between 2.8g and 5.8g. This is much higher than the nutrient value for cabbage (1.19g), peppers (0.67g), blueberries (0.71g) and blackberries (0.69g) (Anonymous, 2002). Schippers (2000) reported that the levels of crude protein and mineral nutrients for vegetable black nightshade were not much affected by drying. He also found that leaves collected during the vegetative stage have a higher protein content than those harvested from flowering onwards.

2.8.2 *Total soluble solid content*

Total Soluble Solids (TSS) is a measurement of the sugar, organic acid and other soluble components in the juice of the fruit. It is measured in terms of percent pure sucrose. TSS can be determined using the Brix hydrometer (measurement of specific gravity) or an Abbe hand refractometer (measure of the refractive index), (Childers and Zutter, 1977).

Few literatures could be found, relating to biochemical analysis of the nightshade complex. Due to the fact that tomatoes also belong within the family Solanaceae, and the morphological similarity to the black nightshades, it is considered reasonable to expect biochemical similarity and to compare the results.

Breeders have spent considerable time and effort trying to breed tomato cultivars with more solids. Success has been minimal, largely due to the complex interactions between the various components of fruits, and between plant and fruit characteristics and composition. Higher solids are not easily attained because high solids are inversely related to other important characteristics (e.g., high yield, concentrated ripening). The best hope for a dramatic improvement in fruit solids is a major gene that will overcome present limitations (Stevens, 1994).

Though there is large genetic variation in soluble solid content of the tomato fruit in wild species, breeders have achieved only limited success, in combining increased levels of soluble solids with high yield in processing cultivars (Berry and Uddin, 1991; Stevens and Rick, 1986). In most circumstances the fruit yield and TSS are inversely related (Stevens, 1986). In general terms the TSS in tomato sap is inversely proportional to the size of the fruit (Hobson, 1995). Stevens and Rick (1986) explained that successful selection for high solid progeny in segregating populations is difficult, because of environmental impact on solid content. They pointed out that the susceptibility to diseases and variation in irrigation and soil texture, which affect the water uptake of the plant, can have a much larger effect on soluble solid content than genotypic variation for fruit solid content.

Kapeliovitch *et al.* (1982) found that the sugar content and acid content of tomatoes increase with ripening. Jones and Scott (1983) as well as Ho and Hewitt (1986) also found that sugar content progressively increases during fruit development and maturation.

2.8.3 *Acids*

The sugar and acid content in the fruit juice of tomatoes mainly determine the basic taste of the fruit. Titratable acidity and pH are both measures of the acid content, and are expected to be highly correlated and inversely related (Hobson and Davies, 1971). However, this relationship has been found to be poor (Paulson and Stevens, 1974). They also stated that it is a complex relationship and is affected by a number of buffers.

2.8.4 *Alkaloids*

An attribute of major importance to the Solanaceae is their content of steroidal alkaloids because few other families have such arrays of these compounds (D'Arcy, 1986). Heywood (1978) stated that steroid alkaloids are characteristic of many *Solanum* species. The alkaloids have been found in more than 350 species and stress or damage to the plants encourages the production. The toxicity arises from impairment of membranes of nerve and muscle cells, often leading to leakage of their contents (D'Arcy, 1986). Thanks to them, they found the fruits and leaves of these plants also have several medicinal properties (Daunay *et al.*, 1995).

The odour in rank-smelling foliage typical in many Solanaceae species, is due to the presence of various alkaloids e.g. scopolamine, nicotine, and atropine. These compounds are toxic to all kinds of organisms, and their hazard to man and livestock is known from early times. The bitterness of the fruit is caused by glycosidal alkaloids. These steroidal compounds disappear or decrease naturally to non-toxic levels in ripe fruits (Daunay *et al.*, 1995).

The effects of solanine poisoning in humans are reported to be nausea, vomiting, diarrhoea, colic, headache, dizziness, loss of speech, fever, sweating and tachycardia, pupil dilation, blindness, mental confusion, convulsions, coma and death (Watt and Breyer-Brandwijk, 1962; Cooper and Johnson, 1984). Even potatoes and tomatoes contain solanine but in very small amounts.

Hyoscyamine, atropine and nicotine occur in different genera throughout the family Solanaceae (Munday, 1988). Various kinds of alkaloids especially the tropane, pyrrolidine and steroid groups occur and the combination of these three types of alkaloid in one family is unique (Henning, 1995). Tropane alkaloids are the most widespread and are known to occur in 21 genera (Hutchings, 1996).

Probably most members of the family Solanaceae should be treated with caution, especially those ones whose toxicity is not known. Fox and Young (1982) warns that there have been cases of death in children after eating some of the berries, probably because they were eaten when green, or like other poisonous plants, they may appear to be harmful to some people and not to others.

2.9 Breeding

2.9.1 *Cytology and polyploidy*

The taxonomic complexity of *Solanum* stems not only from the phenotypic plasticity of many of the species but also hybridization between closely related species as well as the occurrence of polyploidy: confusion is further exacerbate by the large number of specific names that have been applied (Grant, 1971). Identification and inter- relationship of the species can be determined only with the help of cytology, genetics and biosystematics (Beg and Khan, 1989; Singh *et al.*, 1992).

Members of the *Solanum nigrum* complex are highly variable morphologically and constitute a polyploid complex based on x = 12 (Stebbins and Paddock, 1949; Ganapathi and Rao, 1986). Several workers reported that *S. nigrum* L. has three cytotypes (Bhaduri, 1933; Magoon *et al.*, 1962; Tandon and Rao, 1966; Edmonds, 1979a). They are diploid (2n=2x=24), tetraploid (2n=4x=48) and hexaploid (2n=6x=72) (Beg and Khan, 1988; Edmonds and Chweya, 1997). Recent studies have established beyond doubt that the diploid with small bluish-black berries is *S. americanum* Mill., while the tetraploid with orange-red or yellow berries is *S. villosum* Mill. The binomial *S. nigrum* is retained to the hexaploid *S. nigrum* L., which bears large purplish-black berries (Edmonds 1977, 1979b). D'Arcy (1986) stated that there are at least two hexaploids, two tetraploids and probably more than six diploids distributed throughout Africa.

2.9.2 *Pollination*

The plants of the *Solanum nigrum* complex are predominantly self-pollinating (Edmonds and Chweya, 1997) the structure of the flower allows pollen from its own anthers to reach the stigma. This nature in *Solanum* species favours the rapid increase in a population from a few individuals, and confers an evolutionary advantage on these taxa in environments in which populations are frequently destroyed. It therefore partially explains the phenomenal success of the members of this section of *Solanum* as weeds of disturbed habitats, especially in agricultural areas (Rogers and Ogg, 1981). This also explains the high degree of homozygosity and concurrent genetic uniformity of plants both within a population and from generation to generation often encountered within these species (Edmonds and Chweya, 1997).

Some species, however, are highly adapted to cross-pollination. *S. americanum* Mill. notably has styles well beyond the anthers, indicating a higher level of cross-pollination (Schippers, 2000). Some species within the section are also morphologically and physiologically adapted to insect pollination, particularly by bees and Syrphid flies (Edmonds and Chweya, 1997). Buchmann *et al.* (1977) demonstrated that the anthers of the North American diploid *S. douglasii* Dun. are morphologically adapted to such a method of pollination. The flowers, though visually white to pale purple, apparently have a hidden ultraviolet (UV) pattern which changes with the age of the flower. Bees are therefore visually sensitive to the flowers in both visual light and in the UV region of the spectrum.

Cross-pollination will occur when pollen is carried to a flower on a different plant. The most certain way to control pollination in a breeding program is by conducting hand pollination. Although each flower has to be emasculated and pollinated by hand, the effort is profitable because of the large number of seeds contained in each fruit.

Schippers (2000) stated that the lack of a self-incompatibility system, as encountered in all African nightshades, is very useful, since it helps to stabilise any crossing made. After only two or three generations the new population will be sufficiently uniform.

2.9.3 *Hybridization*

Because of man's almost absolute dependence on plants for food, and the pressure on an adequate food supply caused by the constantly increasing population in a world of limited acres, it is important to breed for something "bigger and better" no matter what crop it is. The introduction of hybrid varieties has greatly accelerated plant production for many crop species (Georgiev, 1991). Hybrids allow more rapid utilisation of traits such as disease resistance as well as the exploitation of heterosis for quantitative traits such as quality and yield (Berry and Uddin, 1991).

In general, single-cross hybrids are preferred to double- and triple-cross hybrids in vegetable crops (Kalloo, 1988). Poehlman (1987) reported that single-cross hybrid plants with common parentage would have identical genotypes and appearance, although the plants will be highly heterozygous.

Most taxa belonging to the section *Solanum* can be artificially hybridized with initial success. Genetic breakdown in the F1 or F2 generation, however, usually follows interspecific hybridization (Edmonds and Chweya, 1997). Edmonds (1977) found for both *S. nigrum* L. and *S. villosum* Mill, that crosses within and between the two subspecies of each resulted in morphologically intermediate, extremely vigorous and fertile F1 progeny. Similar successes were recorded within the species *S. chenopodioides* Lam, *S.*

physalifolium var. *nitidibaccatum and S. sarrachoides* Sendtn. (Edmonds, 1977; 1986). However crossings involving variants of the two diploids *S. americanum* Mill. *and S. douglasii* Dun. were not consistently successful.

2.9.4 *Heterosis*

Hybrid vigor, or heterosis is a common phenomenon, it is observed in nearly all F_1 hybrids between parents that are neither closely nor distantly related (Allard, 1960). Heterosis is a function of the degree of dominance and the difference in gene frequency between the parent lines. Yordanov (1983) reported that when suitable pairs with high combining abilities combine, a respective high heterosis effect could be expected. The heterosis effects are normally the highest in the F1 generation and cannot be predicted precisely beforehand.

The high level of autogamy found in some *Solanum* species can be a major asset when attempting to produce F1 hybrids, which are likely to show the heterosis effect. In the F2 and further generations this heterosis effect soon disappears (Omidiji, 1983).

Yordanov (1983) proved that the heterosis effect is higher in tomatoes grown in glasshouses than in the field. He also proved that hybrids endure unfavourable conditions better than the parental cultivars. The environment and stress conditions had a large effect on the combining abilities of the parents, which had a large effect on the heterosis response of the different characteristics measured.

The demand for higher yielding lines with better fruit quality could be addressed by using the heterosis effect in the F1- generation after crossing different lines. Yordanov (1983) points out that heterosis is confirmed more and more as a basic, highly effective breeding method applied in an ever-growing number of agricultural crops. Heterosis as a breeding method offers numerous benefits ranging from early, high-yielding, uniform cultivars which also combines a number of other valuable economic characteristics. The heterosis effect is manifested to a different extent in the individual F1 combinations and cannot be predicted beforehand.

Besides the better yields, hybrid cultivars offer the processing tomato industry other benefits such as better and complex resistance to diseases, early ripeness, uniformity of plants and fruit, improved processing characteristics (solid, colour) and a strong adaptive ability to different environmental conditions (Georgiev, 1991; Boleda, 1992; Stamova *et al.*, 1994).

2.9.5 *Correlation*

The genes of an organism and the environment are mainly responsible for the correlation between characters. In genetic studies, the first problem will always be to distinguish between the genetic and the environmental causes of correlation (Falconer and Mackay, 1996).

Correlation is of interest for three main reasons.

- The genetic causes of correlation through the pleiotropic action of genes – pleiotropy is a common property of major genes, but as yet its effects in quantitative genetics have not been considered.
- The changes brought about by selection – it is important to know how the improvement of one character would cause simultaneous changes in the other characters.
- Natural selection – the relationship between a metric character and fitness is the primary factor that determines the genetic properties of that character in a natural population (Falconer and Mackay, 1996).

The genetic cause of correlation is mainly pleiotropy or closely linked genes. The degree of correlation arising from pleiotropy expresses the extent to which two characters are influenced by the same gene. The correlation resulting from pleiotropy is, however, the overall, or net, effect of all the segregating genes affecting both characters. The environment, however, may be a cause of correlation as far as two characters are influenced by the same differences in environmental conditions. The association between two characters, which can be directly observed, is the phenotypic correlation, or the sum of the genetic and environmental causes of correlation. The genetic correlation is a correlation of the breeding values (Falconer and Mackay, 1996). The correlation observed between characteristics can be negative or positive.

2.10 Genetic markers

The species in the *S. nigrum* complex are notoriously difficult to classify despite having been the subject of frequent study since the eighteenth century. Edmonds (1972) stated that no satisfactory taxonomic treatment has yet been devised. In comparison with morphological markers, deoxyribonucleic acid (DNA) markers offer significant advantage with respect to increased numbers of loci detectable, overall phenotypic neutrality and the ability to score the plant at any developmental stage (Prabhu *et al.*, 1997). Perhaps the use of DNA fingerprinting techniques will contribute greatly to solving this problem once and for all.

The number of advantages marker assisted breeding have over morphological markers include:

- Genotype is readily determined by evaluating appropriate tissue.
- They are usually phenotypically neutral.
- They are dominant or co-dominant, allowing all possible genotypes to be distinguished.
- Epistatic and pleiotropic effects are uncommon (Tanksley, 1983).

In breeding programmes, information on genetic relationships within species is used for organising germplasm collections, identifying heterotic groups and selecting breeding material. Plant breeders face the difficult task of having to select for traits, which are often under complex genetic control and subject to environmental changes. Advances in molecular biology have provided new methodologies, which expand the list of useful genetic markers (Paterson *et al.*, 1991). DNA markers thus provide an opportunity to detect, monitor and manipulate genetic variation more precisely than what is possible with morphologic and biochemical markers (Yamamoto *et al.*, 1994).

2.10.1 *DNA extraction*

The primary objective of the isolation process is to recover the maximum yield of high molecular weight DNA, devoid of protein and other restriction enzyme inhibitors. Any part of a plant can be used to extract the DNA (Sambrook *et al.*, 1989). The most common starting material is young leaves. The leaves can be either fresh, lyophilised, dried in an oven, or in some cases dried at room temperature (Kochert, 1994). Several methods for

DNA extraction have been developed (Murray and Thompson, 1980; Dellaporta *et al.*, 1983; Tai and Tanksley, 1990; Edwards *et al.*, 1991; Lange *et al.*, 1998).

The common methods all have the same goals of simplicity, speed and utilisation of a small amount of starting material (Lamalay *et al.*, 1990). The plant DNA needs to be pure enough so that it will digest reproducibly with restriction enzymes and the resulting preparations can be satisfactorily separated by gel electrophoresis. Pich and Schubert (1993) stated that the preparation of high quality DNA from polyphenol-containing plants, such as tomato (*Lycopersicon esculentum* Mill.) and potato (*Solanum tuberosum* L.) was difficult. The greatest problems in those plants are probably because of DNA degradation, caused by carbohydrates, glycoproteins and secondary plant products such as phenolics, terpenoids and tannins, which may bind to DNA after cell lysis (Pich and Schubert, 1993) which, tend to tonic-purify the plant DNA and prevent proper digestion (Kochert, 1994).

The extraction procedure for plant genomic DNA consists of grinding plant tissue into a powder using either mortar and pestle in liquid nitrogen or a mechanical grinder (Kochert, 1994). This disrupts plant cell walls and cell membranes to release the cell constituents into an extraction buffer, which contains compounds to protect the DNA from the activity of endogenous nucleases (Yu and Pauls, 1994). Different sorts of extraction buffers can be used, but most of them have to maintain a pH at around 8.0. Salts, such as sodium dodecyl sulfate (SDS) have to solubilise the plant membranes and provide the means to rapidly inactivate deoxyribonuclease (DNase). Since DNase requires magnesium ions for activity, ethyl diamine tetra acetic acid (EDTA) is often added to sequester the magnesium ions. Some protein denaturants, such as phenol, chloroform or urea may also be used. Any added detergents or incubation of extracts at elevated temperature will also aid in the inactivation of DNase (Kochert, 1994).

2.10.2 *DNA concentration*

The concentration of DNA solutions often requires adjusting to facilitate efficient handling. Concentrated solutions are viscous and difficult to pipette, this is remedied by adding low-TE buffer or distilled water. The degree of DNA degradation, as evidenced by long strands sheared or digested into smaller pieces, can be estimated by electrophoresis of the sample in an agarose gel. Large molecular weight DNA appears as a band, whereas partially degraded material forms a long smear of large to small fragments. DNA degraded to fragments a few hundred base pairs (bp) or less in length appears as a diffuse spot near the dye fragment. Slight degradation may be difficult to detect and appears only as slightly increased orange-red shading ahead of the main band of high molecular weight DNA. The concentration of fragments of known size after DNA is digested with specific restriction enzymes and hybridized with specific probes also provides an indication of degradation (Kirby, 1992).

2.10.3 *Restriction enzyme*

In 1970, Hamilton Smith identified the first restriction enzyme, Hind II. This discovery was a key factor in the later development of recombinant DNA techniques. Hundreds of endonucleases have been isolated from more than 200 different bacterial species. Each is symbolised by the bacterium of origin and a Roman numeral indicating the series number of the enzyme from the organism. The recognition sites are palindromic: the order of the bases in a segment of one DNA strand is the reverse of that in the complementary strand. The lengths also vary, with 4- and 6- bp sequences being relatively common. The complementary strand cleavage sites may be staggered, as with Eco RI, and form sticky ends, or the opposite, as in Hae III, that forms blunt ends (Kirby, 1992).

Each different restriction enzyme recognises a specific and characteristic nucleotide sequence. Even a single nucleotide alteration can create or destroy a restriction site. These enzymes cut DNA at restriction sites. There is variation – or polymorphism – between individuals in the positions of cutting sites and the lengths of DNA between them, resulting in fragments of different sizes. The range of fragment lengths will be different for different enzymes for example a six-base cutter will generate fewer, and on average larger-sized, fragments than a four-base cutter (Jones *et al.*, 1997b).

The choice of enzyme, according to Kirby (1992) is accomplished by trial and error or by knowledge of the base sequence of the fragment flanking regions. The optimum reaction conditions vary for each enzyme, and this information is provided by the suppliers. The critical features are the digestion temperature and buffer salt concentration. One unit of enzyme is the amount required to digest 1μg of λ DNA in 1h. To ensure complete digestion, both enzyme concentration and reaction time are usually increased. A DNA specimen can be cleaved with more than one enzyme, provided the required buffer salt concentration and reaction temperatures for both enzymes are similar. The reactions are incubated, one-fifth or greater (by volume) of stop/ loading dye is added to stop the reactions and prepare the digest for electrophoresis. Before stopping the reaction, it is prudent to remove an aliquot of the reaction mix for electrophoresis on a mini-gel to ensure that complete DNA digestion has occurred (Kirby, 1992).

2.10.4 *DNA amplification – polymerase chain reaction technique*

Amplification of DNA may be necessary to increase the quantity of sample available for profiling, to reduce the analysis time, or to produce probes for the hybridisation process (Marx, 1988; Mullis 1990). Stretches of nucleotides up to at least 3 000 bp from any DNA containing sample may be efficiently amplified by the polymerase chain reaction (PCR). Microgram quantities of DNA can be produced *in vitro* in only a few hours, by the amplification of picogram starting amounts. Amplified material can also be directly sequenced (Kirby, 1992).

Availability of oligonucleotide primers is the key to the amplification process. One primer is annealed to the flanking end of each DNA target sequence's complementary strand; the thermally stable Taq polymerase is added to mediate the extension. The system requires a reaction buffer, the nucleotides dATP, dCTP, dGTP and dTTP, and a means of thermal cycling of the reagent mix. Twenty-five or more amplification cycles can be performed. Each cycle consists of template denaturation, primer annealing and extension of the region between the primers. Synthesis proceeds across the target sequences flanked by the primers, with the extension products of one primer acting as a template for the other primer. The amount of DNA synthesised in each successive cycle is doubled, resulting in an exponential accumulation (2^n where n = number of cycles). Temperatures up to 95°C are used in the reaction. This is made practical by the use of the highly thermally stable Taq polymerase from the thermophilic bacterium *Thermus aquaticus* (Kirby, 1992).

Since PCR is an extension system, short stretches of template-flanking base sequence must be known, with this information oligonucleotide primers can be synthesised. This requirement limits the universal application of the system at present. However, with the rapid progress in base sequence determination for many animal and plant genomes it is anticipated that this limitation will be short-lived (Kirby, 1992).

During the past few years, the use of the PCR technique has significantly increased the application of DNA markers to genotyping, genome mapping and phylogenetics. DNA-based techniques are more reliable than morphological markers and are an environmentally neutral alternative to detect genetic polymorphisms useful for identification (Kirby, 1992).

2.10.5 *Concept of polymorphism*

Polymorphism refers to different forms of the same basic structure. If modifications of a gene exist at a specific locus in a population, the locus is polymorphic. At the molecular level, polymorphism ranges from a single nucleotide base change to the number of tandem repeats in a repetitive DNA sequence. The changes may be neutral, with no detectable phenotypic effect, or they may result in the production of different forms of the same enzyme (isozymes) active under different environmental conditions, such as pH or temperature. If a specific recognition base sequence is present, the restriction enzyme recognising that site will cleave the DNA molecule and result in fragments of specific base pair lengths. If the site is absent, a different length DNA fragment will be produced (Kirby, 1992).

A survey of genetic relationships using molecular markers provides polymorphism information about a germplasm pool, which is useful for developing, mapping and breeding populations (Beer *et al.*, 1997). The polymorphism information is also useful for selecting parents to be used in a breeding program.

2.10.6 *DNA fingerprinting methods*

The foundation of recombinant DNA analysis was established with the hall mark observation by Wyman and White (1980) of a polymorphic DNA locus, characterised by a number of variable-length restriction fragments called restriction fragment length polymorphism's (RFLPs). The history of DNA fingerprinting as such, is even more recent, dating from 1985. When DNA is isolated, cleaved with a specific enzyme, and hybridized under low-stringency conditions with a probe consisting of the core repeat, a complex ladder of DNA fragments is detected. These profiles appear to be unique for each individual or plant. Other factors favouring DNA analysis include the small sample requirement, the ability to rapidly replicate a sequence a million fold or more *in vitro*, and the relative stability of DNA (Kirby, 1992).

DNA fingerprinting is a technique, which has been widely adopted in order to differentiate among organisms at the species and subspecies levels (Mc Clean *et al.*, 1994). Compared with morphological and biochemical characteristics, the DNA genome provides a significantly more powerful source of genetic polymorphism (Beckmann and Soller, 1986). The advantage of DNA fingerprinting over morphological markers, are the dominance and the absence of pleiotropic effects. The DNA fingerprinting applications are theoretically unlimited and their detection is independent of the plant development (Philipp *et al.*, 1994).

The fingerprints are banding patterns of DNA fragments, which are generated in various ways and then separated by electrophoresis. The DNA fingerprints for closely related organisms are generally very similar. Differences in DNA sequence are observed as the presence or absence of bands. These differences are characteristic and heritable.

In traditional breeding programmes, even when a useful morphological marker is identified, the process of using it can be time consuming and quite costly. Typically, an entire field of a particular strain of crop must be grown and analysed at each step of the selection process. The task of examining the myriad individual plants, to identify the presence or absence of the marker is arduous and time-consuming. Consequently, breeders are abandoning this traditional approach in favour of the much faster, more highly discriminating, and less costly approach of using molecular DNA markers such as RFLPs (restriction fragment length polymorphisms), RAPDs (randomly amplified polymorphic DNAs), SSRs (simple sequence repeats) or AFLPs (amplified fragment length polymorphism).

2.10.6.1 *Amplified fragment length polymorphism (AFLP)*

Molecular DNA markers such as AFLPs offer numerous advantages over markers traditionally used in plant mapping and selective breeding. In the past, breeders have selected among closely related strains based on morphological markers e.g. size and colour, etc. which are readily observable and are inherited with the desired trait e.g. increased yield. Because of the scarcity of such markers, this approach often fails to discriminate between closely related lines, which differ for the trait of interest.

The AFLP technique was developed in 1993 (Vos *et al.*, 1995). Its advantage over other DNA marker techniques includes the detection of a large number of polymorphisms from a single PCR, thus reducing expenses (van Toai *et al.*, 1997).

The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The technique involves three steps:

- restriction of the genomic DNA and ligation of oligonucleotide adaptors,
- selective amplification of a subset of all the fragments in the total digest, and
- gel-based analysis of the amplified fragments.

In this technique, specific double-stranded DNA adapters are ligated to the DNA restriction fragments, so that the sequence of the adapters and the adjacent restriction sites serve as primer-binding sites. The primers are designed to contain the sequences that are complementary to those of adapters and the restriction sites, along with one to three selective bases added at their 3' ends. The use of selective bases allows amplification of only a subset of the restriction fragments, which still generates a large number of bands facilitating the detection of polymorphism (Vos *et al.*, 1995).

The AFLP approach is particularly powerful because it requires no prior sequence characterization of the target genome, and it is readily applicable to a variety of crops. Additionally, it is easily standardised and readily automated for high-throughput application. AFLP technology offers the fastest, most reproducible, and most cost-effective way to generate high-density genetic maps for marker-assisted selection of desirable traits. It is also the ideal tool for determining varietal identity and assessing trueness to type.

This DNA fingerprinting technique (AFLP), detects mostly dominant markers based on the PCR amplification of the genomic restriction fragments (Kiem *et al.*, 1995). AFLP markers combine the best characteristics of the other DNA markers while avoiding their disadvantages. Unlike RFLPs, AFLP technology is PCR-based, requires only minimal amounts of starting DNA, and is readily automatable. Unlike RAPD, AFLP markers have proven to be robust, reliable and reproducible. And, unlike SSRs, AFLP analysis requires no prior sequence knowledge of the target genome and, therefore has no up front characterization costs.

Although some concerns have been raised about the clustering of AFLP markers (Kiem *et al.*, 1995) advantages of the AFLP procedure include the inherent simplicity associated with markers, based on the polymorphisms detected per primer pair. With a small number of primer pairs, it should be possible to assess with adequate precision and reasonable cost the parental genetic contribution to subsequent progeny generations. AFLP markers may also allow breeders to follow changes, which result from selection, genetic drift, seed mixture as well as outcrossing (Van Toai *et al.*, 1997).

The AFLP technique has been shown to be highly effective for detecting large numbers of polymorphisms in species like tomato (Thomas *et al.*, 1995), potato (Van Eck *et al.*, 1995) and many more.

