

DOCTOR OF PHILOSOPHY

Cytology, ploidy and molecular taxonomy of prosopis juliflora DC and prosopis pallida HBK

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ABSTRACT

Prosopis juliflora (Sw.) DC. and *P. pallida* (H.&.B. ex Willd.) H.B.K. occur naturally in the Americas and they are morphologically similar species. They are multipurpose trees and their ability to grow in poor soil conditions has encouraged their introduction throughout the arid regions of the tropics and semi tropics. The taxonomy of the genus *Prosopis* Linnaeus emend. Burkart is unclear and a revision of the genus is suggested by several studies. Due to their similarity, the differentiation of *P. juliflora* and *P. pallida* is difficult. Although there has been some success in differentiating the species using ploidy, leaf morphology and molecular markers, further approaches are required. This study looked for suitable cytological characters and molecular markers that would differentiate the two tropical species. The cytological character analysis allowed for the development of a dichotomous key for their identification based on leaf stomatal characters. Stomatal length and epidermal cell density are the two characters on which the keys were developed. Correct identification of the species will allow for genetic improvement programmes and conservation efforts in the native regions. Multivariate statistical analysis showed that *P. juliflora* and *P. pallida* were separable into two groups. This could help in the classification of unknown herbarium specimens or previously misidentified samples. The phylogenetic relationship of the two species is also disputed and previous RAPD studies show close genetic similarity between the two species, suggesting a reconsideration of the series within section Algarobia. There are also disputes regarding the geographical distribution of both species and naming of new species. In this study, similarity index analysis and cluster analysis on the RAPD data revealed *P. juliflora* to be more closely related to North American *Prosopis* spp. than to *P. pallida*. This result does not agree with other published studies which identifies them

as closely related species, but agrees with the classification of Burkart. In the present study, the transferability of microsatellite markers developed from *P. chilensis* (Molina) Stuntz emend. Burkart and *P. flexuosa* DC. to other *Prosopis* species was studied. The microsatellite markers gave successful amplification profiles in some of the 13 *Prosopis* species analysed. Chloroplast DNA intron region was also utilised in this study to detect potentially informative sequences which could differentiate the two species. The trnL-trnF intergenic spacer region in the cpDNA was utilised. This approach was able to confirm the sequence difference in the spacer region between the two species and future work involving additional DNA regions in the chloroplast gene would provide more insight into the phylogenetic relationship of all the species. This could also clear the taxonomical confusion surrounding the genus as a whole. This study has improved knowledge on the relationship between the morphologically similar tropical *P. juliflora* and *P. pallida*. The differentiation of the two species using cytology and molecular markers has provided an improved understanding of these resourceful species.

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1. INTRODUCTION

1.1 Introducing the genus *Prosopis*

The genus *Prosopis* Linnaeus emend. Burkart, belongs to the family Leguminosae, subfamily Mimosoideae, tribe Mimoseae. Burkart (1976) identified 44 species in this genus, the taxonomic classification based on morphological differences between *Prosopis* specimens. Carolus Linnaeus in 1767 suggested the genus name *Prosopis* in his book *Mantissa Plantarum* for the only species he had seen, *P. spicigera* L. (now *P. cineraria* (L.) Druce. *Prosopis* species are distinguished from other genera of Mimosoideae by their indehiscent fleshy pods and release of pollen in single grains (Burkart 1976, Ffolliott and Thames 1983). Many species of this genus are valued as multipurpose trees with an ability to thrive in physiologically adverse conditions. *Prosopis* species are thought to have evolved from their ancestors to become better adapted to drought conditions by developing deep roots and suitable physiology (Pasiiecznik *et al.* 2001). Species of major economic importance have their pods utilized for food and fodder, and wood for fuel and timber (Pasiiecznik *et al.* 2001) and this has encouraged their introduction to economically poor regions of the world (Hughes 1994).