2.10.6.2 *Simple sequence repeats (SSR)*

Simple sequence repeats (SSR), also called microsatellites, are among the most variable types of tandemly repeated DNA. The fragment polymorphism here relates to total sequence length, as determined by the number of repeat units, and the heterozygote for different fragments in diploid genomes can generally be distinguished (Mc Gregor *et al.*, 2000). The core sequences are regions of short, tandemly repeated DNA motifs and consist of a two to eight bp repeat unit with an overall length in the order of tens of bp, which are found dispersed throughout the genomes of all eukaryotes (Hamada *et al.*, 1982; Tautz and Rentz, 1984). Some core sequences, like GATA, GACA and GGAT, show extensive RFLPs in various eukaryotes, making them suitable for characterization of individuals or varieties (Aggarwal *et al.*, 1994; Vosman *et al.*, 1992; Weising *et al.*, 1995).

Microsatellites are becoming important genetic markers in plants. The main reason for this is that they are co- dominant markers and this is a PCR- based technique, amenable to automation and thus permits analysis of large populations in a relatively short time. Microsatellites as molecular markers are highly polymorphic and generally evenly spread throughout a genome (Areshchenkova and Ganal, 1999). Most importantly, microsatellite markers are multiallelic and detect a much higher level of DNA polymorphism than any other known marker system (Rafalski and Tingey, 1993). The products generated have been found to be highly reproducible (Jones *et al.*, 1997a) and the polymorphisms can easily be detected both by southern hybridisation and by PCR (Arens *et al.*, 1995a).

Although these markers are usually species specific, costly to develop, and prior sequence information is a requirement, once the primers have been developed the system becomes relatively inexpensive (Mc Gregor *et al.*, 2000).

For their use in genetic mapping experiments, it is necessary that microsatellite markers cover the entire genome of a given organism evenly. Unfortunately, for a limited number of plant species, sufficient microsatellite markers have been mapped, to confirm that microsatellites are indeed randomly distributed throughout a genome. In the cases of wheat, barley, maize and rice, microsatellite markers are indeed nearly randomly distributed throughout the genome, based on genetic and physical data (Areshchenkova and Ganal, 1999).

Mapping of GATA- and GACA- containing microsatellite loci in tomato showed that these were not randomly distributed throughout the genome, but were often clustered in the same chromosomal regions, most likely at, or closely linked to the centromeres (Arens *et al.*, 1995b; Broun and Tanksley, 1996; Areshchenkova and Ganal, 1999). Areshchenkova and Ganal (1999) isolated tomato microsatellites containing long arrays (>20 repeats) of the dinucleotide motifs GA, GT, AT, as well as GATA, assessed their variability within *Lycopersicon esculentum* varieties and mapped them onto a genetic map of tomato. The investigated microsatellite markers exhibited between one and five alleles in a diverse set of *L. esculentum* lines.

In recent years, considerable efforts have been undertaken to establish sets of microsatellite markers in important plants such as soybean (Akkaya *et al.*, 1992; Powell *et al.*, 1996), *Arabidopsis* (Bell and Ecker, 1994), wheat (Röder *et al.*, 1995, 1998; Bryan *et al.*, 1997), barley (Liu *et al.*, 1996; Russell *et al.*, 1997), maize (Taramino and Tingey, 1996), rice (Chen *et al.*, 1997), tomato (Smulders *et al.*, 1997), potato (Provan *et al.*, 1996) sunflower (Brunel, 1994) and tropical trees (Condit and Hubbell, 1991) grapevine (Thomas and Scott, 1993) and other. Considerable numbers of markers have been developed to date and used successfully to assess genetic diversity in germplasm.

Chapter 3
Morphological characterization of black nightshade and related species.

3.1 Introduction

The genus *Solanum,* contained in the section Solanum, is composed of a large number of similar species (Edmonds, 1972). The leaves and ripe or unripe fruits of *S. nigrum* L., *S. retroflexum* Dun., *S. scabrum* Mill. and *S. villosum* Mill., are widely used in several tropical and temperate countries of America, Africa and Asia (Heiser, 1969; Rose and Guillarmod, 1974; Fox and Young, 1982; Gbile and Adesina, 1988). The berries are also used in the preparation of preserves (Edmonds, 1972; Palmer, 1985). Many other African *Solanum* species are used medicinally by the local people (Jaeger and Hepper, 1986).

Edmonds and Chweya (1997) stated that no collecting expeditions have been organized, largely because the black nightshades are regarded as weeds or volunteer crops and have not been completely domesticated. In Africa, some local collections are being made, but no records are available on the number of species so far involved. Since many of these species have almost certainly been incorrectly identified, their usefulness as germplasm collections is virtually negligible.

Morphological characters have long been the traditional means of studying genetic variability in plant species, confirming the identity of the plant. Morphological characterization involves the observation, recording and analysis of a number of morphological characters of seed and/ or the growing plant (Mc Donald, 1991). These characters normally include growth habit, branching type, leaf shape and size, floral characters, fruit and seed morphology, and fertility traits. Such characteristics are often controlled by multiple genes and are subject in varying degrees to environmental conditions. Natural specific hybridization and introgression occurring in plants can have an impact upon the accuracy of identifying the plants correctly.

Cultivation practices, however, have a strong influence on the phenotypic expression of the species. Another problem for the plant breeder is the subjective nature of the evaluation of characters, as they are subject to human interpretation. For these reasons, there are limitations on the use of morphological characters. Furthermore, these methods involve a lengthy survey of plant growth, which is costly, labour intensive and susceptible to environmental variations. It is, however, very important to determine the diversity in plants in order to identify potential breeding material. Plant breeders use the knowledge about relationships between species and the genetic diversity in available germplasm when starting a new breeding program and to choose parents for crosses.

Extensive work was done in various morphological characterizing studies, which are reported in Edmonds (1972; 1982; 1983; 1984).

The objectives of this study were to determine and study the morphological characters of the species belonging to the *Solanum nigrum* complex available in South Africa and to analyze the morphological data obtained, and to determine the difference between the species by a dendrogram and select parents to use in a progeny study.

3.2 Materials and methods

3.2.1 *Sources of germplasm*

Seeds of six *Solanum* species and a synthetic hybrid were obtained from Dr. H.J. Meyer at the University of the North on the Qwa-qwa (UniQwa) campus, Phuthaditjaba. The gene bank in Pretoria supplied four different seed accessions. The National Botanical Institute – Kirstenbosch, Cape Town donated one seed accession. A *Solanum* species was collected from the botanical garden of the Department of Plant Sciences at the Free State University (UFS), Bloemfontein.

The accessions obtained from the sources mentioned, with abbreviations used in the study are listed in Table 3.1.

Table 3.1 Accessions and sources of Solanums, obtained with abbreviations used.

ACCESSIONS	ABB.	SOURCE
Solanum americanum Mill.	AME	University of the North - Qwa-qwa
Solanum chenopodioides Lam.	CHE1	University of the North - Qwa-qwa
Solanum chenopodioides Lam.	CHE2	University of the Free State
Solanum kwebense N.E.Br.	KWE	Gene bank – Pretoria
Solanum burbankii Bitter	RET1	University of the North - Qwa-qwa
Solanum retroflexum Dun. (smooth)	RET2	University of the North - Qwa-qwa
Solanum retroflexum Dun. (hairy)	RET3	University of the North - Qwa-qwa
Solanum retroflexum Dun. (smooth)	RET4	Gene bank – Pretoria
Solanum scabrum Mill.	SCA	University of the North - Qwa-qwa
Solanum tomentosum L.	TOM	NIB Kirstenbosch – Cape Town
Solanum villosum Mill.	VIL	University of the North - Qwa-qwa
S. chenopodioides Lam. X *S. americanum* Mill.	CHEXAME	University of the North - Qwa-qwa
Unknown *Solanum*	N22	Gene bank – Pretoria
Unknown *Solanum*	N23	Gene bank – Pretoria

3.2.2 *Plant material*

The seeds received had been stored at room temperature and before use were soaked in distilled water at 4°C for seven days in petri dishes on filter paper. The seeds were then divided into three groups with a different treatment for each group, as found in the literature or as suggested by Dr. Meyer. The treatments were done under non-sterile conditions and were as follow:

1) 8h at 30°C and 16h at 20°C
2) 13h light and 11h dark (photoperiod) at 30°C
3) 25°C in the dark.

Seedling trays were filled with a commercial potting mixture designed for seedlings supplied by Hygrotech Seed (Pty.) Ltd. The germinated seeds form the different treatments were planted in these trays and placed in a heat-controlled glasshouse. The seedlings were watered daily and fertilized every third day with Chemicult (10g/5ℓ) a fertilizer containing all micro- and macro-nutrients. The plants were kept in the trays until a root plug was established and then transplanted into black plastic nursery bags in a heat controlled glasshouse. The plastic bags were filled with a mixture of soil, compost and commercial potting soil. The plants were nurtured in the glasshouse and after 12 weeks they were planted out on the West campus of the UFS to conduct the first set of evaluation of the plants to choose the best parental material for a crossing study (Chapter 5).

Unfortunately not all the species were correctly identified when received for the study. With the help of Edmonds and Chweya's (1997) 'working base' key, photos and drawings in their book, and with the help of Mrs. Kriel at the Geo Potts Herbarium at Dept. Plant Sciences UFS, the species obtained were identified while growing in the field. This identification was confirmed by cytogenetic studies as seen in Chapter 5.

The field experiment was conducted on the campus of the UFS. The experimental design was a complete randomized block with three replications. Each of the plots consisted of three rows with four plants per row. The plants were spaced 1m apart within rows and the blocks were 2m apart. Plants, which died within the first two weeks of the original planting date, were replaced by stock from the glasshouse.

In preparing the field, a 2:3:2 fertilizer mixture was worked into the soil at a rate of 60g/m^2. During the growing season, the plants were watered as required (up to three times a week) and fertilized fortnightly with Chemicult (10g/5ℓ). The plants were sprayed with insecticides when required. Weeds were removed manually. The plants were covered with a net to protect the fruits from being eaten by birds.

3.2.3 *Morphological characters measured*

The accessions were analyzed and data was collected on the following characters.

Stems: Plant height and spread in centimeters (cm) were measured to determine the size of the plant.

Leaves: Leaf area in square centimeters (cm^2) was measured with a CID, Inc. CI-251 Area meter, 10 leaves of each plant were measured and the average leaf area for each plant was determined.

Flowers: Number of days from planting to flowering (anthesis) was recorded for each plant. For the number of flowers formed per bunch, 10 bunches from each plant were randomly chosen and the flowers were counted and the average for each plant was determined.

Fruit: Number of days from flowering to fruit forming and the number of days from fruit forming to fruit ripening was recorded for each plant. The number of fruit formed per bunch was counted by randomly choosing 10 bunches from each plant. The average for each plant was then determined.

Fruit size was determined by measuring the diameter of fruit in millimeters (mm), with a Precision Vernier Caliper RACO expert, 150mm (0.1mm). Fifty randomly chosen fruits from each plant were measured and the average fruit size was determined.

The weight per bunch of fruit in gram (g) was measured with a Scaltec SBC 32 scale (Max 120g Min 0.01g). Ten bunches from each plant were randomly chosen for this measurement and an average was determined.

The weight of a single fruit in gram (g) was measured with the Scaltec SBC 32. For this measurement 50 randomly chosen fruits from each plant were weighed and the average fruit weight was determined.

Seed: Number of seeds in a fruit was counted by squashing 15 randomly chosen fruits from a plant on filter paper. The number of seeds was then counted.

The number of seeds in 0.1g of berries of the same plant was weighed with the Scaltec SBC 32 and counted. Five measurements were done and an average per plant was determined.

Yield characteristics: Total weight was the mass in gram (g) of all the fruit harvested from a plant.

The total yield was the total number of fruits harvested per plant. The berries were harvested when fully matured, with a purple or yellow-orange colour.

The characteristics are summarized in Table 3.2 and the averaged data can be found in Table 3.3.

Table 3.2 Morphological characteristics measured.

QUANTITATIVE TRAITS		QUANTITATIVE TRAITS	
1	Plant height (cm)	9	Flowers formed per bunch
2	Plant spread (cm)	10	Fruit formed per bunch
3	Leaf area (cm^2)	11	Total weight of fruit bunch (g)
4	Days to flowering	12	Weight of single fruit (g)
5	Days to fruit forming	13	Fruit size (mm)
6	Days to fruit ripening	14	Total weight of fruit harvested (g)
7	Number of seeds in a fruit	15	Total number of fruit harvested
8	Number of seeds in 0.1g		

Measurements were done in the field or in the laboratory.

3.2.4 *Statistical analysis*

The data for the 15 morphological characters were subjected to an analysis of variance (ANOVA). The components were calculated using the software program AGROBASE 2000 (Agronomix, Canada). The program also calculated the accession contribution of each characteristic from the ANOVA.

For each morphological characteristic, the data means were calculated and scored as presence (1) or absence (0) and compiled into a binary data matrix (Addendum A). A dendrogram (Figure 3.10) was generated, depicting relationships among the accessions, using the NCSS 2000 (Number Cruncher Statistical Systems; Hintze, 1998) software program. The hierarchical agglomerative clustering method using the Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) was carried out to examine the resemblance and grouping of the accessions (Hintze, 1998).

3.3 Results and discussion

3.3.1 *Germination of seeds*

Germination of the seeds was problematic, possibly due to the poor quality of the seeds received. Different germination procedures were investigated, to find the optimum conditions for germination. The germination took place in plastic petri dishes on filter paper under non-sterile conditions. The best results for seed germination were obtained at 30°C for 16h (light) and 25°C for 8h (dark) in 2.5ml of distilled water. These conditions differed significantly from the conditions stated by Del Monte and Tarquis (1997) and differed slightly from Thullen and Keeley's (1982) 13h photoperiod followed by complete darkness and temperatures of 26.7°C to 32.2°C.

3.3.2 *Morphological characteristics*

Some characteristics and observations of interest, which helped in the identification of the species included in this study, are described on the next few pages and the average morphological data scored for the various species respectively in this study are given in Table 3.3.

Table 3.3 Means of the accessions for the different morphological characteristics measured.

CHARACTERS	CHEXAME	AME	RET1	RET2	RET3	RET4	SCA	VIL	LSD
Plant height	61.67	58.33	21.67	26.67	16.67	20.00	33.33	27.33	8.77
Plant spread	143.33	140.00	110.00	81.67	70.00	100.00	160.00	130.00	50.17
Leaf area	12.08	10.14	2.75	5.87	5.56	5.85	47.10	6.07	13.30
Days to flower	20.33	21.33	19.67	13.67	15.33	14.33	25.00	12.00	3.64
Days to fruit form	12.67	9.67	18.00	17.67	16.67	17.33	11.00	17.67	3.81
Days to fruit ripe	13.33	14.33	17.67	16.33	16.00	16.67	20.33	18.33	3.85
Seeds per fruit	35.00	35.33	31.67	25.33	35.67	27.67	113.00	37.33	8.69
Seeds per 0.1g	119.00	117.67	83.00	87.00	103.33	108.33	94.33	153.67	16.30
Flowers per bunch	4.00	5.00	3.00	3.33	3.00	3.33	6.00	4.33	0.82
Fruit per bunch	4.33	4.00	3.00	3.33	2.67	3.00	5.33	3.33	1.02
Weight per bunch	1.34	1.27	1.09	1.07	1.08	1.08	9.60	0.59	2.44
Weight per fruit	0.26	0.27	0.32	0.31	0.39	0.31	1.49	0.15	0.18
Fruit Size	7.23	7.47	7.93	8.10	8.47	8.07	14.60	5.97	0.59
Total Weight	602.48	360.52	268.21	169.04	54.20	521.43	634.56	207.17	298.42
Total number of fruit harvested	2359	1513	896	644	137	1800	440	1527	642.32

As already stated not all the accessions were correctly identified. Although it was only established that *S. kwebense* N.E.Br, (Figure 3.1), *S. tomentosum* L. (Figure 3.2), as well as the unknown *Solanum* sp. probably *S. lichtensteinii* Willd. (Figure 3.3a and b), did not belong to the *S. nigrum* complex. The plants were already included in the field trail and some characteristics were initially measured. Unfortunately these species produced no berries and they were therefore not depicted in the morphological character dendrogram (Figure 3.10) but were used in the amplified fragment length polymorphism (AFLP) marker study (Chapter 6).

Figure 3.1 *S. kwebense* N.E.Br

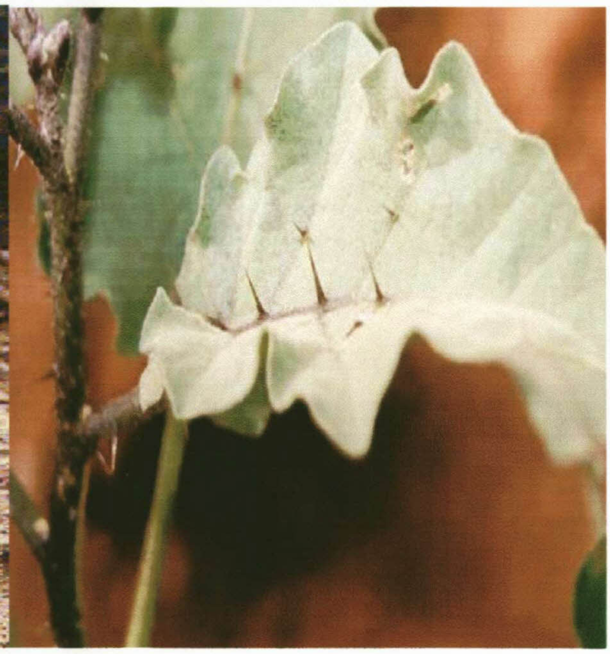


Figure 3.2 *S. tomentosum* L.

Figure 3.3a Unknown *S. sp.* flower.

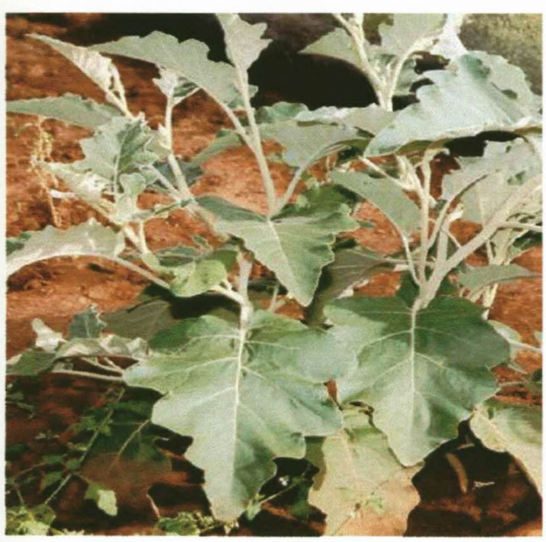


Figure 3.3b Unknown *S. sp.*

S. americanum Mill. (AME) (Figure 3.4a and 3.4b): is an upright growing plant averaging 50 to 65cm high with an average spread of 100 to 160cm. Anthesis took about 21 days with four to six small white flowers per bunch. The anthers were yellow and the style was clearly exserted beyond the anthers. The fruit formed seven to 13 days later and ripened after a further 12 to 16 days. The glossy purple berries ranged from 4.0 to 8.9mm in diameter, averaging 7.5mm. The ripe berries fell from the plant, leaving the calyx behind. There were between 30 and 40 seeds per fruit. According to Edmonds and Chweya (1997) a common synonym for *S. americanum* Mill. is *S. nodiflorum* Jacq.

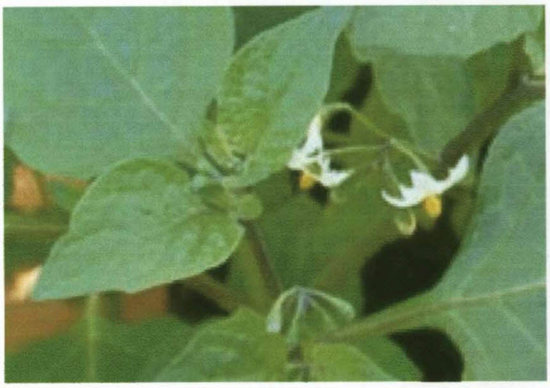


Figure 3.4a *S. americanum* Mill. flower.

Figure 3.4b *S. americanum* Mill. fruit.

Available literature indicates various opinions on the taxonomy of *S. burbankii* Bitter and *S. retroflexum* Dun. Shaw (1971, Appendix B), expressed the opinion that these are indeed two separate species. The leaves are ovate and dentate, but these characteristics are less pronounced in *S. burbankii* Bitter than *S. retroflexum* Dun. A clear bluish-purple line runs down the centre of the corolla lobes of *S. burbankii* Bitter and the plant has purplish stems.

Henderson (1974) stated that although flower colour is phenotypically plastic in some species, it is under genetic control in others and according to Edmonds and Chweya (1997) *S. burbankii* Bitter and *S. retroflexum* Dun are probably synonyms for the same species.

In this study, the accession bearing a clear bluish-purple line down the centre of the corolla were considered to be *S. burbankii* Bitter (RET1).

S. burbankii Bitter (RET1) (Figure 3.5): plants spread 100 to 120cm and were not tall (15 to 30cm). Anthesis took place after 18 to 20 days. Three flowers were formed per bunch and the fruit developed 15 to 20 days later. The dark purple berries had an average diameter of 7.9mm. The berries fell from the plant after ripening and an average of 27 seeds was found in the fruit.

Figure 3.5 *S. burbankii* Bitter with bluish line down corolla.

Three accessions were obtained for *S. retroflexum* Dun. RET2, RET3 and RET4 (Table 3.1). One accession yielded plants which were distinctly hairy (RET3) (Figure 3.6) while the other two accessions yield plants which were morphologically similar, except for the absence of hair (RET2 and RET4). Red spider mite was a problem in the glasshouse, on the species without hair on the leaves. The hairy species (RET3) displayed resistance to insect attacks in the glasshouse and in the field compared to the other plants in the trails.

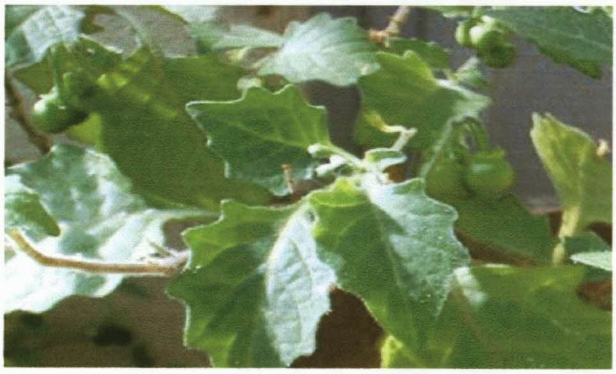


Figure 3.6 *S. retroflexum* Dun. fruit and hairy leaves.

S. retroflexum Dun. (RET4): the plants were not very tall (15 to 25cm) and displayed a spreading nature (75 to 120cm). Anthesis took between 11 and 18 days to occur, with three or four white flowers per bunch. The fruit formed 15 to 20 days later. The fruit ripened between 14 and 20 days after formation. The purple berries ranged from 7.6 to 8.4mm in diameter. There was an average of 25 to 36 seeds per fruit. Red spider mite was problematic on this species. After a period of heavy rain during the field study, the fruit of this species was the only one found to have burst open.

The various accessions of *Solanum chenopodioides* Lam. were only obtained at a later stage. This material could only be used in the AFLP marker study (see Chapter 6) and not in the morphological trial, because the other plants were already established in the field. *S. chenopodioides* Lam. (CHE2) (Figure 3.7): the plants were greyish-green in colour. The anthers were yellow and the style exserted up to 2mm beyond the anthers. The berries fell from the plant with the pedicels still attached.

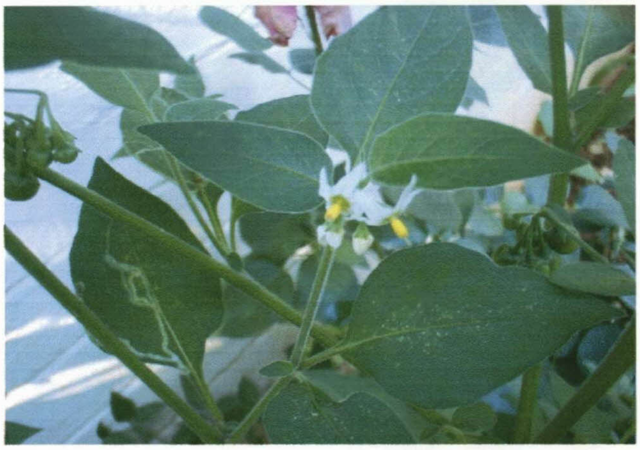


Figure 3.7 *S. chenopodioides* Lam. flower and fruit.

S. scabrum Mill. (SCA) (Figure 3.8): was a robust species and could easily be recognized by its large, dark purple fruit. These plants spread horizontally (120 to 220cm), with strong purplish stems. The plants were on average only 30 to 50cm high. The flowers were white, with purplish brown anthers and took 21 to 28 days to anthesis. Fruit formation took between nine and 14 days. Ripening followed 18 to 20 days later. The deeply purple berries remained on the plant after ripening. The berries had numerous seeds (100 to 124) and berry diameters of between 13.1 and 15.4cm were measured. *S. melanocerasum* All is a common synonym of this species (Edmonds and Chweya, 1997).

Figure 3.8 *S. scabrum* Mill. mature fruit.

S. *villosum* Mill. (VIL) (Figure 3.9): these spreading (90 to 180cm) plants did not grow very tall (17 to 35cm). Anthesis occurred after 11 to 14 days and three to five white flowers formed per bunch. Fruit forming only occurred on average 15 to 21 days later. The berries were elongated, had an orange colour and took between 16 to 21 days to ripen. The berries on these plants were the smallest compared to the others in this study, with a diameter of 5.8 to 6.3mm. The berries fell from the calyces after ripening and only between 33 and 42 seeds per fruit was counted.

Figure 3.9 *S. villosum* Mill. mature fruit.

The flowers for all the species opened at daily intervals. The plants continuously produced flowers under favourable light and temperature conditions, if water was supplied.

3.3.3 *Analysis of variance (ANOVA)*

The mean squares for all measured characteristics except plant spread and days to fruit ripening were significant at $p > 0.01$, and total weight was significant at $p > 0.05$ (Table 3.4).

The genotype contribution to variability (Table 3.4) for most of the morphological characteristics was relatively high thus indicating that the environment contributed only a very small part to the variability. The highest value found for fruit size (99.2%) indicated that the environment's contribution was, in this case insignificant.

Table 3.4 Mean squares of all the morphological characteristics measured and the genotype contribution to variability.