The origin of the genus *Prosopis* is considered to be Africa (Raven and Polhill 1981), though their flavanoid chemistry (Solbrig *et al.* 1977), together with the morphologically diverse South American *Prosopis* species and their similarity to North American and other Old World species (Burkart (1976) supports the consideration of some researchers that South America is the origin (Burkart and Simpson 1977). Most of the species are native to the Americas, with one from tropical Africa and three from South and West Asia and northeastern Africa (Burkart 1976, Hunziker *et al.* 1986).

1.2 Taxonomy of the genus

The genus *Prosopis* is thought to be a primitive one within Mimosoideae (Burkart 1976) with a base chromosome number of 14 (Burkart and Simpson 1977). The taxonomical classification of the genus *Prosopis* by the Argentine botanist Arturo Burkart (1976) is widely accepted as the most complete. He described 44 species of *Prosopis* with many varieties, forms and populations often hybridizing with each other. Predominantly an American genus, about 40 species from *Prosopis* is New World natives (Burkart 1976). 4 of the Old World species are found in Africa and Asia (Ffolliott and Thames 1983). Many of the species he described have overlapping native ranges and may display striking similarity in their morphology. This has led to taxonomic confusion within the genus and much of the debate is concentrated on the ranking and classification of species as described by Burkart (1976). He divided the genus *Prosopis* into five sections (Table 1.1) based on morphological differences, with species from three of these sections, Algarobia, Monilicarpa and Strombocarpa occupying the Americas and two from Africa and Asia.

Prosopis species have been introduced in many countries such as India, Pakistan, Brazil, Hawaii, South Africa, Egypt, Kuwait, and Australia as a source of fuel, firewood, timber, charcoal, gum, production of honey, in controlling erosion and other raw materials (De Loach 1985). In some of the introduced regions they have become noxious weeds, but also noted for their varied types of beneficial values and raw materials (De Loach 1985). About 6 *Prosopis* species have been introduced to southern Africa from the New World (Poynton 1990). Spontaneous hybrids between these introduced species have become invasive weeds colonising a wider range of habitats

than either of the parent species (Poynton 1990). *Prosopis* sp. is highly invasive in Australia, where it was introduced over the last 200 years (Pasiecznik *et al.* 2001). In the Pilbara region of Australia, the invasive *Prosopis* species, which reach high densities and have closed canopies, eventually destroys the herbaceous layer and native shrubs (van Klinken and Campbell 2001).

The section *Algarobia* contains by far the largest number of species. This section accounts for 30 morphologically diverse New World species (Burkart 1976). Most of the species are valuable as multipurpose trees and are widely introduced (Booth and Wickens 1988). Burkart (1976) raised the rank of many species in this section which were previously assigned only varietal rank by Bentham (1875). Burkart (1976) further divided this section into six series, namely, *Sericanthae*, *Ruscifoliae*, *Humiles*, *Denudantes*, *Pallidae* and *Chilenses*. Much of the taxonomic confusion is centred on this section (Pasiecznik *et al.* 2001). The section *Strombocarpa* containing eight species is divided into two series *Strombocarpae* and *Cavenicarpae*, and section *Monilicarpa* is mono-specific. It has been shown that section *Algarobia* can be clearly separated from the other sections based on morphological, biochemical and molecular characters (Martinez 1984, Burghardt and Palacios 1997, Ramírez *et al.* 1999). Although similarities exist in terms of floral and leaf morphologies between the species from sections *Algarobia* and *Strombocarpa*, Burkart (1976) identified certain distinct morphological characters separating the two, for example, the coiling pattern of the fruit and spine. The two sections are also different in terms of genetic variability and adaptive strategy. Hence these sections are suggested to be raised to the level of subgenera (Saidman *et al.* 1996). Despite the wide range of morphological variation in section *Algarobia*, the species hybridise readily. Previous studies have shown a strong degree of

similarity between *Algarobia* species (Martinez 1984, Saidman 1990, Burghardt and Palacios 1997, Ramírez *et al.* 1999).