CHARACTERS	MS FOR ENTRIES	GENOTYPE CONTRIBUTION
Plant height	899.90**	95.9%
Plant spread	3015.33^{NS}	59.6%
Leaf area	631.24**	86.4%
Days to flower	61.28**	89.5%
Days to fruit form	34.83**	79.8%
Days to fruit ripe	14.71^{NS}	51.1%
Seeds per fruit	2478.52**	98.5%
Seeds per 0.1g	1531.66**	91.6%
Flowers per bunch	1.90**	82.8%
Fruit per bunch	4.10**	87.6%
Weight per bunch	27.38**	89.5%
Weight per fruit	0.55**	97.1%
Fruit size	20.13**	99.2%
Total weight	134760.33*	68.0%
Total number of fruit harvested	1451803.12**	86.3%

** Significant at P > 0.01

* Significant at P > 0.05

^{NS} Not Significant

Plant height – The hybrid CHExAME ranked the highest (Table 3.3) and no significant difference was found between this hybrid and the second ranking species AME. Significant differences were found between these two accessions and the other species.

Plant spread – The analysis of variance showed no significant variability for this characteristic (Table 3.4), but the LSD (Table 3.3) indicated significant differences. The genotype contribution to this variability was the second lowest (59.6%) measured, thus indicating that the environment contributed to variability in this characteristic.

The highest ranking species SCA showed no significant difference between itself and the next four accessions on the ranking list. Significant differences were found between SCA and RET4, RET2 and RET3 respectively as well as between AME and RET3 (Table 3.3).

Leaf area – Significant differences were found between the top ranking species SCA and the other accessions. No significant differences were found between the other ranking accessions (Table 3.3).

Days to flowering – The average days to flowering were significantly different ranging from 12 to 25 days for VIL and SCA respectively (Table 3.3). No significant differences were found between VIL, RET2, RET4 and RET3. These species formed flowers within 15 days.

Days to fruit formation – No significant differences were found between AME, SCA and CHExAME. Significant differences were found between these accessions and the other species that took more days to form fruit (Table 3.3).

Days to fruit ripening – The results in Table 3.4 showed no significant variability for this characteristic. The genotype contribution was the lowest with a value of 51.1% thus indicating that the environment contributed significantly to the variability. The species that displayed a greater number of days to ripen were RET1, VIL, and SCA and significant differences were found between them and the other accessions.

Seed per fruit – SCA was the species with the most seeds per fruit (113) and differed significantly from the other accessions (Table 3.3).

Seeds in 0.1g – Significant differences were found between the species. VIL ranked the highest and differed significantly from all the species (Table 3.3).

Flowers per bunch – The hybrid CHExAME and species AME showed the same number of flowers per bunch (4), RET2 and RET4 had an average of 3.33 flowers per bunch and RET1 and RET3 averaged three flowers per bunch. All these species differed significantly from the top ranking species SCA (Table 3.3).

Fruit per bunch – No significant difference was found between SCA and AME. These high ranking species differed significantly from the other accessions.

Weight per bunch – The average weight per bunch ranged from 0.59g for VIL and 9.60g for SCA (Table 3.3). Once again SCA ranked the highest and differed significantly from all the other species.

Weight per fruit – Significant differences between SCA and the other accessions occurred. VIL ranked the lowest with a value of only 0.15g (Table 3.3).

Fruit size - The genotype contribution of the parameter was the highest (99.2%, Table 3.4), indicating that the environment's contribution to this characteristic was insignificant. Significant differences were found between SCA, ranking first once again, and the other accessions (Table 3.3).

Total weight – The analysis of variance results (Table 3.4) indicated a significant variance only at the $p > 0.05$ level for this entry. No significant difference was found between the top ranking species SCA and AME and the hybrid CHExAME. Significant differences were, however, found between SCA and the other accessions.

Total number of fruit harvested – Significant differences were found between CHEXAME ranked first and the other species.

3.3.4 *Dissimilarity measured*

Significant differences were found between the eight accessions for the morphological characteristics measured, with genetic distance ranging from 0.26 to 0.80 (Figure 3.10), reflecting genetic variation. The dendrogram produced, revealed two large clusters (I and II) with two sub-clusters (A and B) each.

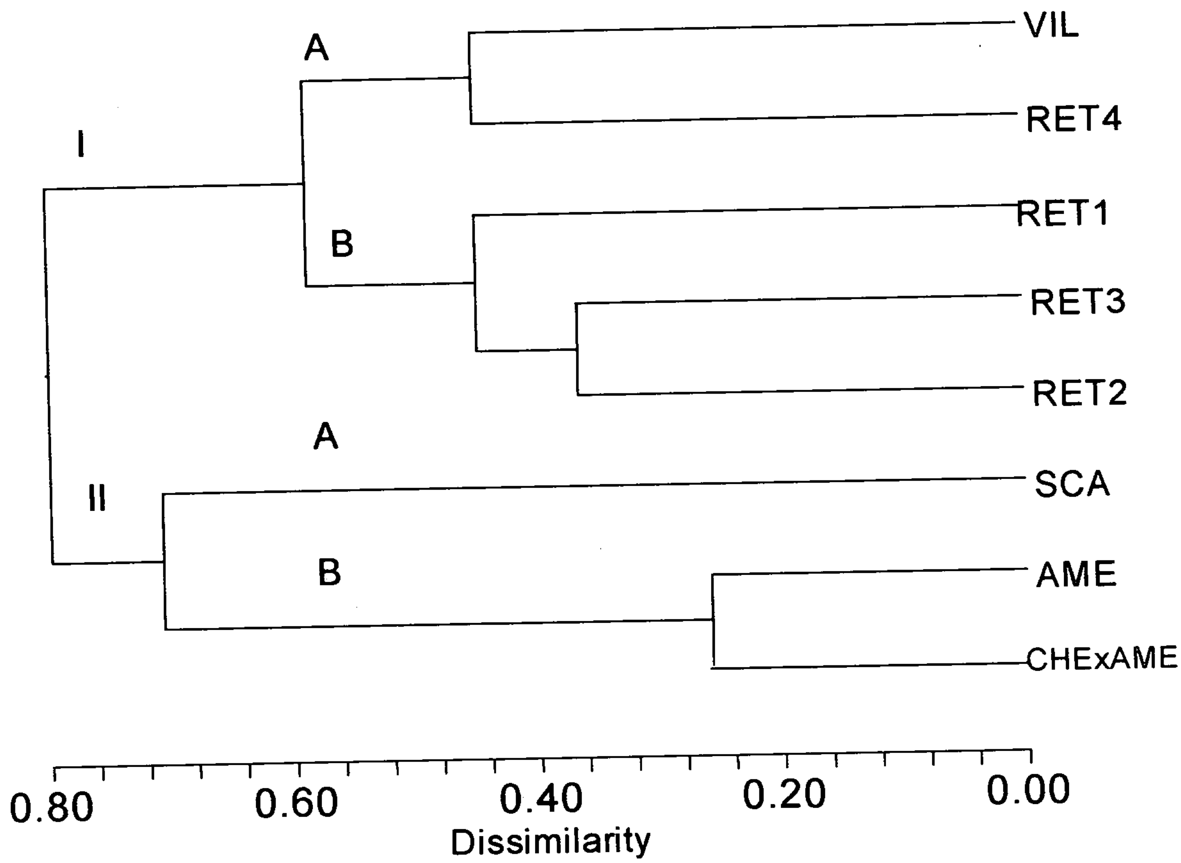


Figure 3.10 Dendrogram for morphological characteristics.
VIL = *Solanum villosum,* RET4 = *Solanum retroflexum* (Pretoria -smooth), RET1 = *Solanum burbankii,* RET3 = *Solanum retroflexum* (hairy), RET2 = *Solanum retroflexum* (smooth), SCA = *Solanum scabrum,* AME = *Solanum americanum,* CHExAME = *Solanum chenopodioides* X *Solanum americanum*

The smooth leaved *S. retroflexum* from Pretoria (RET4) clustered with *S. villosum* (VIL) in the first cluster (IA) with a dissimilarity of 0.44. RET4 clustered separately from the hairy (RET3) and the smooth (RET2) leaved *S. retroflexum*, found in cluster IB with a genetic distance of 0.36. RET2 and RET3 are morphologically very similar, with exception of hairiness. *S. burbankii* (RET1) clustered separately in cluster IB, with a dissimilarity of 0.44. This suggests that they are not the same species, as they differ morphologically, as reflected in the clustering, although they sorted in the same large cluster. All the plants in this cluster are generally characterized by semi-erect growth habit, a small leaf area and small numbers of flowers and fruit per bunch.

S. scabrum (SCA) clustered separately (IIA) with the highest genetic distance of 0.70, while *S. americanum* (AME) and the CHExAME hybrid clustered together with a dissimilarity of only 0.26, the lowest genetic distance in the dendrogram. The CHExAME hybrid was morphologically very similar to the *S. americanum* parent. Clusters IIA and B were characterized by an erect growth habit and a high number of flowers and fruit per bunch.

3.4 Conclusions

According to Edmounds and Chweya (1997), the taxonomy of the African taxa of the species belonging to the section *Solanum* remains to be resolved. To our knowledge, this is the only report on the morphological characters of the *Solanum nigrum* complex species found in South Africa to help the plant breeder choose parental plants for a breeding study. These morphological characteristics measured have proved to be a useful tool in determining the genetic relationship for the accessions studied.

In this study the simple analysis of variance (Table 3.4) showed significant differences for 13 of the 15 morphological characteristics measured. Only plant spread and days to fruit ripening yielded no significant differences.

The dendrogram clearly identified different groupings of the accessions and significant differences were found between the species for the characters studied, reflecting extensive genetic variation as expected between the accessions. Maximum variability for selection in segregating populations may be achieved by utilizing accessions from different clusters as parents for crosses. The species RET4 from cluster IA, RET1 from cluster IB, SCA and AME from cluster IIA and IIB respectively, were chosen as parents in a subsequent crossing study.

Chapter 4

Field evaluation, heterosis and correlation of the morphological characteristics, for the parents and progeny of five accessions in the *Solanum nigrum* complex.

4.1 Introduction

The breeding history of the *Solanum nigrum* complex in South Africa is limited or non-existing. Estimates of genetic variance may be useful for further improvement of this complex. No cultivars for species belonging to the section *Solanum* have yet been developed through conventional plant breeding techniques. Selection of plants exhibiting desirable characteristics is probably the method by which these cultivars will be developed as a crop plant (Edmonds and Chweya, 1997).

There has been much interest in hybrids in many breeding programs, although hybrids have not been exploited optimally in cross-pollinated and especially in self-pollinated vegetable crops (Wehner, 1999). F1 hybrid cultivars are preferred by both plant breeders and seed-producing companies for purely commercial reasons, because they see heterosis as a sure way to preserve their breeders rights on the cultivars which they developed (Yordanov, 1983). Potential increase in yield is one of the main reasons why growers may prefer hybrid cultivars to pure line cultivars (Boleda, 1992).

Allard (1960) defined heterosis as a F1 hybrid that falls outside the range of the parents with respect to some characteristic or characteristics, usually applied to size, rate of growth, or general thriftiness. Falconer and Mackay (1996) describe heterosis as the difference between the hybrid and the mean of the two parents and this is often expressed as a percentage of the mid-parent. The other type of heterosis is high parent or best parent heterosis, which is the difference between the hybrid and the best parent.

Superior-yielding variants could thus be raised through conventional breeding methods with the help of hybrid vigor or heterosis. Heterosis, however, does not occur universally in vegetable crops. Some self-pollinated vegetable crop species do not express any heterosis. Heterosis depends not only on the degree of dominance, but also on the differences in the gene frequencies between the parental lines $H = \Sigma dy^2$ (Falconer and Mackay, 1996).

The association between two characters, which can be directly observed, is the phenotypic correlation, or the sum of the genetic and environmental cause of correlation. The degree of correlation arising from pleiotropy expresses the extent to which two characters are influenced by the same genes. The resulting correlation is thus the overall, or net, effect of all the segregating genes that affect both characters (Falconer and Mackay, 1996). Correlation is of interest to the breeder because the correlation indicates how the improvement of one character could cause simultaneous changes in other characters. The correlation between characters can be positive or negative.

The field performance of five parental species and their progeny over three locations was investigated. The amount of heterosis expressed in the progeny was determined and the estimates of the correlation between characteristics were calculated to determine if the simultaneous selection for more than one characteristic could be advantageous.

4.2 Materials and methods

4.2.1 *Plant material*

Five species were used in the crossing program:

- *S. americanum* Mill. (AME) 2x
- *S. chenopodioides* Lam. (CHE2) 2x
- *S. burbankii* Bitter (RET1) 4x
- *S. retroflexum* Dun. (RET4) 4x
- *S. scabrum* Mill. (SCA) 6x

S. americanum Mill. (AME) was used for its upright growth habit and the ease by which the fruit were harvested. *S. chenopodioides* Lam. (CHE2) was included for it has the best-tasting fruit, however, the fruit are very small (average 6.08mm, Table 4.6). CHE2 also displayed an upright growth habit and was harvested with ease. *S. burbankii* Bitter (RET1) as well as *S. retroflexum* Dun. (RET4) spread a lot and unfortunately have a great number of leaves, however, they are already being used commercially. Fox and Young (1982) states that *S. retroflexum* is the most common species in South Africa. *S. scabrum* Mill. (SCA) was included for its fruit size (up to 15mm) and the fruit was harvested with great ease.

The progeny used in this study were developed by crossing single accessions of each of these chosen parental species as described in Chapter 5.2.2. The F1 plants of *S. chenopodioides* X *S. retroflexum* (CHE2xRET4) and *S. retroflexum* X *S. chenopodioides* (RET4xCHE2) as well as the reciprocal cross between *S. burbankii* (RET1) and *S. chenopodioides* (CHE2) were sterile. These hybrids exhibit vigorous growth. Abundant flowering occurred but no fruits were produced. These hybrids were only used in the analysis of variance for plant height, plant spread, leaf area, days to flower and flowers per bunch.

The successful crosses as seen in Chapter 5 and the parental species were use as experimental material in this study and are presented in Table 4.1

Table 4.1 Parental species and crosses with their abbreviations used in this study

SPECIES OR CROSS	ABB.
Solanum americanum Mill.	AME
Solanum chenopodioides Lam.	CHE2
Solanum burbankii Bitter	RET1
Solanum retroflexum Dun. (smooth)	RET4
Solanum scabrum Mill.	SCA
S. americanum X *S. scabrum*	AMExSCA
S. burbankii X *S. retroflexum*	RET1xRET4
S. chenopodioides X *S. retroflexum*	CHE2xRET4
S. retroflexum X *S. burbankii*	RET4xRET1
S. retroflexum X *S. chenopodioides*	RET4xCHE2
S. scabrum X *S. americanum*	SCAxAME

In each of the crosses listed in Table 4.1 the parent on the left-hand side was the female, while the pollen source was the parent on the right-hand side.

4.2.2 *Experimental design and locations*

The fully matured fruit resulting from the pollination (see Chapter5) were harvested. The hybrid seeds as well as seed from the parents were sown and germinated in a special soil mix for seedlings. This was done in seedling trays in a heat-controlled glasshouse in the spring of 2001 as described in Chapter 3.2.2. The mature seedlings were transferred to black nursery bags and after establishment transplanted to three different locations:

1-Glasshouse (GH)

2-West campus (WEST)

3-Eric Lamb Nursery (EL)

The plants in the GH were planted in 25cm plastic pots. The field experiments conducted on the West-campus of the UFS and at Eric Lamb Nursery, a commercial nursery in Bainsvlei were about 15km apart. These two locations had different soil types. The soil at the UFS had a dark heavy clay content, while the soil at Bainsvlei was a red loamy soil.

The soil in both locations was ploughed and 60g/m^2 2:3:2 fertilizer was worked into the soil. The plants were also fertilized fortnightly during the growing season, with Chemicult (10g/5ℓ). Sufficient water was supplied through an overhead sprinkler system during the period of no rain.

The experimental design for all locations was a complete randomized block design with four replications. Each of the plots consisted of three rows with four plants per row. The plants were spaced 1m within rows and the blocks were 2m apart.

The plants in the field were covered with a net to protect the fruits from birds. Insect and disease control was done by spraying the plants when necessary. The pesticide applied was Metasystox (active ingredient, demeton-S-methyl) which was used to control the black aphid problem in the field trails. In the GH red spider mites were a problem and Talstar (active ingredient, bifenthrin) was sprayed. Weeds were removed manually.

4.2.3 *Morphological characters measured*

The plants were analyzed and data was collected on the 15 characteristics listed in Table 3.2, a detailed description of the characteristics can also be found in Chapter 3.2.3.

4.2.4 *Statistical analysis*

4.2.4.1 *Analysis of Variance (ANOVA)*

The data for the 15 morphological characteristics were subjected to an analysis of variance (ANOVA). The components were calculated using the software program AGROBASE (Agronomix, Canada). Simple ANOVAs were performed separately on the data sets for each location by using the RCBD procedure to assess the performance of each accession (Table 4.2a & 4.2b). A combined analysis of the data across the three locations was also done by using the same procedure (Table 4.10a & 4.10b).

4.2.4.2 *Heterosis*

Heterosis was determined for all progeny as the superiority over the mid-parent and over the high parent. The mid-parent heterosis (average heterosis) was measured as the deviation of the offspring from the mid-parent value, often expressed as a percentage of the mid-parent value.

Mid-parent heterosis was calculated by the formula: $HF_1 = MF_1 - MP/ MP \times 100$

Where: HF_1 is the heterosis for the F_1 cross

MF_1 is the mean performance or value of the F_1 cross

MP is mean of two parents (P) obtained from the mean values (M) of the two parents

High parent heterosis was calculated from the mean values of the F_1 crosses and the better parent, using the formula $HF_1 = MF_1 - MHP / MHP \times 100$

Where: MF_1 is the mean value of the F_1 cross

MHP is the mean value of the better parent.

4.2.4.3 *Correlation*

The correlation coefficients between all the characteristics over all the locations were obtained by using the software program AGROBASE 2000 (Agronomix, Canada) to examine the degree of association among the characteristics.

4.3 Results and discussion

4.3.1 *Simple analysis of variance (ANOVA)*

Highly significant differences between all entries were found for all characteristics measured in each location as depicted in Table 4.2a and Table 4.2b. Entry mean squares (MS) for the ANOVA of the 10 characteristics for the nine accessions that produced fruit are depicted in Table 4.2a. The accessions that produced only flower characteristics were also measured and subject to an ANOVA. The mean square values for the five characteristics measured are included with the other accessions and are presented in Table 4.2b.

Table 4.2a Mean squares and significance of the morphological characteristics measured for nine accessions.

CHARACTERS	MS - ENTRIES GH	MS - ENTRIES WEST	MS - ENTRIES EL
Days to fruit form	128.153**	100.694**	39.757**
Days to fruit ripe	108.049**	39.965**	31.194**
Seeds per fruit	1892.938**	2307.674**	1343.313**
Seeds per 0.1g	10323.688**	3603.132**	6096.569**
Fruit per bunch	15.465**	12.549**	21.340**
Weight per bunch	31.104**	37.703**	11.664**
Weight per fruit	0.359**	0.473**	0.260**
Fruit size	16.293**	20.139**	19.423**
Total weight	79448.374**	688788.647**	9663.406**
Total yield	379745.813**	391060.465**	27272.465**

GH - Glasshouse, WEST - West-campus at UFS, EL - Eric Lamb Nursery.

** Significant at P > 0.01 probability level.

Table 4.2b Mean squares and significance of the five morphological characteristics measured for 11 accessions that form no fruit.

CHARACTERS	MS - ENTRIES GH	MS - ENTRIES WEST	MS - ENTRIES EL
Plant height	580229.091**	279969.468**	423477.614**
Plant spread	130702.273**	279793.064**	124500.455**
Leaf area	325.958**	774.923**	731.872**
Days to flower	635.923**	203.768**	1179.523**
Flowers per bunch	9.923**	9.923**	20.568**

GH - Glasshouse, WEST - West-campus at UFS, EL - Eric Lamb Nursery.

** Significant at P > 0.01 probability level.

4.3.2 *Performance of the accessions*

The mean values for the progeny and corresponding parents were studied and the LSD value was used to identify significant differences among the means of the accessions. The mean values for the locations: GH, WEST, and EL are listed in Table 4.3, 4.4 and 4.5 respectively. The mean values obtained from all three locations are listed in Table 4.6. The means across all locations were used to determine the mid-parent heterosis (Table 4.7) and high parent heterosis (Table 4.8) as well as the correlation that occurred between the characteristics (Table 4.9).

Plant height

Plants were much taller in the glasshouse than at the two field localities. In the glasshouse AMExSCA and SCAxAME grew the tallest, with their parents ranking third and fourth. AMExSCA was significantly higher than both parents and SCAxAME was significantly higher than the lowest parent. At the commercial nursery AME grew the tallest, followed by SCA and then by their hybrid and its reciprocal AMExSCA and SCAxAME. On the West campus AMExSCA was again the tallest entry, with AME ranking second and SCAxAME third, although the difference was not significant. Averaged over all three locations AMExSCA again ranked first, with AME second although the difference was not significant. SCAxAME ranked fourth. The good performance of the AMExSCA hybrid was confirmed with its high mid-parent (Table 4.7) and highest best parent heterosis (Table 4.8). RET1XRET4 was at the lowest three positions at all three localities and across localities. It would therefore seem that there were no big rank differences between the three localities (Table 4.6). Plant height was significantly correlated with important yield attributes like seeds per fruit, fruit per bunch, weight per fruit bunch, single fruit weight, fruit size, and total weight of the fruit harvest (Table 4.9).

Plant spread

Plant spread was the highest at the West campus. In the glasshouse SCAxAME ranked the highest followed by the parent SCA and then the reciprocal AMExSCA (the differences were not significant), and then by RET4. On the West campus SCAxAME again ranked first, followed by the parent SCA and then AMExSCA. At the nursery SCA ranked first followed by SCAxAME, followed by AMExSCA, although differences between them were not significant. Across all three localities SCAxAME ranked first, followed by SCA and then by AMExSCA. The mid-parent heterosis was very low (Table 4.7) and as were expected the highest parent heterosis (Table 4.8) was negative. CHE2xRET4 ranked last across localities and at each locality separately. Once again the rankings seemed to be very stable across different localities. This characteristic was also significantly correlated with the most important yield characteristics, especially total weight of fruit harvest (Table 4.9).

Leaf area

The biggest leaf area was found for SCA growing at the nursery. This was followed by SCAxAME and AMExSCA and then the other parent for these reciprocals, AME, with significant differences between them all. On the West campus SCA had a smaller leaf area than AMExSCA but no significant differences were found between them. The two hybrids SCAxAME and AMExSCA ranked first and second respectively in die glasshouse, and were followed by their parents SCA and AME respectively. Significant differences could only be found between the top ranking hybrid, SCAxAME and its lowest ranking parent AME. Across all three localities SCA ranked first, followed by AMExSCA and then by SCAxAME. CHE2xRET4 ranked the lowest in two of the locations and in the overall average as well. The mid-parent heterosis for AMExSCA and SCAxAME were 18.49% and 17.56% respectively (Table 4.7) and the highest parent heterosis was negative (Table 4.8) as was expected with SCA performance. This characteristic was also significantly correlated with all the important yield characteristics, especially fruit per bunch and total weight of fruit harvest (Table 4.9).

Days to flowering

The hybrid RET4xRET1 took the least number of days to start to form flowers for all three locations and across locations as well. The plants at the nursery flowered first and RET1xRET4 and SCAxAME ranked second and third. No significant differences were found between these top ranked hybrids. On the West campus RET1 and RET1xRET4 followed the top ranking hybrid RET4xRET1. This top ranking hybrid differed significantly from the second ranking parent RET1 and the third ranking hybrid (RET1xRET4) at this location. Significant differences were also found in the glasshouse between RET4xRET1, RET1 and RET1xRET4 ranked first, second and third respectively. SCA took the most days to flower across localities and at the other localities except in the glasshouse and differed significantly from the other genotypes. Small differences were found in the middle ranking orders across the different localities. This characteristic was significantly correlated with most of the important yield characteristics, except total weight of fruit harvest (Table 4.11).

Days to fruit forming

The plants in the glasshouse started forming fruit before the plants in the other locations. RET4xRET1 and RET1xRET4 ranked first and second in the glasshouse, followed by CHE2 but no significant differences were found between them. At the commercial nursery CHE2 and AME ranked first and second followed by the hybrid RET1xRET4. A significant difference was found between the first and third ranking genotypes. On the West campus no significant differences were found between the top four ranking accessions RET4xRET1, CHE2, RET1xRET4 and AME respectively. The plants that took the most days to form fruit were SCA, AMExSCA and SCAxAME which ranked seventh, eighth and ninth over all three localities and across localities. Significant differences were displayed between these genotypes at all the locations and across locations. The heterosis value for the hybrids AMExSCA and SCAxAME was positive with a high mid-parent heterosis for SCAxAME of 67.08% (Table 4.9). This characteristic was significantly correlated with important yield attributes like fruit per bunch, weight per fruit bunch, single fruit weight, fruit size, and total weight of the fruit harvest (Table 4.11).

Days to fruit ripening

The plants at the nursery displayed the least amount of days to fruit ripening. The fruits of AME, CHE2 and RET1 ripened first, second and third, with a significant difference only between the first and third parent. On the West campus fruit of CHE2 ripened first followed by that of AME and RET1. A significant difference was also only found between the first and third parent. In the glasshouse CHE2, RET1 and AME's fruits were the first to ripen respectively, no significant differences were found between these genotypes. Averaged over all three locations CHE2 again ranked first, with AME second although the difference was not significant. RET1 ranked third. SCAxAME and AMExSCA were the hybrids displaying the largest number of days to fruit ripening at the lowest three positions at all three localities and across localities. Again it seemed that there were no big rank differences between the three localities. Days to fruit ripening were significantly correlated with important yield attributes like fruit per bunch, weight per fruit bunch, single fruit weight, fruit size, and total weight of the fruit harvest (Table 4.11).

Seeds per fruit

The fruit from SCA harvested on the West campus contained the most seeds. This parent differed significantly from the hybrids SCAxAME and AMExSCA ranked second and third. In the glasshouse SCA ranked first followed by CHE2 and then the hybrids SCAxAME and AMExSCA ranked third and fourth. The differences between SCA and the other accessions were significant, but no significant differences were found between the other accessions. At the nursery, SCA again ranked first, followed by SCAxAME and then AMExSCA, although differences between the hybrids were not significant, significant differences occurred between the parent SCA and the hybrids. Across all three localities SCA ranked first, followed by SCAxAME and then by AMExSCA with significant differences between SCA and the hybrids. Once again the rankings seemed to be very stable across the different localities, especially where RET1 was found ranking at the bottom of the list for two of the localities and across locations as well. The mid-parent heterosis (Table 4.7) and the highest parent heterosis (Table 4.8) were negative as were expected with the good performance of the SCA parent. Seed per fruit was significantly correlated with the most important yield characteristics, especially with the weight of a bunch of fruit (Table 4.9).

Seeds in 0.1g

CHE2 ranked first for this characteristic in all three the localities and significant differences were found between this parent and all the other genotypes. The highest number of seeds in 0.1g was found for the fruit that formed in the glasshouse and RET4xRET1 and AME were ranked second and third and no significant differences were found between them at this location. At the nursery RET4 and AME were ranked second and third and the differences between them were also not significantly. On the West campus RET4 and SCAxAME ranked second and third respectively, and again no significant differences were found. The averaged value over all three locations displayed CHE as the top ranked genotype, as expected, followed by RET4 and AME with significant differences between the top ranked CHE2 and other accessions. SCA was at the lowest three positions at all three localities and across localities. It seemed that there were no major rank differences between the three localities (Table 4. 6). The amount of heterosis displayed was very low or negative for the mid-parent (Table 4.7) and negative for the best parent as expected (Table 4.8). Seeds per 0.1g were negatively correlated with the other characteristic except plant height (Table 4.9).

Flowers per bunch

The plants at the commercial nursery displayed the highest average number of flowers per bunch. The hybrid SCAxAME ranked the highest followed by the reciprocal AMExSCA and then the parent SCA (the differences between them were significantly). The hybrids RET4xRET1 and CHE2xRET4 as well as the parent RET4 displayed the same number of flowers per bunch. On the West campus as well as in the glasshouse SCA ranked first, followed by AMExSCA and then SCAxAME. The differences between the accessions at both locations were not significant. The hybrids RET4xCHE2 and CHE2xRET4 had the same amount of flowers per bunch and this was also found for the parents RET4 and RET1 in both localities. Across all three localities SCAxAME ranked first, followed by SCA and then by AMExSCA with no significant differences between them. RET1 ranked last across localities and at each locality separately. Once again it seemed that there were no major ranking differences found between the different localities. The mid-parent heterosis for this characteristic was lower than 23% (Table 4.7) and the highest parent heterosis was very low (Table 4.8). This characteristic was also highly correlated with characters like fruit per bunch, weight per bunch and weight per fruit (Table 4.9).

Fruit per bunch

The most fruits per bunch were found for the plants in the glasshouse and no significant differences were found between the top ranking (SCAxAME, SCA and AMExSCA) accessions. The parent RET1 and the hybrid RET1xRET4 produced the same amount of fruit per bunch at this locality. At the nursery RET1, RET1xRET4, RET4xRET1 and RET4 all displayed the same number of fruit per bunch. No significant differences were found between the top ranking hybrids SCAxAME and AMExSCA but significant difference occurred between them and the third ranked parent SCA. On the West campus SCA was ranked first followed by AMExSCA and then SCAxAME (the differences were not significant). At this location CHE2 and AME displayed the same amount of fruit per bunch and so did RET1xRET4 and RET4. Across all three localities SCAxAME ranked first, followed by AMExSCA and then by SCA. RET4 ranked last across localities and at each locality separately as well. Again it seem that there were no big rank differences between the three localities (Table 4.6). The good performance of the hybrids SCAxAME and AMExSCA were confirmed with its high mid-parent heterosis values (Table 4.7) and highest parent heterosis (Table 4.8). This characteristic was significantly correlated with seed per fruit, weight per fruit bunch, single fruit weight, fruit size, and total weight of the fruit harvest (Table 4.9).

Weight per bunch

SCA produced the heaviest bunches of fruit. This parent ranked first in all three the localities, and differed significantly from the other accessions. In the glasshouse and at the nursery SCAxAME and AMExSCA ranked second and third and no significant differences were found between the two hybrids. On the West campus AMExSCA ranked second and its reciprocal SCAxAME was third on the ranking list. The average over all three locations displayed SCA as the top ranked genotype as expected, followed by SCAxAME and AMExSCA with significant differences between the parent and both hybrids. CHE2 ranked at the bottom at all three localities and across localities. It again appeared that there were no big rank differences between the three localities. As expected most of the heterosis values displayed were negative for both mid- and high parent values (Table 4.7 and 4.8). This characteristic was significantly correlated especially with seeds per fruit, weight per fruit and fruit size (Table 4.9).

Weight per fruit

The fruit on the West campus was heavier than that grown at the other two locations. As expected, SCA produced the heaviest fruit, with its hybrids SCAxAME and AMExSCA ranking second and third, with significant differences between the parent and both hybrids. In the glasshouse, SCA once again ranked first, followed by the hybrid AMExSCA and RET1. The hybrid SCAxAME ranked fourth. Significant differences were found between SCA and the other accessions. The plants at the nursery displayed a similar ranking, with the hybrids AMExSCA and SCAxAME ranked second and third. The average over all three locations displayed SCA as top ranked as expected, followed by AMExSCA and SCAxAME, with significant differences between the parent and the hybrids. The hybrids displayed negative heterosis values (Table 4.7 and 4.8) as expected. AME and CHE2 ranked at the bottom at all three localities and across localities. It would therefore seem that there were no major rank differences between the three localities. Weight per fruit was significantly correlated with seeds per fruit, fruit per bunch, weight per fruit bunch, fruit size and total weight of the fruit harvest (Table 4.9).

Fruit size

The fruit on the West campus was slightly bigger than that at the other two locations. SCA produced significantly bigger fruit than the hybrids SCAxAME and AMExSCA which were ranked second and third. At the commercial nursery and in the glasshouse, SCA once again produced significantly bigger fruit, followed by the hybrid AMExSCA and its reciprocal SCAxAME. Averaged over all three locations SCA ranked first as expected with AMExSCA second and SCAxAME ranked third, with significant differences between the parent and the hybrids. It was expected for the hybrids to display a negative mid-parent and highest parent heterosis as seen in Table 4.7 and Table 4.8. CHE2 was at the lowest ranking position at all three localities and across localities. It seemed once again that there were no big rank differences between the three localities (Table 4.6). Fruit size was significantly correlated with important yield attributes like seeds per fruit, fruit per bunch, weight per fruit bunch, single fruit weight and total weight of the fruit harvest (Table 4.9).

Total weight

Plants on the West campus yielded the highest average total weight. SCA as expected ranked first and differed significantly from SCAxAME and AMExSCA that ranked second and third. In the glasshouse no significant differences were found between SCA, RET1 and AMExSCA that ranked first, second and third. The hybrid SCAxAME ranked fourth and differ significantly from the high ranked parent SCA. At the commercial nursery, SCA was followed by AMExSCA and then by SCAxAME, with no significant differences between the hybrids but significant differences between the hybrids and the top ranking parent SCA. For the average over all three locations, as expected, SCA again ranked first, with SCAxAME second with a significant difference between the two genotypes. AMExSCA ranked third. The good performance of the parent SCA explained the negative mid-parent (Table 4.7) and highest parent heterosis (Table 4.8). Changes did occur in the ranking of the rest of the genotypes and differences were found between the three localities (Table 4. 6). Total weight was significantly correlated with important yield attributes especially with weight per fruit bunch and single fruit weight (Table 4.9).

Total number of fruit harvested

Total yield was the highest in the glasshouse. Here CHE2 yielded the most fruit and was followed by SCAxAME, RET1 and AMExSCA (the differences between all four were not significant). On the West campus SCAxAME ranked first, followed by the parent SCA and no significant difference was found between them. RET4xRET1 and RET4 followed this with significant differences between them and the top ranking genotypes but not between themselves. At the nursery SCAxAME ranked first followed by AMExSCA and the difference between them were not significant. CHE2 and SCA that differ significantly from these top ranking hybrids ranked thirds and fourth. Across all three localities SCAxAME ranked first, followed by AMExSCA and then by RET1. RET1xRET4 was at the lowest three positions at all three localities and across localities. It seems that there were no big ranking differences between the three localities. The percentage mid-parent and highest parent heterosis for SCAxAME was the highest (Table 4.7 and 4.8). This characteristic was significantly correlated with the important yield characteristics, such as fruit per bunch, weight per fruit bunch and total weight of fruit harvested (Table 4.9).

Table 4.3 Means of the different morphological characteristics for the accessions tested in the glasshouse.

CHARACTERS	AME	RET1	RET4	CHE2	SCA	AMEX SCA	SCAX AME	CHE2X RET4	RET4X CHE2	RET1X RET4	RET4X RET1	LSD*
Plant height	1017.50	412.50	405.00	797.50	1115.00	1300.00	1145.00	320.00	495.00	350.00	337.50	164.6192
Plant spread	837.50	755.00	1075.00	1010.00	1205.00	1080.00	1277.50	790.00	767.50	902.50	862.50	241.6143
Leaf area	20.21	11.95	7.89	4.05	23.43	25.89	30.96	7.09	5.07	15.45	13.45	7.2425
Days to flower	32.75	20.25	23.50	36.75	36.75	33.00	33.25	62.75	37.50	22.00	16.00	1.8267
Days to fruit form	13.00	13.75	15.25	11.75	16.00	18.50	28.50	-	-	11.50	9.25	3.2039
Days to fruit ripe	18.25	17.75	20.50	15.75	21.75	25.25	29.25	-	-	22.50	31.00	5.9714
Seeds per fruit	33.25	31.25	31.25	42.75	98.75	36.25	40.75	-	-	32.50	30.50	7.91110
Seeds per 0.1g	113.75	79.75	108.25	252.00	96.75	111.75	96.00	-	-	104.50	118.75	18.8572
Flower per bunch	5.50	4.25	4.25	5.00	8.50	8.00	7.75	5.25	5.25	4.50	4.75	0.8677
Fruit per bunch	6.00	4.75	3.75	5.25	8.25	8.25	9.00	-	-	4.75	4.25	1.1714
Weight per bunch	1.85	2.04	1.28	0.95	9.79	3.84	4.14	-	-	1.36	1.56	0.9361
Weight per fruit	0.26	0.44	0.30	0.14	1.18	0.47	0.42	-	-	0.30	0.33	0.0670
Fruit size	7.30	8.88	8.08	6.58	13.58	9.73	9.33	-	-	8.08	8.15	0.5099
Total weight	133.45	414.81	181.50	160.27	476.32	346.74	214.47	-	-	77.49	125.98	151.1685
Total number of fruit harvested	542.50	942.00	654.50	1158.75	416.25	771.25	983.75	-	-	244.75	392.75	393.7306

* $p = 0.05$

Table 4.4 Means of the different morphological characteristics for the accessions tested on the West-campus.

CHARACTERS	AME	RET1	RET4	CHE2	SCA	AMEX SCA	SCAX AME	RET4X CHE2	CHE2X RET4	RET1X RET4	RET4X RET1	LSD*
Plant height	820.00	317.50	175.00	617.50	452.50	945.75	626.25	242.00	222.00	240.00	290.00	90.4033
Plant spread	1396.25	1140.00	1191.00	1116.25	1577.50	1416.25	1585.00	836.25	800.00	1396.00	1300.00	304.8442
Leaf area	25.25	7.22	8.03	8.21	37.66	40.14	32.06	5.68	4.85	6.61	10.45	7.9210
Days to flower	27.00	22.25	27.25	26.25	42.25	36.00	34.50	35.25	35.00	23.75	19.00	2.3316
Days to fruit form	14.00	15.50	17.75	12.50	18.50	21.00	27.50	-	-	13.00	12.00	2.5076
Days to fruit ripe	16.75	18.25	19.50	14.75	24.25	23.75	22.50	-	-	20.25	19.75	3.0562
Seeds per fruit	37.25	32.00	36.50	39.00	108.00	51.00	56.75	-	-	34.25	35.50	3.8092
Seeds per 0.1g	140.50	141.75	150.25	212.00	113.75	116.75	148.75	-	-	138.50	114.75	7.5157
Flower per bunch	5.50	4.25	4.25	5.00	8.50	8.00	7.75	5.25	5.25	4.50	4.75	0.8677
Fruit per bunch	5.00	3.75	3.50	5.00	8.00	7.75	6.75	-	-	3.50	4.50	1.2497
Weight per bunch	1.12	1.42	1.35	0.67	10.50	3.59	3.54	-	-	1.45	1.49	0.6854
Weight per fruit	0.22	0.37	0.32	0.15	1.30	0.45	0.58	-	-	0.28	0.31	0.1227
Fruit size	6.65	8.33	7.95	6.08	13.68	9.20	9.78	-	-	7.43	7.73	0.7118
Total weight of fruit harvest	51.21	216.75	231.82	26.23	1336.22	306.66	706.77	-	-	148.64	245.13	114.8202
Total number of fruit harvested	239.50	581.00	729.00	175.75	1026.50	679.00	1103.25	-	-	538.75	781.50	190.6982

* $p = 0.05$

Table 4.5 Means of the different morphological characteristics for the accessions tested at the commercial nursery.

CHARACTERS	AME	RET1	RET4	CHE2	SCA	AMEX SCA	SCAX AME	CHE2X RET4	RET4X CHE2	RET1X RET4	RET4X RET1	LSD*
Plant height	972.50	250.00	260.00	532.50	942.50	933.75	675.00	237.50	245.00	182.50	245.00	251.5416
Plant spread	682.50	470.00	795.00	717.50	867.50	850.00	852.50	345.00	465.00	700.00	687.50	178.9706
Leaf area	13.79	4.72	4.83	5.88	41.08	29.62	31.87	2.64	5.28	6.37	8.22	4.9880
Days to flower	25.50	20.75	26.00	25.50	77.75	28.50	19.25	22.00	25.50	15.75	16.25	8.7148
Days to fruit form	13.25	17.25	17.00	10.75	18.00	18.50	21.50	-	-	14.50	16.50	3.4390
Days to fruit ripe	14.00	18.00	18.25	14.75	21.25	22.50	20.50	-	-	18.25	18.00	3.1539
Seeds per fruit	36.75	25.75	39.00	37.25	88.50	41.75	45.24	-	-	31.75	32.75	8.6440
Seeds per 0.1g	142.75	118.00	156.25	239.50	117.75	127.25	131.00	-	-	121.50	118.25	23.7511
Flower per bunch	5.50	3.50	4.00	4.25	7.75	8.75	9.75	4.00	4.25	3.50	4.00	0.7161
Fruit per bunch	5.00	3.00	3.00	4.25	6.75	8.00	8.75	-	-	3.00	3.00	0.8128
Weight per bunch	0.93	0.73	0.87	0.42	5.80	2.41	2.73	-	-	0.81	1.10	0.4927
Weight per fruit	0.19	0.22	0.24	0.10	0.98	0.43	0.34	-	-	0.28	0.30	0.0984
Fruit size	6.60	7.05	7.30	5.60	13.33	9.08	8.43	-	-	7.73	7.78	0.8730
Total weight	26.96	24.89	16.31	21.66	140.36	107.86	105.68	-	-	18.89	22.06	25.4090
Total number of fruit harvested	139.50	115.00	68.50	196.00	152.50	259.75	290.75	-	-	70.00	72.25	61.5160

* $p = 0.05$

Table 4.6 Means of the different morphological characteristics for the accessions tested across localities.

CHARACTERS	AME	RET1	RET4	CHE2	SCA	AMEX SCA	SCAX AME	CHE2X RET4	RET4X CHE2	RET1X RET4	RET4X RET1	LSD*
Plant height	936.67	326.67	280.00	649.17	836.67	1059.83	815.42	259.83	327.33	257.50	290.83	102.4635
Plant spread	972.08	788.33	1020.33	947.92	1216.67	1115.42	1238.33	645.00	689.58	999.50	950.00	139.7582
Leaf area	19.75	7.96	6.92	6.05	34.06	31.88	31.63	4.86	5.34	9.48	10.71	3.8631
Days to flower	28.42	21.08	25.58	29.50	52.25	32.50	29.00	39.92	92.75	20.67	16.92	3.0043
Days to fruit form	13.42	15.50	16.67	11.67	17.50	19.33	25.83	-	-	13.00	12.58	1.7295
Days to fruit ripe	16.33	18.00	19.42	15.08	22.42	23.83	24.08	-	-	20.33	22.92	2.4064
Seeds per fruit	35.75	29.67	35.58	39.67	98.42	43.00	47.58	-	-	33.17	32.58	4.0001
Seeds per 0.1g	132.33	113.17	138.25	234.50	109.42	118.58	125.25	-	-	121.50	117.25	10.1433
Flower per bunch	5.50	4.00	4.17	4.75	8.25	8.25	8.42	4.83	4.92	4.17	4.50	0.4637
Fruit per bunch	5.33	3.83	3.42	4.83	7.67	8.00	8.17	-	-	3.75	3.92	0.6155
Weight per bunch	1.30	1.39	1.17	0.68	8.70	3.28	3.47	-	-	1.21	1.39	0.4092
Weight per fruit	0.22	0.34	0.29	0.13	1.15	0.45	0.45	-	-	0.28	0.31	0.0555
Fruit size	6.85	8.08	7.78	6.08	13.53	9.33	9.18	-	-	7.74	7.88	0.4014
Total weight of fruit harvest	70.54	218.82	143.21	69.38	650.96	253.75	342.31	-	-	81.67	131.06	62.1773
Total number of fruit harvested	307.17	546.00	483.67	510.17	531.75	570.00	792.58	-	-	284.50	415.50	143.4237

* $p = 0.05$

Table 4.7 Heterosis of the F1 hybrids calculated from the mid-parent values.

	PH	PS	LA	D/FL	D/FF	D/FR	S/F	S/G	FL/B	FR/B	W/B	W/F	FS	TW	TY
AMEx SCA	19.53	1.92	18.49	-19.42	25.03	22.99	-35.90	-1.90	20.00	23.08	-34.40	-34.31	-8.44	-29.66	35.89
SCAx AME	-8.04	13.15	17.56	-28.10	67.08	24.28	-29.08	3.62	22.47	25.69	-30.60	-34.31	-9.91	-5.11	88.95
CHE2x RET4	-44.07	-34.46	-25.06	44.95					8.30						
RET4x CHE2	-29.54	-29.93	-17.66	236.7					10.31						
RET1x RET4	-15.11	10.52	27.42	-11.40	-19.18	8.66	1.67	-3.35	2.08	3.45	-5.47	-11.11	-2.40	-54.88	-44.74
RET4x RET1	-4.12	5.05	43.95	-27.48	-21.79	22.50	-0.14	-6.73	10.16	8.14	8.59	-1.59	-0.63	-27.60	-19.29

Table 4.8 Heterosis of the F1 hybrids calculated from the best-parent values.

	PH	PS	LA	D/FL	D/FF	D/FR	S/F	S/G	FL/B	FR/B	W/B	W/F	FS	TW	TY
AMEx SCA	13.15	-8.32	-6.40	-37.80	10.46	6.29	-56.31	-10.39	0.00	4.30	-62.30	-60.87	-31.04	-61.02	7.19
SCAx AME	-12.94	1.78	-7.13	-44.50	47.60	7.40	-51.66	-5.35	2.06	6.52	-60.11	-60.87	-32.15	-47.41	49.05
CHE2x RET4	-59.98	-36.79	-29.77	35.32					1.68						
RET4x CHE2	-49.58	-32.42	-22.83	214.4					3.58						
RET1x RET4	-8.04	-2.04	19.10	-19.19	-22.02	4.69	-6.77	-12.12	0.00	-2.09	-12.95	-17.65	-4.21	-62.68	-47.89
RET4x RET1	3.87	-6.89	34.55	-33.85	-24.54	18.02	-8.43	-15.19	7.91	2.35	0.00	-8.82	-2.48	-40.11	-23.90

Plant Height (PH), Plant Spread (PS), Leaf Area (LA), Days to Flowering (D/FL), Days to Fruit Forming (D/FF), Days to Fruit Ripening (D/FR), Number of Seeds in a Fruit (S/F), Number of Seeds Weighing 0.1g (S/G), Flowers per Bunch (FL/B), Fruit per Bunch (FR/B), Weight per Fruit Bunch (W/B), Weight of a Single Fruit (W/F), Fruit Size (FS), Total Weight of Fruit Harvest (TW) and Total Number of Fruit Harvested (TY)

Table 4.9 Linear correlation matrix for all the characters measured over all the locations.

	PH	PS	LA	D/FL	D/FF	D/FR	S/F	S/G	FL/B	FR/B	W/B	W/F	FS	TW
PS	0.1608	-												
LA	0.5653**	0.4047**	-											
D/FL	0.2522**	0.1100	0.3685**	-										
D/FF	0.4055**	0.2407	0.5192**	0.0239	-									
D/FR	0.0781	0.3771**	0.3525**	0.2100	0.2989**	-								
S/F	0.4150**	0.2820**	0.5118**	0.4747**	0.2351	0.2173	-							
S/G	0.0810	-0.1867	-0.2965**	-0.1053	-0.2628**	-0.4034**	-0.1135	-						
FL/B	0.5874**	0.3573**	0.7706**	0.3227**	0.4843**	0.3420**	0.5656**	-0.2151	-					
FR/B	0.5513**	0.4487**	0.7251**	0.2765**	0.4572**	0.3681**	0.5106**	-0.1916	0.8662**	-				
W/B	0.3690**	0.4372**	0.6227**	0.4034**	0.3449**	0.3949**	0.9003**	-0.3514**	0.6443**	0.6202**	-			
W/F	0.3057**	0.3621**	0.5498**	0.4525**	0.3131**	0.3639**	0.8959**	-0.3922**	0.5380**	0.4833**	0.9495**	-		
FS	0.2815**	0.3654**	0.5992**	0.4946**	0.3745**	0.4333**	0.8302**	-0.4688**	0.5761**	0.5289**	0.9063**	0.9593**	-	
TW	0.3630**	0.3940**	0.3347**	0.1817	0.3233**	0.3260**	0.6406**	-0.2160	0.4317**	0.3906**	0.7509**	0.7343**	0.6556**	-
TH	0.2214	0.4763**	0.1796	0.0834	0.3040**	0.3041**	0.1498	-0.0128	0.2160	0.2943**	0.2862**	0.2242	0.2311	0.6597**

Plant Height (PH), Plant Spread (PS), Leaf Area (LA), Days to Flowering (D/FL), Days to Fruit Forming (D/FF), Days to Fruit Ripening (D/FR), Number of Seeds in a Fruit (S/F), Number of Seeds Weighing 0.1g (S/G), Flowers per Bunch (FL/B), Fruit per Bunch (FR/B), Weight per Fruit Bunch (W/B), Weight of a Single Fruit (W/F), Fruit Size (FS), Total Weight of Fruit Harvest (TW) and Total Number of Fruit Harvested (TH)

** Significant at p = 0.01

4.3.3 *Combined ANOVA*

Combined analyses were done for the trails over all the locations. The results listed in Table 4.10a were the analysis done for 10 of the characteristics measured on the nine accessions that produced fruit. Table 4.10b depicted the rest of the characteristics (5) measured on all 11 accessions. All the characters in both tables displayed highly significant differences (p>0.01) for all the entries. The effect of the environment (locations) was highly significant for all the characteristics except days to fruit form (Table 4.10a) and leaf area (Table 4.10b). The genotype x environment interaction was significant for most characteristics except for fruit per bunch and fruit size (Table 4.10a) as well as plant spread (Table 4.10b).

Table 4. 10a Mean squares for the morphological characteristics measured for nine of the accessions over all locations.

CHARACTER	ENTRIES	LOCATION	LOC BY ENTRIES
Days to fruit form	234.938**	23.583*	16.833**
Days to fruit ripe	130.704**	150.398**	24.252*
Seeds per fruit	5378.704**	404.704**	82.610**
Seeds per 0.1g	17862.750**	5528.028**	1080.319**
Fruit per bunch	46.766**	10.481**	1.294
Weight per bunch	75.883**	15.721**	2.294**
Weight per fruit	1.060**	0.120**	0.016**
Fruit size	54.927**	5.179**	0.464
Total weight	419634.511**	871211.236**	179132.958**
Total number of fruit harvested	276572.975**	3165269.009**	260752.884**

*, ** Significant at P> 0.05 and P> 0.01 probability levels respectively.

Table 4. 10b Mean squares for the five morphological characteristics measured for 11 accessions over all locations.

CHARACTER	ENTRIES	LOCATION	LOC BY ENTRIES
Plant height	1177401.883**	772402.053**	53137.145**
Plant spread	449837.718**	3633542.303**	42579.036
Leaf area	1663.386**	95.001*	84.684**
Days to flower	1161.706**	243.462**	428.754**
Flower per bunch	37.971**	1.705*	1.221**

*, ** Significant at P> 0.05 and P> 0.01 probability levels respectively.

4.4 Discussion and conclusions

Edmonds and Chweya (1997) stated that no cultivars for species belonging to the *Solanum nigrum* complex have yet been developed through conventional plant breeding techniques. In this study five parental species and their progenies were studied, and 15 morphological characteristics were measured. Morphological characterization continues to be the first step for the description and classification of the newly formed hybrids in any program. Statistical methods are also important and can be used as a useful tool to screen the new accessions and make certain predictions.

In this study, the simple and combined analysis of variance was done on the characteristics and both showed significant differences for entries. Significant differences were also found among the accessions reflecting the extensive genetic variation between them. SCA ranked first for important characteristics such as seeds per fruit, weight per bunch, weight of a single fruit, fruit size and total weight harvested. One of the hybrids with SCA as female parent (SCAxAME) performed well in most of the other characteristics measured such as flowers per bunch, fruit per bunch and total yield. These results were confirmed by the high mid-parent heterosis values calculated for these characteristics.

In the combined analysis the mean squares for locality by entry was significant for most characteristics, which indicates that they were influenced by the environment. Despite this, the rankings did not vary that much between the localities. Fruit per bunch, fruit size and plant spread were not influenced by the environment, and are therefore probably highly heritable. Selection for these characteristics should lead to rapid genetic improvement in plants. Plant spread and fruit per bunch were both significantly correlated with total yield, and selection for these characteristics will lead to a yield increase.

CHE2 was included because of its tasty fruit. This parent was, however, incompatible with the other parents and yielded no progeny. SCA was excellent in terms of all the most important characteristics, and this was expressed in its progenies, especially the SCAxAME cross. AME was also found to bear tasty fruit (Chapter 8). Selecting the best progeny from the SCAxAME may therefore be a good starting point for combining good production with a good taste in the fruit. This cross and its reciprocal also showed very high mid and higher parent heterosis for total yield, and can therefore be used for hybrid breeding purposes.

Based on the correlation values, the most important yield components were weight per fruit bunch, single fruit weight, fruit size and total weight of fruit harvest. Selection of characteristic correlated with total yield such as fruit per bunch, weight per bunch and total weight will probably result in a plant with a much higher yield because of the relatively small environmental influence.

In conclusion, we believe that hybrids with valuable morphological traits can be developed and that parents like SCA and AME can be employed in a breeding program.

Chapter 5

Cytogenetic and crossability studies in five species of the *Solanum nigrum* complex

5.1 Introduction

Solanum section *Solanum*, also known as the *S. nigrum* complex, is a cosmopolitan group of plants of about 30 perennial herbaceous species (Schilling, 1981). Members of this complex are highly variable phenotypically and form a polyploid series, with diploid (2n=2x=24), tetraploid (2n=4x=48) and hexaploid (2n=6x=72) species (Stebbins and Paddock, 1949; Edmonds and Chweya, 1997). These ploidy differences may provide a barrier to hybridization between morphologically similar plants leading to cyto-races difficult to differentiate using classical methods (Edmonds, 1977).

The limited knowledge of the evolutionary relationships between species of *Solanum* and the lack of defined boundaries between most species has posed problems for taxonomists (López and Hawkes, 1991). Daunay *et al.* (1995) also stated that the nightshades are not well known and their genetic resources are not organized. Biosystematic studies by Jörgensen (1928), Henderson (1974) and Khan *et al.* (1977) have also shown the complexity of this group.

Species belonging to the complex are predominantly self-pollinating, but out and cross breeding does occur (Edmonds, 1979a). A number of researchers reported inter- and intra-specific hybridization, especially among the smaller-flowered diploids (Edmonds and Chweya, 1997). Venkateswarlu and Rao (1972) reported natural hybrids, also at higher ploidy levels, e.g. intra-specific hybrids of the hexaploid *S. nigrum*. Where accession distribution overlaps, natural hybrids were also reported among the *Solanum nigrum* complex, despite their different ploidy levels, such as between the hexaploid *S. scabrum* and the diploid *S. americanum* (Henderson, 1974). Edmonds and Chweya (1997) stated that natural hybridization is probably more widespread in this section of *Solanum* than generally supposed and that it is probably followed by subsequent genetic breakdown in the F1 and F2 generations.

Failure to obtain F1 hybrids for some accessions can result from genetic or from cytoplasmic incompatibilities that are expressed either in failure of fertilization or in death of the zygote at any stage between early cleavage divisions and maturity (Allard, 1960). Hawkes (1958) reported that among the intrinsic factors that reduce crossability in *Solanum* are sterility barriers or mechanisms such as gametic sterility, self-incompatibility, one way incompatibility or endosperm and embryo abortion. Extrinsic factors such as temperature, humidity, light intensity, day length etc. must also be considered to play a role in a breeding program (Hawkes, 1958). Cribb and Hawkes (1986) reported that high temperature and low moisture, or sudden fluctuations of both caused bud and/ or flower dropping.

No cultivars have yet been developed through conventional plant breeding techniques, but local variants or landraces may have been selected in some of the regions in which these plants are utilized as food and/or medicinal plants (Edmonds and Chweya, 1997).

The identity of the genotypes included in this study was not absolutely certain, although they were distinguishable by means of morphological characters. Singh *et al.* (1992) stated that identification and inter- relationship of species can be determined adequately only with the help of cytology, genetics and biosystematics. The purpose of this work was to verify the species by chromosome numbers and to investigate the crossability of the species.

5.2 Materials and methods

5.2.1 *Mitosis*

Randomly selected root tips from each species listed in Table 5.1 were used for chromosome counting. The seeds were germinated in petri dishes under non-sterile conditions. Ten actively growing root tips of each species were cut and pre-treated in cold water at 4°C for 24h. The tips were then transferred to Carnoy's fixative (6:3:1 ethanol/ chloroform/ acetic acid) for 48h. The tips were hydrolysed in 1N HCl at 60°C for 7min. The root tips were then rinsed in distilled water and stained in leuco-basic fuchsin for at least 2h at 4°C, refrigerated in the dark. Approximately 2mm of the stained root tips were squashed in a small drop of aceto-orcein. Photographs were taken through a Nikon microscope.

Table 5.1 *Solanum* species and abbreviations used in this study.

SPECIES	ABB.
Solanum americanum Mill.	AME
Solanum burbankii Bitter	RET1
Solanum retroflexum Dun. (smooth)	RET4
Solanum chenopodioides Lam.	CHE2
Solanum scabrum Mill.	SCA

5.2.2 *Hybridization*

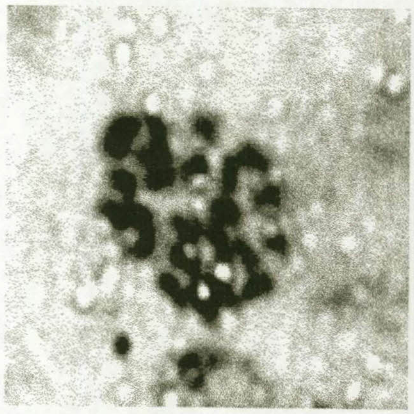
The *Solanum* species listed in Table 5.1 and seeds from two cocktail tomatoes (SUN and JOS) were included in the hybridization study. Mayfords, a seed company supplied the tomato seeds. These plants selected as parents for this crossability study were raised from seeds, in a glasshouse under uniform experimental conditions. Cross-pollinations were made reciprocally between the five species (Table 5.1) and the two tomato cultivars (SUN and JOS). The young buds were emasculated one day prior to anthesis as recommended by Tigchelaar (1986), to avoid accidental self-pollination. The stigmas remain receptive for three to five days after the opening of the flower buds, and are thus unaffected by early emasculation (Edmonds and Chweya, 1997). The emasculated flowers were bagged to prevent pollination by any unknown source. Pollen was transferred from the male donor plant to the female, by removing the flower of the donor and tapping the pollen out over the stigma of the female plant. The female flower was then covered again with a small paper bag, to prevent any other pollen to get in contact with the stigma. Each flower was labeled, showing the cross details. All the utensils were sterilized with 70% ethanol between each pollination, to prevent cross-contamination.

5.3 Results and discussion

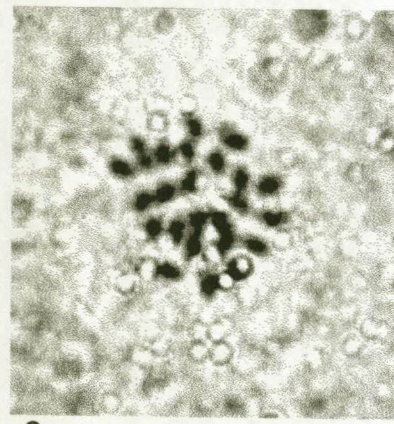
5.3.1 *Cytology of the Solanum species*

Cytological analyses of the five *Solanum* species listed in Table 5.1 were carried out. Only chromosome number was taken into account in this study (Figure 5.1). With regard to the chromosome numbers in the species accessions, no variations were found. The species were found to fit into a normal euploid series 2x-4x-6x.

Two species *S. americanum* Mill. (AME) and *S. chenopodioides* Lam. (CHE2) were diploid (2n=2x=24). *S. retroflexum* Dun. (RET4) and *S. burbankii* Bitter (RET1) were both tetraploid (2n=4x=48). *S. scabrum* L. (SCA) was hexaploid (2n=6x=72). The results helped in confirming the species identity. Similar results were reported by Horsman *et al.* (1997).



1



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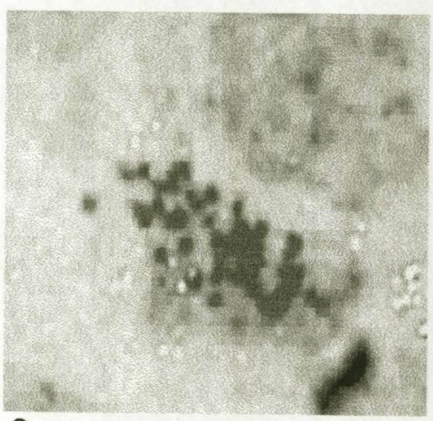

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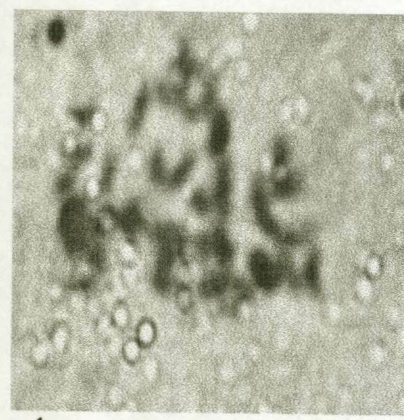

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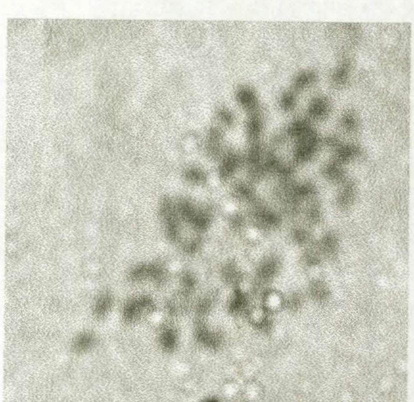

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Figure 5.1 Chromosomes of 1 - *S. americanum* Mill. (AME), 2 - *S. chenopodioides* Lam. (CHE2), 3 - *S. retroflexum* Dun. (RET4), 4 - *S. burbankii* Bitter (RET1), 5 - *S. scabrum* L. (SCA).

5.3.2 *Hybridization*

Crosses were made between the species shown in Table 5.2. For each combination 20 emasculated flowers were pollinated with another species.

Table 5.2 Number of berries, seeds and germination (%) obtained from the various crosses.

CROSS	BERRIES	SEEDS	GERMINATION %
SUN x AME	1	4	0
SUN x RET1	1	8	0
SUN x CHE2	1	13	0
SUN x RET4	1	9	0
SUN x SCA	2	0	0
JOS x AME	1	10	0
JOS x RET1	1	12	0
JOS x CHE2	1	68	0
JOS x RET4	1	15	0
JOS x SCA	1	34	0
AME x RET1	1	2	0
AME x CHE2	6	0	0
AME x RET4	5	0	0
AME x SCA	7	250	85
AME x SUN	1	0	0
AME x JOS	1	0	0
RET1 X AME	2	0	0
RET1 X CHE2	6	49	78
RET1 X RET4	5	55	49
RET1 X SCA	2	0	0
RET1 x SUN	1	0	0
RET1 x JOS	1	0	0
CHE2 X AME	6	19	0
CHE2 X RET1	5	15	38
CHE2 X RET4	9	121	80
CHE2 X SCA	9	51	10
CHE2 x SUN	1	0	0
CHE2 x JOS	1	0	0
RET4 X AME	6	0	0
RET4 X CHE2	4	43	84
RET4 X RET1	6	86	70
RET4 X SCA	10	0	0
RET4 x SUN	1	0	0
RET4 x JOS	1	0	0
SCA X AME	5	51	80
SCA x RET1	3	0	0
SCA x CHE2	8	0	0
SCA x RET4	3	0	0
SCA x SUN	1	0	0
SCA x JOS	1	0	0

For each cross in Table 5.2 the female parent is on the left and pollen source on the right.

Not all the crosses were successful, especially the crosses between the *Solanum* species and the cocktail tomatoes (SUN and JOS). Where the tomatoes were emasculated and use as female plants, fertilization by *Solanum* pollen resulted in berry set but the seeds from the crosses failed to germinate. Where the tomatoes were used as pollen parent tiny fruits formed for all the *Solanum* species but the fruit aborted and fell off long before maturity.

All the crosses made between the *Solanum* species, resulted in berry set. Some of the combinations, however, only resulted in a 5% berry set (Table 5.2). Only 50% of the crosses formed seeds but only 22.5% of these germinated and produced healthy seedlings. The mean number of seeds per fruit, was found to be less than that compared with those of their parents, this was also reported by Ganapathi and Rao (1985) in their study on diploid species of the *Solanum nigrum* complex.

Though the diploids (AME and CHE2) were crossed with each other in both directions, no progeny were obtained. The diploid CHE2 crossed with the tetraploids, and progeny were obtained for the reciprocal crosses as well. The diploid AME crossed with tetraploids, however, resulted in no progeny. The crosses between the diploids and hexaploid (SCA) resulted in hybrids but only the reciprocal cross between SCA X AME yielded progeny. The crosses and reciprocal crosses between the two tetraploids (RET1 and RET4) readily produced progeny. Progeny between the tetraploids and hexaploids could not be obtained.

The following crosses and reciprocal crosses were successful:

- *S. americanum* X *S. scabrum* and *S. scabrum* X *S. americanum;*
- *S. burbankii* X *S. chenopodioides* and *S. chenopodioides* X *S. burbankii*;
- *S. burbankii* X *S. retroflexum* and *S. retroflexum* X *S. burbankii*
- *S. chenopodioides* X *S. retroflexum* and *S. retroflexum* X *S. chenopodioides*.

The only other cross that produced mature progeny was *S. chenopodioides* X *S. scabrum*.

The F1 plants of *S. burbankii* X *S. chenopodioides; S. chenopodioides* X *S. burbankii*; and *S. chenopodioides* X *S. retroflexum*; *S. retroflexum* X *S. chenopodioides* were fully sterile. The hybrids exhibited vigorous growth with abundant flowering but failed to produce fruits in all cases.

5.4 Conclusions

Although hybridization attempts often appeared successful, the berries that formed were often found to be empty or to contain non-viable seeds. Edmonds (1977) also found similar results in the study on *Solanum*. Beg *et al.* (1989) stated that the production of sterile hybrids indicated that the species were not closely related to each other.

Crosses between diploids and tetraploids were only successful in some combinations (Table 5.2), thus showing that the two diploid species exhibited a difference in their crossability to the same tetraploid. Venkateswarlu and Roa (1972) stated in their study that it is probable that the genomes of the two diploids (AME and CHE2) also differ in genes, which control crossability with tetraploids. Thus all diploids are not isolated from the tetraploids to the same extent; some species seemed to have diverged enough as to be unable to cross as seed parents while others still remained at a lower level of differentiation retaining the ability to cross with tetraploids either as seed or pollen parents.

Hybrid sterility seems to be the major hurdle for gene exchange between the diploid and tetraploid species.

Some of the smaller to medium-flowered diploids, such as *S. americanum,* are generally considered to be "species in the making" which have not yet developed complete isolatory barriers to hybridization (Stebbins and Paddock, 1949). Contrasting with the diploids, the tetraploid and hexaploid species of this section seem to be more genetically isolated from one another, with genetic breakdown occurring at various stages from pollination to the maturation of the progeny (Edmonds, 1977).

The occurrence of polyploidy in the section is probably the most efficient barrier to natural hybridization between these species. Successful crosses are more difficult between taxa of differing ploidy levels than they are between taxa of the same chromosome number, with interploidy crosses leading to the development of morphologically intermediate but sterile progeny. In contrast with some reports (Rao *et al.,* 1971; Tandon and Rao, 1974), Edmonds (1977) found that such interploidy crosses were not dependent on the use of the higher ploidy level as the maternal parent. As found for the crosses between AMExSCA and CHE2xSCA and CHE2xRET4 in this study as well.

Despite considerable effort and success, the *Solanum* nigrum complex remains a rich, largely untapped source of new characteristics to further improve the crop and hopefully other species in the Solanaceae family.

Chapter 6

Genetic distance analysis of *Solanum species* using Amplified Fragment Length Polymorphism (AFLP) markers

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6.1 Introduction

Advances in molecular biology have provided new methods, which extend the range of useful genetic markers (Paterson *et al.*, 1991). Beckmann and Soller (1986) proved that compared to morphological and biochemical characteristics, the DNA genome provides a significantly more powerful source of genetic differences. Phillipp *et al.* (1994) also pointed out that the advantage of DNA fingerprinting over morphological markers is the dominance and the absence of pleiotropic effects. The DNA - based techniques are theoretically unlimited and also more reliable and are an environmentally neutral alternative to detect genetic polymorphisms.

The AFLP technique involves the selective amplification of specific restriction fragments resulting from single or double digestion of genomic DNA. This provides numerous informative bands and can be sized accurately using fluorochrome-labeled primers and an automated sequencing scanner. The number of selective nucleotides in the primers for the selective amplification as well as the complexity of the genomic DNA determines the number of amplified DNA fragments (Lin and Kuo, 1995). The AFLP technique was designed as a sensitive method, which allows high numbers of polymorphic genetic markers to be identified (Vos *et al.*, 1995).

In comparison with RFLPs or RAPDs, the AFLP technique is an effective way of finding polymorphisms since it allows the detection of a large number of bands, typically 50-100, with a single primer pair (Vos *et al.*, 1995). This technique detects mostly dominant markers based on PCR amplification of the genomic restriction fragments (Kiem *et al.*, 1995). Variations in the DNA sequence of any origin or complexity can be detected by the AFLP technique and the procedure is independent of environmental influences and the tissue type. It uses small amounts of DNA (e.g. 50 to 500 ng), and does not require prior sequence information (Vos *et al.*, 1995).

One of the few negative points of the technique is that very high-quality genomic DNA is necessary for AFLP fingerprinting to ensure complete digestion by the restriction enzymes (Lin and Kuo, 1995).

AFLP is thus a robust and rapid technique for displaying large numbers of DNA polymorphisms. This technique is being used extensively for genetic mapping and fingerprinting in plants (Vos *et al.*, 1995).

The objectives of this study were to determine the genetic relationships between 10 different *Solanum* species belonging to the Section *Solanum* and 4 species beloning to other sections found in South Africa, using the AFLP technique.

6.2 Materials and methods

6.2.1 *Plant material*

Seeds for the accessions were obtained from several sources as detailed in Chapter 3.2. They were germinated in seedling trays. The seedling trays were filled with a commercial seedling mixture and placed in a temperature-controlled glasshouse. The seedlings were watered daily and fertilized with Chemicult (10g/5ℓ) every third day. The plants were kept in the seedling trays until a well-established root plug formed and were then transplanted into black plastic nursery bags. The nursery bags were filled with a mixture of soil, compost and a commercial potting soil. The plants were nurtured and grown into strong plants. The young leaves of the 14 accessions were harvested for DNA extraction. The accessions used in the study and abbreviations are listed in Table 6.1.

Table 6.1 Accessions and abbreviations used in this study.

ACCESSIONS	ABB.
Solanum americanum Mill.	AME
Solanum chenopodioides Lam.	CHE1
Solanum chenopodioides Lam.	CHE2
Solanum burbankii Bitter	RET1
Solanum retroflexum Dun. (smooth)	RET2
Solanum retroflexum Dun. (hairy)	RET3
Solanum retroflexum Dun. (smooth)	RET4
Solanum scabrum Mill.	SCA
Solanum villosum Mill.	VIL
S. chenopodioides Lam. X *S. americanum* Mill.	CHExAME
Unknown *Solanum*	N22
Unknown *Solanum*	N23
Solanum kwebense N.E.Br.	KWE
Solanum tomentosum L.	TOM

6.2.2 *DNA extraction*

The genomic DNA was extracted from approximately 1g fresh leaf tissue of the individual genotypes by using a modified monocot extraction procedure developed by Edwards *et al.* (1991). The leaves from one plant of each genotype were collected on ice for transporting to the laboratory. The leaves were then ground with a cold mortar and pestle to a fine powder in liquid nitrogen. The DNA was isolated from this powder by placing it into a cold 50ml centrifuge tube and 10ml warm (65°C) extraction buffer (5M NaCl, 0.5M Tris-HCl, 0.25M EDTA and 20% SDS at pH 8) was added and mixed very well. It was then placed in a preheated water bath (65°C) for 30min and inverted every 10min. One ml Cetyl triethyl ammonium bromide (CTAB) (10%, w/v) and 2ml 5M NaCl was added to the homogenate as clean up step and the mixture was incubated in a water bath for an hour. Ten ml Chloroform-isoamylalkohol (24:1) was added, the mixtures were then centrifuged for 15min at 10 000rpm. The aqueous layer (top layer containing the DNA) was removed with a Pasteur pipette and placed into a clean 50ml centrifuge tube. Ice cold absolute ethanol (100%) was added (1:2) for the precipitation of the DNA and refrigerated for up to 12h. The precipitated DNA that formed was scooped out and washed three times in 70% (v/v) ethanol to remove residual salts before being dissolved in sterile water. The DNA concentration was determined with a Hitachi U-2000 spectrophotometer. Finally the concentration of the DNA was confirmed by agarose electrophoresis and visualized with ethidium bromide staining under UV light.

6.2.3 *AFLP analysis*

The AFLP technique was performed as described by Vos *et al.* (1995) and according to the manufacturer instructions (Gibco BRL) with minor modifications.

6.2.3.1 *Restriction endonuclease digestion of the DNA and ligation adapters*

Approximately 250ng of genomic DNA was double digested with the two restriction endonucleases: a frequent cutter, the 4bp restriction enzyme *Mse* I and a rare cutter, the 6bp restriction enzyme *EcoR* I. This generates small DNA fragments (<1 kb) that amplify well. Following heat inactivation of the restriction endonucleases (incubation of 15min at 70°C), the genomic DNA fragments were ligated. Ligation to *EcoR* I and *Mse* I adapters (Table 6.2) forming common adapter sequences flanked by variable genomic DNA which serve as primer binding sites for amplification in subsequent reactions to generate the template for amplification.

Table 6.2 Adapter and primer sequence used in the AFLP reactions.

***MSE* I – ADAPTER**	***ECOR* I – ADAPTER**
5'-GACGATGAGTCCTGAG-3'	5'-CTCGTAGACTGCGTACC-3'
Mse I – primers	*EcoR* I – primers
(5'-GATGAGTCCTGAGTAA-3'	5'-GATGCGTACCAATTC-3'
Mse I + CAA	*EcoR* I + ACA (FAM)
Mse I + CTA	
Mse I + CTT	

6.2.3.2 *Amplification of restriction fragments*

PCR is performed in two consecutive amplification reactions. In the first reaction, called pre-amplification, the genomic DNA was amplified with AFLP primers having one selective nucleotide each. Pre-amplifications of the restriction fragments templates were performed for 20 cycles with the following cycle profile: a 30sec DNA denaturation step at 94°C, a 60sec annealing step at 56°C and a 60sec extension step at 72°C.

The PCR products of the pre-amplification reaction were diluted and used as a template. This product was diluted 50 fold in a 1:50 dilution by transferring 3µl to a 1.5ml microcentrifuge tube containing 147µl TE buffer. The selective AFLP amplifications of the pre-amplified fragment templates, with a pair of AFLP primers each having three selective nucleotides, were performed in a 20µl PCR reaction containing, 5µl of the dilutes pre-selective reaction, 4.5µl of the MSE I + 3 primer, 1µl EcoR I + 3 primer (Fluorescently labelled) 2µl of 10 X PCR buffer and 5U Taq DNA polymerase. The following three primer combinations with three selective nucleotides on both primers were used: E-ACA/M-CAA, E-ACA/M-CTA and E-ACA/M-CTT (Table 6.2). *EcoR* I primer (PE Biosystems) was labelled with FAM, a fluorescent label. The 14 *Solanum* accessions were screened individually with each of the primer combinations.

The reactions were performed as follows: the first cycle consisted of a 30sec DNA denaturation step at 94°C, a 30sec annealing step at 65°C and a 60sec extension step at 72°C. The annealing step temperature was subsequently reduced by 0.7°C during 12 cycles. This gives a touch down phase of 13 cycles. The 23 remaining cycles had the following cycle profile: a 30sec DNA denaturation step at 94°C, a 30sec annealing step at 56°C and a 60sec extension step at 72°C. All amplification reactions were performed in a Perkin-Elmer 9600 thermal cycler (Perkin Elmer Corp., Norwalk, CT, USA).

After amplification 5µl of each of the selective reactions were added to a new tube containing 24µl of formamide dye (98% formamide, 10mM EDTA, bromophenol blue, xylene cyanol) and 1µl ROX 300 standard size marker. This was denatured at 94°C for 5min and immediately placed on ice slush and run on a Perkin Elmer ABI Prism 310 Automated capillary sequencer (PE Biosystems). The Gene Scan® analysis software analysed the raw data collected from the ABI Prism. The application has been designed for fragment analysis and identifies, quantifies and sizes each DNA fragment.

6.2.4 *Statistical analysis*

Each distinct band appearing was scored as presence (1) or absence (0) of each DNA fragment and compiled into a binary data matrix (Addendum D). Both polymorphic and monomorphic fragments from the three primer combinations were used in the analysis.

The NCSS 2000 (Number Cruncher Statistical System; Hintze, 1998) Statistical System for Windows was used to build a cluster hierarchy that is commonly displayed as a tree diagram called a dendrogram, depicting relationships among the species. The hierarchical agglomerative clustering method using the Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) was carried out and similarity between all pairs of accessions were estimated and converted to dissimilarity and expressed as Euclidean genetic distances. (Hintze, 1998).

6.3 Results and discussion

The number of amplified DNA polymorphic bands detected by the individual primer combinations was dependent on the primer combination and the species. A minimum number of 10 (primer combination E-ACA/M-CTA) and a maximum of 43 (primer combination E-ACA/M-CAA) polymorphic bands were observed. Although the three primer combinations tested differed from each other by substitution of only one to three nucleotides, unique AFLP DNA patterns or fingerprints were generated by each primer combination. Of a total of 359 bands generated by the three primer combinations, 222 (62%) were clearly polymorphic. All 14 accessions were distinguished by any one primer combination.

The 14 accessions separated clearly into two major clusters (I and II) (Figure 6.1) with two sub-clusters (A and B) each. The genetic distance over all accessions ranged from 0.23 to 0.75.

The species totally distant from the *Solanum nigrum* complex (*S. tomentosum* L. (TOM), *S. kwebense* N.E.Br. (KWE), N22 and N23) were clearly clustered separately, in cluster IA and cluster IB, from those falling within the complex, as was expected.

It was very interesting that the eight accessions evaluated for morphological data all clustered in IIA. *S. burbankii* - smooth (RET1), *S. retroflexum* – hairy (RET3), *S. retroflexum* - smooth (RET4), and *S. retroflexum* – smooth (RET2) all clustered in cluster IIAi. With a genetic distances of 0.34 between RET1 and RET3, and a genetic distance of 0.32 between RET4 and RET2. *S. villosum* (VIL), clustered separately from the others with a dissimilarity of 0.54 in the same large cluster.

The CHExAME hybrid and *S. americanum* clustered together in cluster IIAii with a genetic distance of 0.33. *S. scabrum* (SCA) clustered alone with a dissimilarity of 0.53 in cluster IIAii. Within sub-cluster IIB, *S. chenopodioides* (CHE1) and *S. chenopodioides* (CHE2) clustered together with a genetic distance of 0.37.

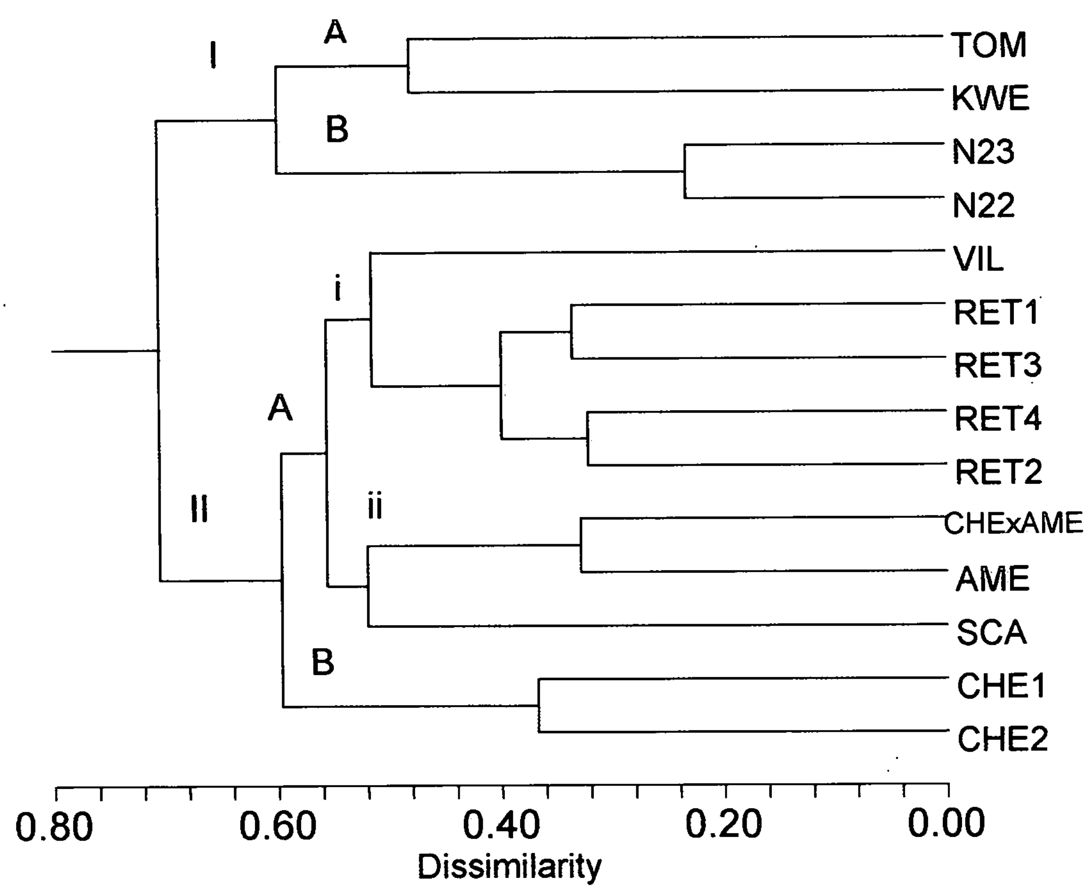

Figure 6.1 Dendrogram for AFLP data.

TOM = *Solanum tomentosum,* KWE = *Solanum kwebense,* N23 = *unknown Solanum,* N22 = *unknown Solanum,* VIL = *Solanum villosum,* RET1 = *Solanum burbankii,* RET3 = *Solanum retroflexum (hairy),* RET4 = *Solanum retroflexum (Pretoria-smooth),* RET2 = *Solanum retroflexum (smooth),* CHExAME = *Solanum chenopodioides X Solanum americanum,* AME = *Solanum americanum,* SCA = *Solanum scabrum,* CHE1 = *Solanum chenopodioides (UniQwa),* CHE2 = *Solanum chenopodioides (UF)*

6.4 Conclusions

Published information on the levels of polymorphism in the black nightshade group and the utility of AFLP markers to assess relationships between species is limited or non-existing. The number of amplified DNA polymorphic bands detected in this study by the individual primer combinations ranged from 10 to 43. Although the three primer combinations tested, differed from each other by substitution of only one to three nucleotides, unique AFLP DNA patterns or fingerprints were generated by each primer combination. These results indicate that the AFLP assay is able to detect single base pair changes in genomic DNA. For their work on potatoes, Kim *et al.* (1998) also found this. Out of a total of 359 bands generated by the three primer combinations 222 (62%) were clearly polymorphic.

The AFLP fingerprinting technique could be used to predict genetic relationships between germplasm of the different accessions analysed. It also showed adequate resolution of the different accessions to be used for breeding purposes.

Biochemical and morphological genetic markers are scare or non existent in the *Solanum nigrum* complex. Therefore the AFLP DNA pattern or fingerprint should be a valuable tool for plant breeders and the knowledge of the genetic relationships generated in this investigation will be of value in directing the exploitation of available germplasm.

Chapter 7

Simple Sequence Repeats (SSR) for germplasm analysis in *Solanum retroflexum* and four related species and their progeny

7.1 Introduction

Species in the *Solanum nigrum* complex can play an important role in nutrition and generating an income in developing African countries. Information regarding the complex's genomic organization is limited. Molecular markers could help to gain a better understanding of the black nightshade genetics and the application of SSR or microsatellites could improve the genetic analysis of this plant species, and this information could facilitate future breeding programs.

Microsatellites can be used for identification in a single locus approach via PCR (Beckmann and Soller, 1990). A primer pair flanking a microsatellite enables amplification of this microsatellite, and any polymorphism in the length of the microsatellite between cultivars can thus be detected (Thomas and Scott, 1993). One of the main features of microsatellites is thus the high level of variation among cultivars, expressed as the variable copy number of the short sequence motifs that occur as tandem repeats. These repeats are highly polymorphic even among closely related cultivars.

Microsatellites have a high polymorphic information content and are especially crucial for genetic comparison of organisms with a narrow genetic base (Smulders *et al.*, 1997). The co-dominant, mode of inheritance of microsatellites gives them an advantage over dominant marker types as easy transferable markers between different crosses (Thomas and Scott, 1993), this also allows one to discriminate between homo- and heterozygous states, and it increases the efficiency of genetic mapping and population genetic studies.

This polymorphic nature of microsatellites, together with their abundance and even genomic distribution, are responsible for their emergence as ubiquitous genetic markers (Love *et al.*, 1990; Weissenbach *et al.*, 1992). Microsatellite length polymorphisms are typically detected by PCR amplification of genomic DNA, using specific oligonucleotide primers, which are complementary to the regions flanking the repeats (Akkaya *et al.*, 1992). Each allele is usually represented by a number of bands that presumably arise from mechanisms such as strand slippage during replication (Tautz, 1989; Luty *et al.*, 1990; Schlötterer and Tautz, 1992) and the terminal transferase activity of *Taq* DNA polymerase (Clark, 1988).

In plants, it has been demonstrated that microsatellites are highly informative and locus specific and this has been reported for many plant species including tomatoes (Arens *et al.*, 1995a and b), peppers (Sanwen *et al.*, 2000), potatoes (Provan *et al.*, 1996), sorghum (Djè *et al.*, 2000) and many others. Microsatellite markers have also been successfully used in characterization of germplasm collections of different species (Lamboy and Alpha, 1998; Hokanson *et al.*, 1998) and in genome mapping (Tautz, 1989; Taramino and Tingey, 1996 and Röder *et al.*, 1998).

The advances of DNA fingerprinting over morphological markers are clearly stated as superior and theoretically unlimited and more reliable than morphological studies, this is also clearly stated in Chapter 6. The objective of this study was to assess the genetic diversity within and among five *Solanum* genotypes and their progeny by using microsatellite markers.

7.2 Materials and methods

7.2.1 *Plant materials and DNA extraction*

Seeds from five of the species obtained for this study and their progeny (Table 7.1) were germinated and the seedlings were grown into healthy plants as described in Chapter 3.2.2. The accessions were then planted in the field for the morphological study as described in Chapter 4.2 and the young leaves of these plants were harvested for DNA extraction. The extraction of DNA was from the fresh young leave tissue by a modified monocot extraction procedure developed by Edwards *et al.* (1991) and fully described in Chapter 6.2.2.

The precipitated DNA was resuspended in 200μl sterile water and the DNA concentrations were determent spectrophotometrically by using a Hitachi U-2000 spectrophotometer at 260nm using the formula: [DNA] = Optical density (OD_{260}) x dilution x constant (50μg/ml). The DNA solution was diluted to a working concentration of 50ng/μl and stored at 4°C. Finally the DNA concentration was confirmed by agarose electrophoresis and visualized with ethidium bromide staining under UV light.

Table 7.1 Parental species and progeny and the abbreviations used in this study.

ACCESSIONS	ABB.
Solanum americanum	AME
Solanum burbankii	RET1
Solanum retroflexum (smooth)	RET4
Solanum chenopodioides	CHE2
Solanum scabrum	SCA
S. americanum X *S. scabrum*	AMExSCA
S. scabrum X *S. americanum*	SCAxAME
S. burbankii X *S. chenopodioides*	RET1xCHE2
S. chenopodioides X *S. burbankii*	CHE2xRET1
S. burbankii X *S. retroflexum*	RET1xRET4
S. retroflexum X *S. burbankii*	RET4xRET1
S. chenopodioides X *S. retroflexum*	CHE2XRET4
S. retroflexum X *S. chenopodioides*	RET4xCHE2
S. chenopodioides X *S. scabrum*	CHE2xSCA

7.2.2 *SSR analysis*

7.2.2.1 *Polymerase chain reactions (PCR)*

PCR amplification was used to detect the loci using different primer sets. The reactions were carried out in a total reaction volume of 25μl containing: 2.5μl 10 X PCR buffer (20mM Tris-HCl (pH8.4), 50mM KCl), 1.5mM $MgCl_2$ 0.25mM deoxyribonucleotids (dNTP), and 0.5U Taq DNA polymerase (Roche), 10pmoles of each primer in the set (Table 7.2) and 50ng genomic DNA. Amplifications were performed in Eppendorf tubes using an AB Applied Biosystems GeneAmp® PCR System 2700.

Standard cycling consisted of: initial denaturation of the template DNA at 94°C for 5min, followed by 35 cycles of 95°C for 1min, 45°C (annealing temperature) for 2min and 72°C for 2min. In the final PCR cycle the extension time at 72°C was increased to 10min. In some cases the annealing temperature was modified as described in the results.

7.2.2.2 *Detection of SSR*

After PCR amplification, 10μl aliquots of the reaction mixture were mixed with gel-loading buffer and loaded on 2% agarose gels. The gels were electrophoresed for 2h at a constant currant of approximately 80V in a 0.5 X TAE buffer (0.438g/ℓ Tris (pH8), 0.11ml/ℓ Acetic acid and 0.029g/ℓ EDTA). A 100bp DNA ladder (Promega) was used as marker to estimate the size of the amplification products. Finally the DNA bands were visualized with ethidium bromide under UV light and the Gel Doc 1000™ image analysis system (Biorad) and the Molecular Analyst fingerprinting software program.

7.2.2.3 *Primer sets*

The cost and research effort required to clone and sequence SSR-containing DNA fragments from the accessions lead to the decision to screen available primer sets. Primer pairs designed for potatoes, sorghum, peppers and tomatoes were tested for the ability to amplify SSRs in black nightshades. Table 7.2 lists the sequences of all the primers tested, along with the recommended annealing temperatures and the nature of the repeats.

Table 7.2 Microsatellite primer sequences used in the screening process.

NAME	PRIMER FLANKING SEQUENCE (5'-3')	Tm °C	REPEAT	PRODUCT SIZE (bp)
STS 1 + 2	TCT CTT GAC ACG TGT CAC TGA AAC TCA CCG ATT ACA GTA GGC AAG AGA	59	$(TCAC)_m$	<100 – 500
STS 1+ 3	TCT CTT GAC ACG TGT CAC TGA AAC TTG CCA TGT GAT GTG TGG TCT AGA A	60	$(TCAC)_M(CTT)_n$	350 – 700
STWIN 12 G	TGT TGA TTG TGG TGA TAA TGT TGG ACG TGA CTT GTA	49	$(TGAAA)_2(ATA)_6$	<100 – 400
ST iika A_1 : A_2	TTC GTT GCT TAC CTA CTA CCC AAG ATT ACC ACA TTC	48	-	-

ST GB SS1:2	AAT CGG TGA TAA ATG TGA ATG C ATG CTT GCC ATG TGA TGT GT	55	-	-
SB KAFGK[*]	GCT TTC GGC GAG CAT CTT ACA A GCG GTT GGA TTC GCC ATG	54	$(AAC)_9$	280-320
SB1-10[*]	GTG CCG CTT TGC TCG CA TGC TAT GTT GTT TGC TTC TCC CTT CTC	65	$(AG)_{27}$	350-400
SB4-15[*]	GCT GCT AAG CCG TGC TGA TTA TTT GGG TGA AGT AGA GGT GAA CA	57	$(AG)_{16}$	120-130
SB4-22[*]	TGA GCC GAA AAC CGT GAG CCC AAA ACC AAG AGG GAA GG	59	$(ACGAC)_4/(AG)_6$	270-300
SB4-32[*]	CTC GGC GGT TAG CAC AGT CAC GCC CAT AGA CAG ACA GCA AAG CC	59	$(AG)_{15}$	160-180
SB4-121[*]	GAA AAA TCT CCG TCA ATC CCA AAA TAA CGC TGA ACA ACG AAA GGA ATA AGT G	60	$(AC)_{14}$	200-225
SB5-85[*]	AGA CGC TTT TCT CTC TCT CTC TCT CTC TCT TAG CCC TGC CGC ATA CTG AAT	60	$(AG)_{12}$	200-225
SB5-236[*]	GCC AAG AGA AAC ACA AAC AA AGC AAT GTA TTT AGG CAA CAC A	57	$(AG)_{20}$	165-185
SB6-36[*]	GCA TAA TGA CGG CGT GCT CTT CCA AGT GAA AGA AAC CAT CA	60	$(AG)_{19}$	155-190
SB6 –57[*]	ACA GGG CTT TAG GGA AAT CG CCA TCA CCG TCG GCA TCT	60	$(AG)_{18}$	285-305
SB6-84[*]	CGC TCT CGG GAT GAA TGA TAA CGG ACC ACT AAC AAA TGA TT	58	$(AG)_{14}$	170-190
SB6-325[*]	AGC GCA GGA GCG CGA A TCA TCC GCT ACT ACC GTC AGA AA	56	$(AAG)_{22}$	110-140
SB6-342[*]	TGC TTG TGA GAG TGC CTC CCT GTG AAC CTG CTG CTT TAG TCG ATG	56	$(AC)_{25}$	250-320
CM2[***]	GTA CCT ATG GGA ATA AGC AAA CCA ATT TGT CTG AAG TTG AGT	52	$(AT)_6$	160
CM5[***]	CAT GAC CAC CAT GAG GAT A GAT AGC CAC GAG CAT AGT ATT	52	$(CCA)_8$	160
CM6[***]	AGT TAA CAA CTT TGG TGC TGT TAA TAT GGT AAG CAC ATT CCA	54	$(AG)_5$	130
CM8[***]	ATA GCT CAC ATG CCC TAT AAA AAT CTT GAG CAA TAA TTG GAC	52	$(ATATA)_5$	195
CM11[***]	TCT GCT TTA AAA ACA CAT ACA T CAT TCT AAC TGA AAT TGC ATG	50	$(AC)_5(TA)_8$	116
TMS2[**]	TCT TTC ATT TCA TGT CAC GA AGG AGA CCT TAT GAT TCA AGG	55	$(GT)_{41}(TA)_6(CT)_9$	387
TMS22[**]	TGT TGG TTG GAG AAA CTC CC AGG CAT TTA AAC CAA TAG GTA GC	55	$(GT)_9(AT)_8(AC)_{13}(GA)_{12}$ imp.	180
TMS29[**]	AGC CAC CCA TCA CAA AGA TT GTC GCA CTA TCG GTC ACG TA	55	$(CT)_3(C)_{14}(CT)_{23}$	354
TMS37[**]	CCT TGC AGT TGA GGT GAA TT TCA AGC ACC TAC AAT CAA TCA	55	$(GA)_{21}(TA)_{20}$	193
TMS39[**]	CGG CGT ATT CAA ACT CTT GG GCG GAC CTT TGT TTT GGT AA	60	$(AT)_{29}$ imp.	120
TMS 45[**]	CCG TCC AGA AGA CGA TGT AA CAA AGT CTT GCC AAC AAT CC	55	$(GA)_{17}(GT)_8$ imp.	248

[*] Brown *et al.* (1996); [**] Areshchenkova and Ganal (1999); [***] Sanwen *et al.* (2000).

7.2.3 *Data analysis*

Each DNA profile was documented and the bands scored as presence (1) or absence (0) and compiled into a binary data matrix (Addendum E). This matrix was used to construct a dendrogram (Figure 7.1) depicting the genetic distance between the accessions tested. The most commonly used hierarchical clustering algorithm of Unweighted Pair-Group Method using an Arithmetic Mean (UPGMA) cluster analysis of the genetic distance matrix by NCSS 2000 Statistical System for Windows (Hintze, 1998) was used.

7.3 Results and discussion

Amplification was initially carried out on the genomic DNA of test parents (RET1 and/or SCA or CHE) under standard PCR conditions. PCR products were analyzed on 1% agarose gels with 0.8µl ethidium bromide to visualize the DNA under UV light. SB4-32, SB6-36, TMS29 and TMS37 showed amplification. For the other 26 primer sets listed in Table 7.2 the amplification conditions were modified by altering the annealing temperature to 50°C. Only five of the primer sets tested showed slight smears and very light bands, only one primer set SB6-57 showed good amplification products. The other sets were tested by altering the annealing temperature to 55°C. Two primer sets, STWIN12G and SB6-84 showed amplification. Only these seven primer sets that showed amplification among the tests parents were used on all the genomic DNA of parents and progeny to find polymorphisms between the genotypes.

Problems have been experienced with spurious high molecular weight bands that may be PCR artifacts or the results of nonspecific priming. It appears that all loci scored in this study were highly polymorphic with many alleles and a wide range of product sizes. (Table 7.3). Null alleles were also detected in some of the individuals, which could result from nucleotide sequence polymorphisms occurring only at one of the primer sites as stated by Taramino and Tingey (1996).

Table 7.3 SSR polymorphism and product size of accessions tested.

PRIMER	REPEATS	Tm °C	PRODUCT SIZE (bp)
SB4-32	$(AG)_{15}$	45	100-900
SB6-36	$(AG)_{19}$	45	130-800
TMS29	$(CT)_3(C)_{14}(CT)_{23}$	45	180-800
TMS37	$(GA)_{21}(TA)_{20}$	45	300-1000
SB6-57	$(AG)_{18}$	50	750-2800
STWIN12G	$(TGAAA)_2(ATA)_6$	55	100-1000
SB6-84	$(AG)_{14}$	55	100-300

Genetic distances were calculated to generate a dendrogram. The goodness of fit of the dendrogram constructed was confirmed by the co-phenetic correlation r = 0.86. Table 7.4 depicted the genetic distances found between all the accessions tested. The cluster analysis used for the genetic distance determination of the five parental species and progeny identified two major clusters (Figure 7.1). Cluster I was further sub divided into two clusters as seen in Figure 7.1 and both these clusters were again divided into two clusters each.

AME and RET1 were clustered alone in cluster II, with a genetic distance of 0.48 (Table 7.4). In cluster Bi and Bii most of CHE2's progeny were found. The parent CHE2 clustered with RET1xCHE2 with a genetic distance of 0.44 in Bii. In cluster Aii the other parents (SCA and RET4) displayed a distance of 0.42 (Table 7.4). SCA and one of its hybrids AMExSCA cluster together in this same cluster with a distance of 0.36. Cluster Ai depicted RET4xCHE2 and CHE2xSCA with a distance of 0.34. SCAxAME clustered separately from the others in this with a genetic distance of 0.42.

Table 7.4 Genetic distances.

	RET1	CHE2	RET4	SCA	AMEX SCA	RET1X CHE2	RET1X RET4	CHE2X RET1	CHE2X RET4	CHE2X SCA	RET4X CHE2	RET4X RET1	SCAX AME
AME	0.480	0.530	0.489	0.521	0.516	0.530	0.547	0.547	0.526	0.475	0.503	0.530	0.494
RET1		0.503	0.490	0.494	0.490	0.485	0.494	0.521	0.490	0.455	0.465	0.503	0.494
CHE2			0.435	0.440	0.455	0.440	0.490	0.460	0.424	0.429	0.429	0.480	0.460
RET4				0.424	0.418	0.475	0.494	0.485	0.440	0.435	0.435	0.465	0.455
SCA					0.366	0.470	0.480	0.48	0.455	0.429	0.429	0.470	0.460
AMEX SCA						0.435	0.465	0.475	0.440	0.424	0.413	0.455	0.465
RET1X CHE2							0.418	0.480	0.445	0.460	0.450	0.490	0.480
RET1X RET4								0.33	0.435	0.450	0.450	0.490	0.480
CHE2X RET1									0.490	0.440	0.430	0.480	0.470
CHE2X RET4										0.390	0.390	0.455	0.435
CHE2X SCA											0.346	0.418	0.407
RET4X CHE2												0.360	0.372
RET4X RET1													0.418

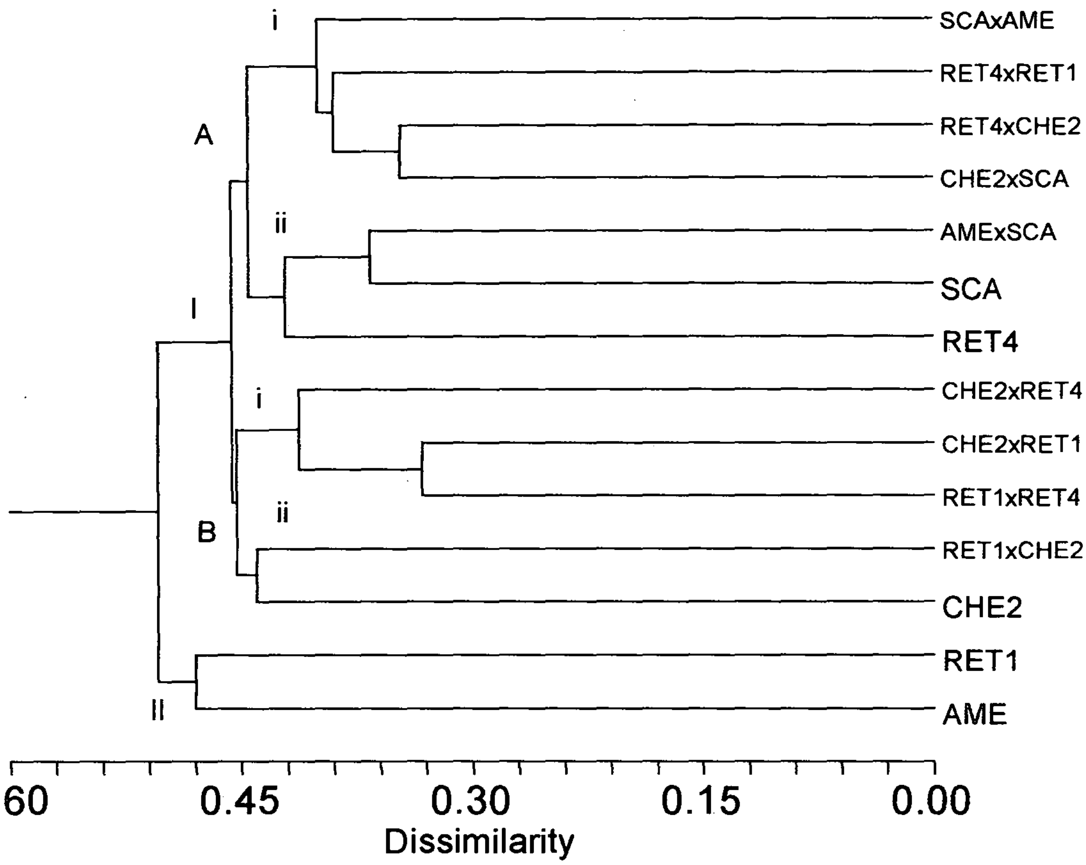

Figure 7.1 Dendrogram for SSR data.

SCAxAME = *S. scabrum* X *S. americanum*, RET4xRET1 = *S. retroflexum* X *S. burbankii*, RET4xCHE2 = *S. retroflexum* X *S. chenopodioides*, CHE2xSCA = *S. chenopodioides* X *S. scabrum*, AMExSCA = *S. americanum* X *S. scabrum*, SCA = *Solanum scabrum* Mill, RET4 = *Solanum retroflexum* Dun. (smooth), CHE2xRET4 = *S. chenopodioides* X *S. retroflexum*, CHE2xRET1 = *S. chenopodioides* X *S. burbankii*, RET1xRET4 = *S. burbankii* X *S. retroflexum*, RET1xCHE2 = *S. burbankii* X *S. chenopodioides*, CHE2 = *Solanum chenopodioides* Lam., RET1 = *Solanum burbankii* Bitter, AME = *Solanum americanum* Mill.,

7.4 Conclusions

Limited or non-existing published information on the levels of polymorphism in the black nightshade group and the utility of SSR markers to assess relationships between accessions was available. Some of the primers developed for soybean, tomato, pepper and potato were used to assess genetic diversity in the germplasm of five *Solanum nigrum* species and their progeny. Twenty-nine random sets of primers were selected for screening and seven had been used to amplify each of the parents and their progeny.

Database screening was the least costly in terms of time and resources and has been used as an entry point for the development of SSR markers in several plant species (Brown *et al.* 1996). One of the most important parameters of a given marker is the degree of polymorphism it defines (Taramino *et al.*, 1997). It seems that in the case of SSRs the polymorphisms increase with an increasing number of repeat units (Weber, 1990). This was also found in this study and the results revealed a high degree of polymorphism.

Optimization of the PCR conditions was important. The optimum conditions were not the same for the various primer pairs with annealing temperatures of 45, 50 and 55°C for the different primers. The genetic distance value amongst the species and their progeny varied from 0.33 to 0.54 as indicated in Table 7.4 clearly indicates the diversity among the accessions tested showing the close relationship and confirm the fact that the Solanum *nigrum* complex is a group of plants closely related.

The level of variation with careful selected microsatellites indicates that this could represent a contribution to the genetic characterization of *Solanum* for high level of polymorphism, it is very likely that this marker system will have the advantage of distinguishing closely related accessions arising from a similar pedigree. The use of this new kind of molecular marker could facilitate molecular breeding practices in the crop.

Chapter 8

Assessment of the total protein, total soluble solids and acid content in five genotypes of the *Solanum nigrum* complex

8.1 Introduction

In Africa, hunger and malnutrition are common. There are an estimated 25 to 30 million malnourished children on the continent. Millions of people across the continent are regularly threatened by food insecurity. A further tragedy is that millions of people are forced to live below their full potential because they lack the energy and good health to function at their best (Anonymous, 2003).

Good nutrition is thus an indispensable part of health and growth and man is thus dependent on plant proteins for many of their amino acids. We use these amino acids to build our own proteins and thus as an energy source. The composition of seed, leaf and stem proteins as well as the protein quantity in plants is thus important, due to the nutritional value of the protein to our diet. It seems probable that the pressure of world populations will lead to an increase in the direct consumption of plant proteins by humans, especially those individuals who cannot afford to purchase adequate quantities of animal protein.

It was found that people of higher income groups in developing countries are able to supplement their starchy diet with meat, eggs and/ or milk, which the people in the low income groups are not able to consume regularly (Sreeramulu, 1982). Keller *et al.* (1969) stated that the edible wild leafy vegetables have an important role to play in African agricultural and in the nutritional systems for the people living on this continent. Edmonds and Chweya (1997) also stated that *Solanum nigrum* L. and its related species play a significant role in the nutrition and food supply in subsistence farming societies in Africa. Apart from being a chief source of minerals and vitamins, the leafy vegetables are also cheap sources of protein and supply carbohydrates (Sreeramulu, 1982).

Many diets could thus be improved if the quantity and /or quality of the protein in these utilised crops were enhanced. There are two possible ways of accomplishing this: one, by breeding programmes, and two, by improved management, e.g. fertilizer treatments for those soils which limit the amount and quality of the protein of the plants grown in them.

For these programmes to be successful, satisfactory screening methods for the total protein and protein quality must be available (Evans and Boulter, 1974). These methods must be simple, fast and must not require expensive equipment or much plant material. Several methods exist for determining total protein (Boulter and Derbyshire, 1978). The three most commonly used of these methods are: the Biuret, Lowry and Bradford assays (Copeland, 1994).

Lowry's assay is widely used for the determination of total proteins from plant tissues and has been found to be a most reliable and satisfactory method for quantitation of soluble proteins. The procedure of Lowry *et al.* (1951) generates sensitivity, which is reasonably constant from protein to protein, down to about 0.01 mg of protein/ml, and is best used on solutions with concentrations in the range of 0.01 – 1.0 mg/ml of protein.

The assay is based on the peptide bonds reacting with an alkaline cupric tartrate reagent, producing Cu^{+}, which in tum reacts with the phenol reagent and forms a purple-blue colour. The protein estimations are a completely acceptable altemative to a rigorous absolute determination in almost all circumstances where protein mixtures or crude extracts are involved (Waterborg and Matthews, 1996). The procedure for the protein assay is based on the micro-Lowry method as modified by Peterson (1983), and utilizes sodium dodecyl sulfate (SDS), to facilitate the dissolution of relatively insoluble lipoproteins.

One disadvantage of this method is the fact that a range of substances interferes with this assay, including buffers, nucleic acids and sugars. Waterborg and Matthews (1996) reported that in many cases, diluting the substances can minimize the effects of these agents, assuming that the protein concentration is still sufficiently high to be detected after dilution. The assay procedure followed in this study included a precipitation step, which allowed the separation of the protein sample from interfering substances and also consequently concentrated the protein sample, allowing the determination of proteins in the dilute solutions according to Peterson's (1983) modifications. In spite of these drawbacks, the Lowry assay is still routinely used because of its reliability (Schuler and Zielinski, 1989).

The soluble solids and acid contents is also an important aspect of fruit quality and mainly determine the basic taste of the fruits (Hobson and Davies, 1971). Although it is generally accepted that good taste is determined by the ratio of sugars to acids, this has not yet been quantified (Ho, 1995). Attempts to increase the fruit soluble solids content and to alter the acid content in tomatoes had limited success. This may be attributed to the complex interactions between the various components involved (Stevens, 1994). Davies *et al.* (1958) and Hewitt and Stevens (1981) reported that the sugar concentration of fruit juice is high in fruit with a high number of leaves per plant. Further, the sugar levels in fruit are maximised only when the fruit are allowed to ripen fully on the plant (Davies and Hobson, 1981; Ho and Hewitt, 1986).

The potential of black nightshade as a food source has not yet fully been established and published information is limited. The author believes that this food source could provide nutrition for many people where it is propagated in developing countries. The objective of this study was to determine the levels of the total proteins and the total soluble solids (TSS) as well as the acid content in the fruits of the *Solanum* species used as parents in the crossing study and to establish the nutritional value, of this crop.

8.2 Materials and methods

8.2.1 Plant material

Seeds for the species used as parents (Table 8.1) in the crossing study were germinated, and grown in a temperature-controlled glasshouse to healthy plants, as described in Chapter 3.2.2. The young leaves and ripe fruit of the five species were harvested from mature plants and used in the protein assay and to assess the TSS and acid content.

Table 8.1 *Solanum* species and abbreviations used for the protein assay and determination of the TSS and acid content.

SPECIES	ABB.
Solanum americanum Mill.	AME
Solanum burbankii Bitter	RET1
Solanum retroflexum Dun. (smooth)	RET4
Solanum chenopodioides Lam.	CHE2
Solanum scabrum Mill.	SCA

8.2.2 *Total protein extraction and determination of concentration*

The total protein was extracted from ±0.4g fresh leaf tissue and compared to the total protein extracted from ±0.4g ripe fruit. Three replicates were assayed for each species. The material from each species was placed in liquid nitrogen and ground to a powder with a cold mortar and pestle. Protein was isolated from this powder by placing it into a 5ml Eppendorf and 1.2ml extraction buffer was added and mixed very well. The extraction buffer contained 50mM Tris- HCl (pH 7.5), 0.1mM Phenylmethylsulfonyl fluoride (PMSF) and 0.1g Polyvinylpurrolidone (PVP) per ml buffer. The PVP was added to the buffer just before use. The mixtures were then centrifuged for 15min at 10 000rpm.

One ml of the supernatant, which contained the proteins, was poured into a clean Eppendorf and used in the assay. An aqueous solution (0.1ml) of sodium deoxycholate, (DOC, 1.5mg/ml) was added to each sample and vortexed very well. The samples were then incubated at room temperature for 10min. A Trichloroacetic acid solution (TCA, 72% w/v) of 0.1ml was added and the samples were vortexed again. The samples were then centrifuged for 10min at 10 000rpm to pellet the precipitates. The supernatants were decanted and blotted away.

The precipitated pellet, which formed for each sample was dissolved in 1.0ml Lowry's reagent solution, from the Sigma Protein assay kit. Samples were incubated for 20min at room temperature. Folin and Ciocalteu's phenol reagent working solution (0.5ml) from the kit was rapidly added to each sample and mixed immediately. Colour development was allowed for 30min.

This colorimetric assay is based on the fact that certain metal ions and dyes bind to the protein in a specific mass ratio, and upon binding become intensely coloured. Within a specific range of protein concentration, these reagents will give rise to an absorption band whose intensity is linearly proportional to the protein concentration of a solution (Copeland, 1994). Waterborg and Matthews (1994) stated that the vortex step is critical for obtaining reproducible results. The folin reagent is only reactive for a short time under the alkaline conditions, and great care should therefore be taken to ensure thorough mixing.

The choice of protein standards could have a dramatic effect on the concentration estimates obtained for the unknown samples. Bovine serum albumin (BSA) tends to be a sensitive standard and is commonly used because of the low cost (Copeland, 1994).

After the 30min-colour development, the solutions were transferred to cuvets and the absorbance of the samples was measured at 540nm and at 750nm for greater sensitivity as described by Copeland (1994). The absorbance values of the standards (BSA) vs. their corresponding protein concentration were plotted to prepare a calibration curve. The protein concentration of the unknown samples was determined from this calibration curve.

8.2.3 *Determination of total soluble solid content*

Mature fruit from each species in Table 8.1 were picked and stored open at room temperature. These randomly picked fruit were squashed on the lens of an ATAGO hand held refractometer, which was used to determine the sugar content. The instrument was calibrated with distilled water, and the sugar content was measured at day one, four, eight, 12 and 16 (after harvest) with five replications for each species.

The refractometer converts the refractive index of the sample to %Brix and then displays the value. Brix is based on a scale for measuring the density of sugar solutions and represents the percentage by weight of sugar in a solution at a certain temperature.

8.2.4 *pH*

Twenty ripe berries from the species listed in Table 8.1 were randomly picked and squashed in a beaker. The pH reading was made on the unfiltered fruit juice using a digital pH-meter from inoLab pH/cond Level1 WTW. Two replications were done for each species.

8.3 Results and discussion

8.3.1 *Total protein concentration*

The results of the protein content found in the leaves of the species are presented in Table 8.2 and that found in the fruit are reported in Table 8.3. The data represents the average values of three replicates with the standard deviation (SD) value in brackets in each table. The standard calibration curves were used to determine the protein concentrations of the unknown samples and the correlation coefficient (r) are reported in the tables. From the results it was clear that the 750nm readings were more sensitive as stated by Copeland (1994) and the readings showed a higher absorbance and resulted in a lower protein percentage for the plant material tested. From these results it is quite clear that the precise method of determination play a major role in the end results.

Table 8.2 Protein concentration and wet weight percentage of the *Solanum* species leaves assayed.

NAME	FRESH WEIGHT (mg)	ABSORBANCE 540nm	750nm	[PROTEIN] mg/l 540nm	750nm	% PROTEIN at 540nm	750nm
AME	370.1 (± 0.056)	0.4580	0.4890	0.262	0.134	0.071	0.036
RET1	412.2 (± 0.008)	0.3390	0.3650	0.176	0.077	0.043	0.019
RET4	416.5 (± 0.007)	0.5093	0.5080	0.295	0.144	0.071	0.035
CHE2	405.5 (± 0.002)	0.6840	0.7540	0.427	0.268	0.015	0.066
SCA	443.7 (± 0.017)	0.8220	0.8143	0.527	0.299	0.029	0.067
correlation coefficient (r)		0.9810	0.9739				

Table 8.3 Protein concentration and wet weight percentage of the *Solanum* species fruit assayed.

NAME	FRESH WEIGHT (mg)	ABSORBANCE 540nm	750nm	[PROTEIN] mg/l 540nm	750nm	% PROTEIN AT 540nm	750nm
AME	418.3 (± 0.021)	0.5830	0.7273	0.241	0.197	0.058	0.047
RET1	461.3 (± 0.028)	0.7670	0.7823	0.263	0.214	0.057	0.046
RET4	428.3 (± 0.072)	0.5737	0.7933	0.239	0.216	0.056	0.050
CHE2	424.4 (± 0.011)	0.8047	0.8493	0.394	0.281	0.093	0.066
SCA	514.3 (± 0.075)	0.5460	0.6103	0.263	0.193	0.051	0.038
correlation coefficient (r)		0.9873	0.9860				

As seen in Table 8.2 the highest protein percentage extracted from the ±0.4g leaves was for SCA and then CHE2 with no significant difference at 750nm. For the mature fruit (Table 8.3) CHE2 reported the highest protein percentage at 540nm followed by AME and RET1 and RET4 with no significant difference between the last three mentioned species.

When comparing the protein percentage (at the most sensitive level) the protein percentage in the fruit was in most cases higher as seen for AME, RET1 and RET4. The protein percentage for CHE2 leaves and fruit displayed the same value. The protein percentage found in the leaves of SCA was much higher than the percentage protein in the species fruit. SCA is according to Schippers (2000), one of the species often use as leafy vegetables.

8.3.2 *Total soluble solid content*

The TSS content measured remained relatively constant for SCA (SD, 0.24) throughout the 16 days of development, as seen in Table 8.4 and Figure 8.1. The TSS for the species RET1 differed slightly over the 16 day period with a SD value of 0.51. The TSS for RET4 declined from 16.40% to 14.08% (SD, 1.07) and it is clear that the %Brix increased from 15.76% to 20.80% for AME (SD, 1.84), the values for CHE2 increased as well from 16.52% to 18.04% (SD, 0.65). The TSS in the fruit juice was inversely proportional to the size of the fruit as clearly depicted for SCA, with an average fruit size of 14.6mm and AME 7.47mm (Table 3.4). These results agree with those reported by Hobson and Kilby (1985) and Stevens (1986) on tomatoes.

Table 8.4 Averaged %Brix values for species measured over 16 days.

	AME	RET1	RET4	CHE2	SCA	LSD
DAY 1	15.76	12.52	16.40	16.52	6.76	1.3151
DAY 4	17.74	11.84	14.76	16.60	6.60	1.4238
DAY 8	17.57	13.32	14.28	16.16	6.40	1.3449
DAY 12	20.14	12.44	13.52	16.48	6.04	1.2285
DAY 16	20.80	13.00	14.08	18.04	6.38	1.7478

No significant differences were found between CHE2, RET4 and AME for the first day but on the 12th and 16th day significant differences were found between the genotypes. Day *et al.* (1990) found in their study on blueberries that the TSS remained at the same level throughout storage. El-Kazzaz *et al.* (1983) also reported no significant difference in TSS contents in their study among strawberries.

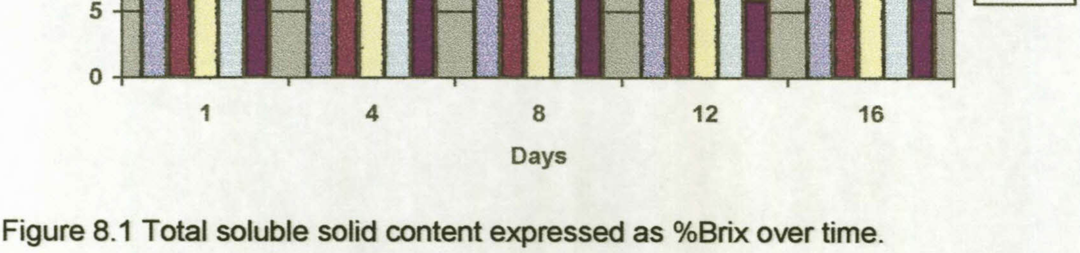

Figure 8.1 Total soluble solid content expressed as %Brix over time.

8.3.3 *pH measurement*

All the pH values for the five species measured are listed in Table 8.5. These values were measured on the juice of 20 freshly picked mature fruit and the data represents the average values of two replications for each species. Significant differences were only found between CHE2 and SCA, RET4 and RET1 with the LSD value of 0.4085. AME only differed significantly from RET4 and RET1.

Table 8.5 Acid content measured as pH from species tested.

SPECIES	ABB.	pH
Solanum americanum Mill.	AME	4.26
Solanum burbankii Bitter	RET1	3.87
Solanum retroflexum Dun. (smooth)	RET4	3.95
Solanum chenopodioides Lam.	CHE2	4.83
Solanum scabrum Mill.	SCA	4.21

TSS and acid content is at best only a rough guide to the taste of the fruit. More satisfactory information may be gained by using quick and inexpensive methods for quantitating the acidity of the fruit juice (Hobson and Kilby, 1985). Stevens (1986) stated that acidity contributes to taste more intensely than sugars. The best tasting species were CHE2 and AME and the results in this study confirm this as seen in Fig. 8.1 and Table 8.5.

8.4 Conclusions

Africa ranks lowest in the world in terms of the quantity and quality of available food. Malnutrition still has a grip on its people and good nutrition is the corner stone for survival, health and development. Black nightshade leaves, used as vegetables, can provide significant amounts of protein to the diet, and contribute several other minerals and vitamins (Fortuin and Omta, 1980; FAO, 1988) as well as the fresh and processed fruits and thus help in providing food for the people. Sreeramula (1982), also stated that the daily consumption of leafy vegetables in sufficient quantities would meet a large proportion of an individual's protein requirements.

The determination of the total protein indicates that the Lowry assay could be used successfully in determining the concentrations for the species tested. The percent for all species were low but the type of assay does play a role in the screening of the proteins. Schippers (2000) also reported that the leaves collected during the vegetative stage have a higher protein content than those harvested from flowering onwards. The leaves in this study were harvested during flowering, and this may be the reason for the low protein percentage found. The nutrient values may vary with soil fertility and plant age as well, as stated by Chweya (1997).

The protein concentrations in this study were found to be higher in the leaves than in the fruit of SCA, making the species valuable as a crop with significant potential as it is already being used as vegetable crop.

There was a clear inverse relationship between the TSS contents of the juice and fruit size, which correlates with results found in tomatoes (Hobson and Kilby, 1985; Stevens 1986). It was also found that the sugar concentration of fruit juice was higher in fruit with a high number of leaves per plant as was found for CHE2 (Fig. 8.1). Work done by Davies *et al.* (1958) and Hewitt and Stevens (1981) on tomatoes has shown the same results. CHE2 was the most tasteful species and shown the highest pH value (Fig.8.2).

Biochemical data may be superior taxonomic characters because they are not significantly affected by the environment (Gotlieb, 1977; Smith and Smith, 1986) and have a demonstrated ability to distinguish between different elite germplasm (Smith and Smith, 1989). It is also important that the growing conditions must be optimised as well to get a good balance between yield and quality.

Chapter 9

General conclusions

For the plant breeder morphological characters such as growth habit, leaf size, floral characters, fruit and seed morphology and fertility traits, have long been the traditional means for the evaluation of plants. Unfortunately not all the species received for this study were correctly identified and with the help of Edmonds and Chweya's (1997) 'working base' the species were identified. The identities of the species were also confirmed by cytogenetic studies and the chromosome numbers establish this identities of the species studied.

The 15 morphological characteristics measured proved to be a useful tool in determining the genetic relationship of the species studied as significant differences were found between the species for the characters studied, reflecting extensive genetic differences between the accessions. The morphological variation identified valuable traits usable in a breeding program and assist selection of species from different clusters as parents in a crossing block.

The occurrence of polyploidy is probably the most efficient barrier to natural hybridization between genotypes in this genus. Successful crosses were more difficult between taxa of different ploidy levels than they are between taxa of the same chromosome number, with interploidy crosses leading to the development of morphologically intermediate but sterile progeny. In the hybridization attempts some berries that formed were found to be empty or to contain non-viable seeds.

Extensive differences for almost all characteristics were found between species. *Solanum scabrum* ranked first for important morphological characteristics such as seeds per fruit, weight per bunch, weight of a single fruit, fruit size and total weight harvested. One of the hybrids with *Solanum scabrum* as female parent (SCAxAME) performed well in most of the other characteristics measured. These results were confirmed by the high mid-parent heterosis values calculated for these characteristics.

The ranking order of the accessions for most characteristics seemed nearly the same. Based on the correlation values, the most important yield components were weight per fruit bunch, single fruit weight, fruit size and total weight of fruit harvest. Selection of characteristic correlated with total yield such as fruit per bunch, weight per bunch and total weight would probably result in a plant with a much higher yield because of the relatively small environmental influence.

Apart from DNA, the biochemical and morphological genetic markers are scarce in the *Solanum nigrum* complex. Knowledge of the DNA distance between species should thus be useful in any breeding program because it facilitates efficient sampling and utilization of germplasm resources. The breeder can use genetic distance information to make informed decisions regarding the choice of species to cross for the development of populations, or to facilitate the identification of diverse parents to cross in hybrid combinations in order to maximize the expression of heterosis (Smith *et al.*, 1990).

DNA fingerprint patterns is a valuable tool for plant breeders and the knowledge of the genetic relationships generated in this investigation will be of value in directing the exploitation of available germplasm. *S. kwebense* N.E.Br, *S. tomentosum* L. as well as the unknown *Solanum* sp. did not belong to the *S. nigrum* complex as clearly seen in the AFLP marker study. AFLP results showed adequate resolution of the different species; the number of amplified polymorphic bands was dependent on the primer combination and the species.

Database screening was used as an entry point for the development of SSR markers in this study. The optimization of the PCR conditions was important and was not the same for the various primer pairs. The genetic distance values amongst the accessions clearly indicate the diversity among the accessions tested showing the relationship and confirmed the fact that the Solanum *nigrum* complex is a group of plants closely related.

The level of variation with careful selected microsatellites indicates that this could represent a contribution to the genetic characterization of *Solanum* for high level of polymorphism, it is very likely that this marker system will have the advantage of distinguishing closely related accessions arising from a similar pedigree. The use of this kind of molecular marker could facilitate molecular breeding practices in the crop.

There was some similarity between the clustering of species for morphological and for DNA data. Morphological data is often seen as subjective and unreliable (Smith & Smith, 1988). The AFLP, SSR and morphology dendrograms were able to clearly distinguish between the tested accessions. However, the AFLP dendrogram can be assumed as superior.

Solanum nigrum L. and its related species play a significant role in the nutritional needs of the people of subsistence farming societies in Africa. Several species are collected and frequently cultivated as a vegetable. Unfortunately very limited information is available on this crop's germplasm and to our knowledge, no known cultivars have been developed through conventional plant breeding methods (Edmonds and Chweya, 1997; Schippers, 2000).

Good nutrition is the cornerstone for survival, health and development and black nightshade leaves, can provide significant amounts of protein to the diet, and contribute several other minerals and vitamins (Fortuin and Omta, 1980; FAO, 1988) as well as the fresh and processed fruits and thus help in providing food for the people.

The percentage of the total protein for all the species were low but the type of assay does play a role in the screening of the proteins. Schippers (2000) also reported that the leaves collected during the vegetative stage have a higher protein content than those harvested from flowering onwards. The leaves in this study were all harvested during flowering this might be the reason for the low protein percentage found. The nutrient values may vary with soil fertility and plant age as well (Chweya, 1997).

To improve the understanding of the crop for better yield and quality, nutrient demand should be further investigated. Growing conditions should be optimized for a good balance between yield and quality.

Future research can be focused on large-scale production of the plants to help feeding people were it is really needed. Despite considerable effort and success, the *Solanum* nigrum complex remains a rich, largely untapped source of new characteristics to further improve this crop and hopefully other species in the Solanaceae family.

Chapter 10

Summary

- *Solanum nigrum* L. and its related species can play a significant role in the food supply and nutrition of people in subsistence farming societies in Africa.
- The objectives of this study were to conduct morphological trials on the parent material and progeny and to determine heterosis and correlation of the progeny to identify desirable characters that can be used in further breeding studies. The identification of the species was confirmed with the help of cytogenetics and the crossability of five species was investigated. The genetic relationships between the different species and progeny were established. The total protein percentage, sugar content and pH of the different parental species was also determinate.
- Seeds were obtained from several sources and seedlings were grown in a heat-controlled glasshouse. Established plants were planted out in a glasshouse and two field locations, these plants were used in the various investigations. The experimental designs were complete randomized blocks with three or four replications.
- The 15 morphological characters studied were subjected to an ANOVA and significant differences were found between the accessions tested.
- Cytological analyses were carried out and regard to the chromosome numbers the species seem to fit into the normal euploid series.
- The ANOVA on the parents and progeny showed significant difference reflecting the extensive genetic differences between them. *Solanum scabrum* was an excellent parent and selections for characteristics like fruit per bunch and fruit size should lead to improved plants.
- AFLP markers were used to distinguish polymorphisms between 14 species in this study. Three primer combinations between *EcoR* I and *MSE,* were used and multiple polymorphisms (62%) were detected.
- Seven independent sequences and their reverse sequences were selected for use as primers in the SSR study. The genetic distance value amongst the species and their progeny indicates the diversity but close relationship among the species tested.
- The young leaves and fruit of the parental species used in the crossing study were used in the assessments of the protein percentage, sugar and acid content of the plants.

- The protein percentages were in most cases higher in the fruit than the leaves except for *Solanum scabrum*; one of the species often used as leafy vegetable in Africa.
- The TSS value in the fruit juice was inversely proportional to the size of the fruit.
- The *Solanum nigrum* complex and related species is a rich, untapped source of characteristics and could be used to improve the crop and probably other species in the Solanaceae family.

Opsomming

- *Solanum nigrum* L. en verwante spesies in die kompleks kan 'n noemenswaardige rol as voedingsbron speel vir mense in Afrika, veral vir die in klein boerdery gemeenskappe.
- Die doel van die studie was: om morfologiese studies op die ouers en nageslag uit te voer; en om die heterose en korrelasie van die nageslag te bepaal om sodoende vas te stel watter eienskappe in 'n teelprogram gebruik kan word. Die spesies is deur middel van sitogenetiese studies ondersoek om hul positief te identifiseer en 'n kruisingsstudie tussen vyf spesies is uitgevoer. Die genetiese afstande en verwantskappe tussen die spesies is ook bepaal. Die totale protein, suiker en suur inhoud van die ouers is ook ondersoek.
- Sade afkomstig van verskeie bronne is ontkiem en die saailinge is in 'n temperatuur beheerde glashuis gekweek. Die volwasse plante wat in verskeie ondersoeke gebruik is was in die glashuis as ook in twee ander veld lokaliteite uitgeplant. Die eksperimentele ontwerp was 'n volledige gerandomiseerde blok ontwerp met drie of vier herhalings.
- Die 15 morfologiese karakters was onderhewig aan 'n ANOVA en betekenisvolle verskille is tussen die lyne waargeneem.
- Sitologiese studies is op die ouer spesies, wat in die kruisingsstudie gebruik is, uitgevoer en met betrekking tot die chromosoom getalle kon die spesies in die normale euploid reeks ingedeel word.
- Die ANOVA vir die ouers en nageslag toon merkwaardige verskille en dit dui dus op die genetiese verskille tussen die lyne. *Solanum scarbrum* was 'n uitstekende ouer en seleksies vir karakters soos vrugte per tros en vruggrootte kan tot verbeterde plante lei.
- AFLP merkers is gebruik om die verskillende vlakke van polimorfisme tussen die 14 spesies te onderskei. Drie priemstuk kombinasies tussen *EcoR* I en *MSE* is gebruik en 62% polimorfismes is waargeneem.
- Sewe onafhanklike priemstuk volgordes en hul omgekeerde volgordes is gebruik in die microsatelliet (SSR) studie. Die genetiese afstande tussen die spesies en hul nageslag dui diversiteit aan maar daar is tog 'n baie nou verwantskap tussen die ouer spesies.

- Die protein persentasies in die jong blare en vrugte as ook die suiker inhoud en suur gehalte van die vrugte is bepaal. Die protein persentasie was in die meeste gevalle hoër in die vrugte as in die blare behalwe vir *Solanum scabrum,* 'n spesie wat alreeds as blaargroente gebruik word.
- Die suiker inhoud in die vrugte was omgekeerd eweredig aan die vruggrootte.
- Die *Solanum nigrum* kompleks en verwante spesies is 'n ryk, maar onontginde bron en kan sekerlik gebruik word om hierdie gewas te verbeter en heel moontlik ook vir ander spesies in die familie Solanaceae.

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Addendum A

Binary data matrix for morphological characteristics measured.

CHARACTERS	CHEXAME	AME	RET1	RET2	RET3	RET4	SCA	VIL
Plant height	1	1	0	0	0	0	1	0
Plant spread	1	1	0	0	0	0	1	1
Leaf area	1	0	0	0	0	0	1	0
Days to flower	1	1	1	0	0	0	1	0
Days to fruit form	0	0	1	1	1	1	0	1
Days to fruit ripe	0	0	1	0	0	1	1	1
Seeds per fruit	0	0	0	0	0	0	1	0
Seeds per 0.1g	1	1	0	0	0	1	0	1
Flowers per bunch	1	1	0	0	0	0	1	1
Fruit per bunch	1	1	0	0	0	0	1	0
Weight per bunch	0	0	0	0	0	0	1	0
Weight per fruit	0	0	0	0	0	0	1	0
Fruit Size	0	0	0	0	1	0	1	0
Total Weight	1	1	0	0	0	1	0	1
Total number of fruit harvested	1	1	0	0	0	1	1	0

Addendum B

RHODES UNIVERSITY
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DEPARTMENT OF Botany and Micrbiology.

24th November, 1971.

Dr. J.A. Coetzee,
Department of Botany,
University of the Orange Free State,
Bloemfontein.

Dear Dr. Coetzee,

Many thanks for the loan of the Solanum nigrum complex specimens. They proved to be of much help in my project.

I have placed tentative names, following the scheme of C.B. Heiser (1961), on each of the sheets. The distinguishing morphological features of each of the most common groups within the complex are as follows:

S.retroflexum: Ovate, deeply dentate leaves. Strongly reflexed calyx (in fruit) and corolla lobes.
S.gracile: Lanceolate, slightly dentate leaves. Calyx and corolla lobes not markedly reflexed.
S.nodiflorum: Ovate, entire leaves. Calyx and corolla lobes not markedly reflexed.
S.burbankii: Ovate, dentate leaves (not to the same extent as in S.retroflexum). Calyx and corolla lobes not markedly reflexed. Bluish line down centre of corolla lobes. Purplish stems.

I hope this information will be of use to you.

Thanking you,
Yours faithfully,

I.M. Shaw.

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Genetic relationships between Southern African *Solanum retroflexum* Dun. and other related species measured by morphological and DNA markers

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Key words: AFLP, genetic relationships, morphological characteristics, *Solanum nigrum* complex, *Solanum retroflexum* Dun.

Summary

In this study, the genetic relationship between 14 genotypes of black nightshade, most which were part of the *Solanum nigrum* complex, was investigated. Fifteen morphological characters were measured and used to compile a dendrogram. Amplified fragment length polymorphism (AFLP) markers were also used to assess the level of polymorphism between the 14 *Solanum* genotypes. Three *EcoR* I/*Mse* I primer combinations with three selective nucleotides per primer were used for screening the respective genotypes. Multiple polymorphisms could be detected to the extent that all the genotypes studied could be distinguished, using any single primer combination, thus showing the usefulness of AFLP's for this purpose. Up to 43 polymorphic bands were detected with a single primer combination among the 14 different genotypes. The three primer combinations generated a total of 359 bands, of which 222 (62%) were clearly polymorphic. This data was used to compile a dendrogram. Both the morphological and AFLP marker analysis clearly separated the different genotypes into similar groups.

Introduction

One of the most widespread and variable species groups of the genus *Solanum* L., is that contained in the section *Solanum*, centering around the type species *Solanum nigrum* L., the black nightshade. This section is composed of a large number of similar species. Although these species are seen as weeds, especially in Europe and North America, many of them are minor food plants in various developing countries such as South Africa. The stems and leaves are used as pot-herbs and vegetables, and the berries as fruit and in the preparation of preserves. The plants are also used for medicinal purposes (Edmonds, 1972; Edmonds & Chweya, 1997).

Morphological characters have long been the means of studying genetic variability in plant species. Morphological data are affected by environmental interaction and thus descriptions must be made with sufficient replication and valid comparisons are only possible for descriptions taken at the same location during the same season (Smith & Smith, 1988).

Beckmann & Soller (1986) stated that compared to morphological and biochemical characteristics, the DNA genome provides a significantly more powerful source of genetic differences. Phillipp et al. (1994) also pointed out that the advantage of DNA fingerprinting over morphological markers is the dominance and the absence of pleiotropic effects. The DNA polymorphisms are theoretically unlimited and their detection is independent of the plant's development and the environment.

The AFLP technique detects mostly dominant markers based on polymerase chain reaction (PCR) amplification of genomic restriction fragments. This technique is designed as a sensitive method, which allows high numbers of polymorphic genetic markers to be identified, independent of environmental influences and tissue type (Vos et al., 1995).

The objectives of this study were to determine the genetic relationships between different *Solanum* genotypes belonging to the Section *Solanum* found in South Africa, using morphological data and the AFLP technique.

Materials and methods

Plant material

In Table 1 all the genotypes used, and their sources are given. Both entries from *S. chenopodioides* Lam. were only obtained at a later stage. This material could only be used in the AFLP marker study and not in the morphological trial because the other plants were already established in the field.

The material was established in the glasshouse at the end of the 1999 season and was transferred to the field early 2000. Field experiments were conducted on the campus of the University of the Free State. The experimental design was a complete randomised block with three replications. Each of the plots consisted of three rows with four plants per row. The plants were spaced 1 m within rows and the blocks were 2 m apart. Plants that died within two weeks of the original planting date were replaced by stock from the greenhouse.

Morphological characters measured

Solanum kwebenza N.E.Br., *S. tomentosum* L. as well as the *Solanum* sp. supplied by the gene bank in Pretoria do not belong to the *S. nigrum* complex and did not produce any berries and are therefore not included in the morphological character study. The other eight genotypes were analysed and data was collected on the following characteristics: plant height, plant spread, leaf area, days to flowering, days to fruit forming, days to fruit ripening, number of seeds in a fruit, number of seeds weighing 0.1g, flowers per bunch, fruit per bunch, weight per fruit bunch, weight of a single fruit, fruit size, total weight of all fruit harvest and total number of fruit harvested.

DNA extraction and amplification

Genomic DNA was extracted from approximately 1 g fresh leaf tissue of individual plants by using a modified extraction procedure developed by Edwards et al. (1991). The leaves were collected on ice and ground with a cold mortar and pestle to a fine powder in liquid nitrogen. The DNA was isolated from this powder by placing it in a cold 50 ml centrifuge tube and 10 ml warm (65 °C) extraction buffer (5 M NaCl, 0.5 M Tris-HCl, 0.25 M EDTA and 20% SDS at pH8) was added and mixed well. It was then placed in a preheated water bath (65 °C) for 30 min and inverted every 10 min. 1ml CTAB and 2ml NaCl was added as a clean up step and the mixture stood in the water bath for an hour. 10 ml chloroform-isoamylalkohol (24:1) was added, the mixtures were then centrifuged for 15 min at 10 000 rpm. The upper liquid was removed with a Pasteur pipette and placed into a clean 50 ml centrifuge tube. Ice cold 100% absolute ethanol was added (1:2) and refrigerated for up to 12 hours. The DNA that formed was scooped out and washed three times in 70% ethanol and then dissolved in sterile water. The DNA concentration was determined with a Hitachi U-2000 spectrophotometer.

The AFLP method was performed as described by Vos et al. (1995) and according to the manufacturers instructions (Gibco BRL) with some modifications. Approximately 250ng of genomic DNA was double digested with two restriction enzymes (*EcoR* I and *Mse* I), and then restriction fragments were ligated with *EcoR* I and *Mse* I adapters.

Pre-amplifications of the restriction fragments templates, were performed for 20 cycles with the following cycle profile: a 30s DNA denaturation step at 94 °C, a 60s annealing step at 56 °C and a 60s extension step at 72 °C. Pre-selective PCR products were diluted 50 fold in 1/50 TE buffer.

Selective AFLP amplifications were performed in a 20μl PCR reaction containing 5μl of the diluted pre-selective reaction, 4.5μl of the *Mse* I +3 primer, 1μl *EcoR* I +3 (fluorescently labelled) primer, 2μl of 10 × PCR buffer and 5U of Ampli Taq DNA polymerase.

The following three primer combinations with three selective nucleotides on both primers were used: E-ACA/M-CAA, E-ACA/M-CTA, E-ACA/M-CTT. Each one of the 14 *Solanum* genotypes were screened by the primer combinations. The *EcoR* I primer was labelled with FAM, a fluorescent label (PE Biosystems). Reactions were performed as follows. The first cycle consisted of a 30s DNA denaturation step at 94 °C, a 30s annealing step at 65 °C and a 60s extension step at 72 °C. The annealing step temperature was subsequently reduced by 0.7 °C during 12 cycles. This gives a touch down phase of 13 cycles. The 23 cycle will have the following cycle

Table 1. Genotypes and source with abbreviations used

Genotype	Abb.	Source
Solanum americanum Mill.	AME	University of the North – Qwa-qwa
Solanum burbankii Bitter (smooth)	RET1	University of the North – Qwa-qwa
Solanum retroflexum Dun. (smooth)	RET2	University of the North – Qwa-qwa
Solanum retroflexum Dun. (hairy)	RET3	University of the North – Qwa-qwa
Solanum scabrum Mill.	SCA	University of the North – Qwa-qwa
Solanum villosum Mill.	VIL	University of the North – Qwa-qwa
Solanum chenopodioides Lam. X *Solanum americanum* Mill.	CHE × AME	University of the North – Qwa-qwa
Solanum sp.	N22	Gene bank – Pretoria
Solanum sp.	N23	Gene bank – Pretoria
Solanum retroflexum Dun. (Pretoria, smooth)	RET4	Gene bank – Pretoria
Solanum kwebense N.E.Br.	KWE	Gene bank – Pretoria
Solanum tomentosum L.	TOM	NIB Kirstenboch – Cape Town
Solanum chenopodioides Lam.	CHE1	University of the North – Qwa-qwa
Solanum chenopodioides Lam. from UFS botanical garden	CHE2	University of the Free State

profile: a 30s DNA denaturation step at 94 °C, a 30s annealing step at 56 °C and a 60s extension step at 72 °C. All amplification reactions were performed in a Perkin-Elmer 9600 thermal cycler (Perkin Elmer Corp., Norwalk, CT, USA).

After amplification, 5 μl of each of the selective reactions were added to a new tube containing 24 μl of formamide and 1 μl of Rox 500 (PE Biosystems) standard size marker, denatured at 94 °C for 5 min and run on a Perkin Elmer ABI Prism 310 Automated capillary sequencer (PE Biosystems).

Statistical analysis

For each morphological characteristic the data was averaged (Table 2) and scored as presence (1) or absence (0). The data was scored as 0 when the value was below the average of all the genotypes and as 1 when it was above the average, in order to generate binary data to create the dendrogram, using the NCSS 2000 (Number Cruncher Statistical Systems) software programme and to enable us to compare the results with the AFLP analysis.

Analysis for the AFLP results was also conducted to generate a dendrogram, depicting relationships among the species by using the NCSS 2000 software program. Data was scored as presence (1) or absence (0) of each DNA fragment and compiled into a binary data matrix. Both polymorphic and monomorphic fragments from the three primer combinations were used in the analysis. Estimates of similarity between all pairs of accessions were converted to dissimilarity and expressed as Euclidean genetic distances.

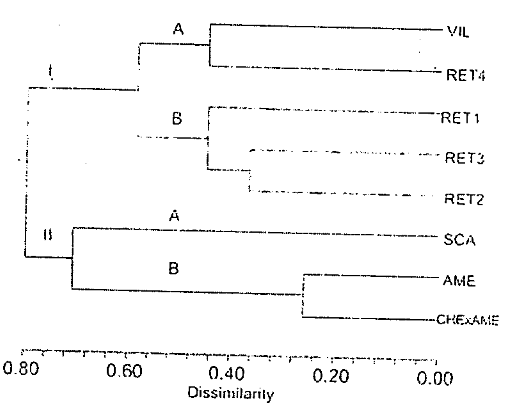

Figure 1. Dendrogram for morphological characteristics VIL = *Solanum villosum*, RET4 = *Solanum retroflexum* (Pretoria -smooth), RET1 = *Solanum burbankii* (smooth), RET3 = *Solanum retroflexum* (hairy), RET2 = *Solanum retroflexum* (smooth), SCA = *Solanum scabrum*, AME = *Solanum americanum*, CHExAME = *Solanum chenopodioides* X *Solanum americanum*.

Results and discussion

Morphological data

Significant differences were found between the eight genotypes for the morphological characteristics measured, with genetic distance ranged from 0.26 to 0.80

Table 2. Averaged data of the different morphological characteristics tested

	CHEX AME	AME	SCA	RET2	RET3	RET1	RET4	VIL
PH (cm)	62	58	33	27	17	22	20	27
PS (cm)	143	140	160	82	70	110	100	130
LA(cm^2)	12.08	10.14	47.10	5.87	5.56	2.75	5.85	6.07
D-Flo (days)	20	21	25	14	15	20	14	12
D-FF (days)	13	10	11	18	17	18	17	18
D-FR (days)	13	14	20	16	16	18	17	18
S/F	35	35	113	25	36	27	28	37
S/0.1 g	119	118	94	87	103	83	108	154
Flo/B	4	5	6	3	3	3	3	4
Fru/B	4	4	5	3	3	3	3	3
W/B (g)	1.34	1.27	9.60	1.07	1.08	1.09	1.08	0.59
W/F (g)	0.26	0.27	1.49	0.31	0.39	0.32	0.31	0.15
F-size (mm)	7.2	7.5	14.6	8.1	8.5	7.9	8.1	6.0
TW (g)	573.52	314.80	634.56	88.65	40.01	262.46	308.41	182.81
TY	2218	1355	440	303	98	880	1134	1372

Plant Height (PH), Plant Spread (PS), Leaf Area (LA), Days to Flowering (D-Flo), Days to Fruit Forming (D-FF), Days to Fruit Ripening (D-FR), Number of Seeds in a Fruit (S/F), Number of Seeds Weighing 0.1g (S/0.1g), Flowers per Bunch (Flo/B), Fruit per Bunch (Fru/B), Weight per Fruit Bunch (W/B), Weight of a Single Fruit (W/F), Fruit Size (F-size), Total weight of fruit harvest (TW) and Total Number of Fruit Harvested (TY).

(Figure 1), reflecting genetic variation. The dendrogram produced, revealed two large clusters (I and II) with two sub-clusters (A and B) each (Figure 1).

The smooth leaved *S. retroflexum* from Pretoria (RET4) clustered with *S. villosum* (VIL) in the first cluster (IA) with a dissimilarity of 0.44. Rao and Kumar (1981) also demonstrated a close genetic relationship between these two species. RET4 clustered separately from the hairy (RET3) and the smooth (RET2) leaved *S. retroflexum*, found in cluster IB with a genetic distance of 0.36. RET2 and RET3 are morphologically very similar, with exception of hairiness. *S. burbankii* (RET1) clustered separately in cluster IB, with a dissimilarity of 0.44 to RET2,3. All the plants in this cluster I are generally characterised by semi-erect growth habit, a small leaf area and small amount of flowers and fruit per bunch.

In the second major cluster (IIA) *S. scabrum* (SCA) clustered separately with the highest genetic distance of 0.70, while *S. americanum* (AME) and the CHExAME hybrid clustered together with a dissimilarity of only 0.26, the lowest genetic distance in the dendrogram. This clustering might be incidental because the AME genotype examined here was only a genotype from the parental species and variation in the parental species is assumed. Cluster II is characterised by an erect growth habit, a high number of flowers and fruit per bunch.

AFLP data

The number of amplified DNA polymorphic bands detected by the individual primer combinations ranged from 10 to 43. The number of amplified DNA polymorphic bands was dependent on the primer combination and the species. A minimum number of 10 (primer combination E-ACA/M-CTA) and a maximum of 43 (primer combination E-ACA/M-CAA) polymorphic bands were observed. Although the three primer combinations tested differed from each other by substitution of only one to three nucleotides, unique AFLP DNA patterns or fingerprints were generated by each primer combination. In their work on potatoes, Kim et al. (1998) reported similar findings. Of a total of 359 bands generated by the three primer combinations, 222 (62%) were clearly polymorphic. All 14 genotypes were distinguished by any one primer combination.

The genotypes separated into two major clusters (I and II) (Figure 2) with two sub-clusters (A and B) each. The species falling out of the *Solanum nigrum* complex (*S. tomentosum* L. (TOM),*S. kwebenza* N.E.Br. (KWE), N22 and N23) were clearly separate (IA and IB) from those falling within the complex, as was expected.

It was very interesting that the eight genotypes evaluated for morphological data clustered all together in IIA. *S. burbankii* - smooth (RET1), *S. retroflexum* – hairy (RET3), *S. retroflexum* – smooth (RET4), and

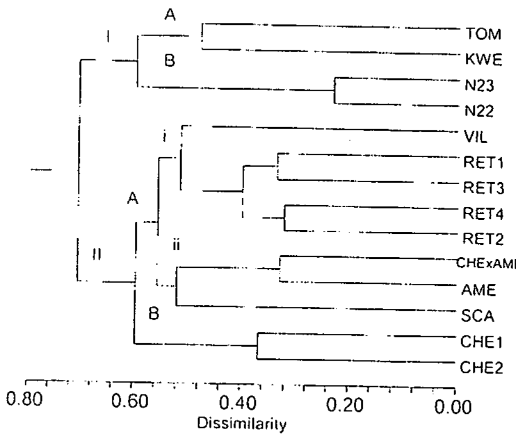

Figure 2. Dendrogram for AFLP data. TOM = *Solanum tomentosum*. KWE = *Solanum kwebenza*. N23 = unknown *Solanum*. N22 = unknown *Solanum*. VIL = *Solanum villiosum*. RET1 = *Solanum burbankii* (smooth). RET3 = *Solanum retroflexum* (hairy). RET4 = *Solanum retroflexum* (Pretoria-smooth). RET2 = *Solanum retroflexum* (smooth). CHExAME = *Solanum chenopodioides X Solanum americanum*. AME = *Solanum americanum*. SCA = *Solanum scabrum*. CHE1 = *Solanum chenopodioides (UN)*. CHE2 = *Solanum chenopodioides (UFS)*.

S. retroflexum – smooth (RET2) all clustered in IIAi. With a genetic distances of 0.34 between RET1 and RET3, and a genetic distance of 0.32 between RET4 and RET2. *S. villosum* (VIL) clustered separately from the others with a dissimilarity of 0.51 in the same large cluster. This grouping differed from that found for the morphological analysis and is an example of the possibly miss comprehensiveness of morphological traits.

The CHExAME hybrid and *S. americanum* genotype clustered together in IIAii with a genetic distance of 0.34. *S. scabrum* clustered alone with a dissimilarity of 0.52 in the same cluster (IIAii). Within sub-cluster IIB, *S. chenopodioides* (CHE1) and *S. chenopodioides* (CHE2) clustered together with a genetic distance of 0.37.

There was similarity between the clustering of species for morphological and for DNA data. Morphological data is often seen as subjective and unreliable (Smith & Smith, 1988). The AFLP technique and morphology were able to clearly distinguish between the tested genotypes. However, with respect to the above discussion, the AFLP dendrogram can be assumed as superior to the morphology based dendrogram.

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Addendum D

Binary data matrix for the AFLP data

AME
0100100010001000110100100000000010000110000001100010001000000000100100
0001001000010001001010000000100001010010010000100000000000000000001000
0001000000101000000000000000000011100000000000011100000100010100000101
0000001000000000001111000000010100100000000000000000010001000100010100
0001100100110010011000001010010000001001010000010010000000000000001011
01010001010000101011010001000000000000

CHE1
0111000000001001010001000000000100010100000011000101000000000000010000
0100010000000100110000000000100001000010000100010000000000000000000000
0011000000000000100000000010001001000000100000100000000000000000100011
1000001000000000010000000000010000000000100000000101001000000000000000
0100000000000000000000000000000000001000000000000000000000000000000000
00000000000000000000000000000000000000

CHE2
1100000000001001010100100000000010001010000001100011000000111001000010
0110100000000100000100000000010000100000000000100111000000010101000100
0001100000000000000000000010001001000000100000100100000000000000100001
1000001000010000010000000000010000000000000000001001001010010100100000
0000000000000000000000000000000000000000000000000000000000000000000000
00000000000000000000000000000000000000

KWE
0110010100010110000000000100111001000011010000000110000010010001001000
0010000100000000010000011110000110111000001000010000110100001011001010
0101000010111000100100010100100010000110100001001001010010000101000000
0000100100001001001010001101000000100000011010000000100001000000111010
0000000000000000000000000000000000100000000100100000000000000000000000
00000000000000000000000000000000100001000

RET1
1100000000001000110000000000000000000010000000000000000000000000000010
1111010001010100000000000000000000000000000000100000000000000000000000
0011000100001000000000000000000010100000000001001000000011011010000001
0000001000000000000000000000000000000000000000010000000000000000001
0000000000000000000000000000000000000000000000000000000000000010000000
00000000000000000000000000000000000000

RET2
0110000100101001010101110000000101000000000010100000000100000100011000
0101010000001000100001010000000000101000000000001000000000000000000000
0011000101011000000010010000000101000000000000010000000110010100000001
0000001000000000000000000000000000000000000010101000100001100000000100
0000000000100000000110000000010000000000110000000000001000000000010000
000010001000100000001000000000000000000

RET3
01001000100110001100001100000001000001000001010000000100100100010010
1001010000010001000000000000000000000000000000000000000000000000
00010011010010000000100100000001010100000001001000000011001010000001
0000001000000000000010000000000000000000000000000000001000000000000000
00000000000000010000000001000000000010000000000100000000001000000000
0100001000100000001000100000000000000000

RET4
01100001000010010100010001000001010001000001010000000100000000011010
01110100000100000000101000000000001000000000000100000000000000000000
000100010000100001000001000000010100000000000010010000011001010000001
000000100000000000010000000000000000000000001000000010000010000000000
000000000000000000001000000000100000000010000000000000100000000010000
0000100010001000000010100000000000000000

SCA
01010010000010100100001000000000001000100000001100010000100000010001000
00010100000001001000000000000000000000000000000100000000000000000000
00110001000110010001010000100001010001000001001001000010000100100101
000000100000000001100000100001100000000000000000000010000000100010001
0001000100010100001000100000100000101001000000000010100000000000001100
11010000001010011100001000100000001000000

TOM
01100101000101100010100000011010100010100100011101001011000000000001
00100001000000100001000010000001000001001000000000000100001000000001
1001010000101000000100100000001000000000110000010000101000101000100000
00001000000000101000000000000000000000000000000000010000000001000010
000001001000000000001000000010001000101100000111100010001000011000000
0001000000000000000000000000101010110100

VIL
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000100001000010000000000000000000000000000000001000000000000000000000
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0000000000000000000000000000000000000000000000000000000000000000000000
0000000000000000000000000000000000000000

CHExAME
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N22
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0000000000000000000000000000000000000000

N23
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0010010000100100000010001001001010001011

Addendum E

Binary data matrix for SSR data.

AME
000000001000000010010010010000000000001000000101000000000000001000000
000000100000000000000100010001010001000001000100011001000000000001000
010001101101010110000000100000001000000010000000000000100000100001011 0
001010000100

RET1
000001000000000100000000000000000000000000000100000000000010000000 0
0000000100000000000000100000000000000000000010000000100101100010000 0
100100101001000000000001000000010001000100000100000010010010100010 11
0101001000000

RET4
0000001000000100001000010100100000000100010000100000000000000000000
000000000000100000000000000010010000100000000000100010000000101000000
000000000000000010100100000000100100001000000000000000001000000000100000
00000000001

CHE2
000100100000000100010000100000010000000100000100000000000000000000001100
000001000000000000001000000000000010000000010000000000001000000000000
000000000000000000000000100000010000000010000001000001000000000100101
000000010010

SCA
000000100000000000000000110010000000000010010000000000000000000000000000
000100000000000000010000100010000000000100000100000000000000000000000000
000000000000000000100000101010000100000000100000000000001000100000010000
000100001010

AMExSCA
000000100010000001000000010000000000001000000000010000000000000000100000
000000000000100100000010011010100010000100000000000000000000000001000010
000000000000000000000000100000000100000001000000000000100001000000010000
000100000010

SCAxAME
0000000000001000000010000000000000100000000000100100010000010000000010
000000000100000000000001000000101000000000100000000000000000000000000000
000000000000000000000000000100100000010000100010010000000000000010000000
00000000000

RET1xCHE2
001000100000000100100001000001000000000100001001000000000000000000001000000
01000001000110000000000000000000001000000100000000110000100000001000100000
000100000000000000000000000000000000000100010000000001000010000000010100
000000100010

CHE2xRET1
0010000000001000000000000000110001000100001000010010001010100000010010
0001000010001000100000000000000010000000100000010010000000000000000000
0000000000000000000000000000000001000000001001000100000000000001000000
0000000000000

RET1xRET4
1000000000001000001000100001100010010000000010000100010101000000010010
0001000010001000100000000000000000000000100000001010000000000010000000
0000000000000000000000000000000000000000000000100000011000010000001000
0000000100000

RET4xRET1
0000000000100000000000000001001001000000000000001000100101010000000000
1000010000010000000000000000000100001000000000000000000000000000000000
0000000000000000000010100000010001001000000100110011000000000000100000
0000000000000

CHE2xRET4
0000000001000000000000000000000000000000000000000100000100000000000010000
0010000000100100001100000000000101000000010000000000000000000000000000
0000000000000000110000000000000010100000100000001000000000000001000000
0010000000000

RET4xCHE2
0000000000100000000000000000000000000000000000001000100000010001000000
0100000001001000000010000000001000000000010000100000000000000000000000
0000000000000000000000000000010000000100000001010000000000000100000
0000000000000

CHE2xSCA
0000100000000000000000000000000000000000000000000000000001000000000
0100000000010000000000000000010000000100000000000000000000100000001
0100010001001100000000000000000000000000010000000000000000000000000100
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Key words

Amplified Fragment Length Polymorphism

Cytogenetic

DNA fingerprinting

Genetic diversity

Hybridization

Morphological characterization

Nutritional value

Simple Sequence Repeats

Solanum nigrum complex

Solanum retroflexum