Identifying taxonomically good species and grouping them into complexes on the basis of their distinguishable morphological characters, genetic similarities and geographical proximities would help in clarifying the existing debates on this section and the genus as a whole (Pasiiecznik *et al.* 2001). However, assigning ranks to the different forms and habits of species is complicated within this section (Pasiiecznik *et al.* 2001). For example, *P. glandulosa* Torrey exists in prostrate and bushy forms. Burkart (1976) assigned varietal rank to the prostrate form of *P. glandulosa* that Johnston (1962) identified in North America. But a similar case observed with *P. laevigata* (H. & B. ex. Willd.) was not assigned a varietal rank (Johnston 1962, Burkart 1976). Ramírez *et al.* (1999) also suggested regrouping the species in this section by identifying molecular markers which occur commonly in species of *Algarobia*. Pasiiecznik *et al.* (2001) suggested grouping the species of the genus into ‘complexes’ or ‘species groups’ based on morphological and molecular similarities, environmental adaptations and resource characteristics. One such complex, the *P. juliflora*-*P. pallida* complex, has generated special interest over the past few years owing to their resource value and similarities. The origins of the two species can be traced back to the tropical regions of Peru and Central America (Pasiiecznik *et al.* 2001). *P. pallida* is native to Peru, Ecuador and Colombia while *P. juliflora* is native to Central America, from Mexico to Panama, Caribbean islands (introduced from the mainland and subsequently naturalized) and to South America, from Venezuela to Peru (Burkart 1976). Naturalised populations of *P. juliflora* also occur on the Galapagos Islands (Wiggins and Porter 1971).

Table 1.1: The 44 species of *Prosopis* L. as described by Burkart (1976)I. Section *PROSOPIS*

1. *P. cineraria* (L.) Druce
2. *P. farcta* (Solander ex Russell) MacBride
var. *farcta*
var. *glabra* Burkart
3. *P. koelziana* Burkart

II. Section *ANONYCHIUM*

4. *P. africana* (Guill., Perr. & Rich.) Taubert

III. Section *STROMBOCARPA*

Series Strombocarpae

5. *P. strombulifera* (Lam.) Benth
var. *strombulifera*
var. *ruiziana* Burkart
6. *P. reptans* Benth
var. *reptans*
var. *cinerascens* (A. Gray) Benth
7. *P. abbreviata* Benth
8. *P. torquata* (Cavanilles ex Lagasca) DC.
9. *P. pubescens* Benth
10. *P. palmeri* S. Watson
11. *P. burkartii* Muñoz

Series Cavenicarpae

12. *P. ferox* Grisebach
13. *P. tamarugo* F. Phillipi

IV. Section *MONILICARPA*

14. *P. argentina* Burkart

V. Section *ALGAROBIA*

Series Sericanthae

15. *P. sericantha* Gillies ex Hooker & Arnott
16. *P. kunzei* Harms

Series Ruscifoliae

17. *P. ruscifolia* Grisebach
18. *P. fiebrigii* Harms
19. *P. vinalillo* Stuckert
20. *P. hassleri* Harms
var. *hassleri*
var. *nigroides* Burkart

Series Denudantes

21. *P. denudans* Benth
var. *denudans*
var. *patagonica* (Spegazzini) Burkart
var. *stenocarpa* Burkart
22. *P. ruizleali* Burkart
23. *P. castellanosii* Burkart
24. *P. calingastana* Burkart

Series Humiles

25. *P. humilis* Gillies ex Hooker & Arnott
26. *P. rojasiana* Burkart

Series Pallidae

27. *P. rubriflora* E. Hassler
28. *P. campestris* Grisebach
29. *P. pallida* (Humboldt & Bonpland ex Willd.) H.B.K.
30. *P. affinis* Sprengel
31. *P. articulata* S. Watson
32. *P. elata* (Burkart) Burkart
33. *P. tamaulipana* Burkart

Series Chilenses

34. *P. chilensis* (Molina) Stuntz emend. Burkart
var. *chilensis*
var. *riojana* Burkart
var. *catamarcana* Burkart
35. *P. juliflora* Swartz DC.
var. *juliflora*
var. *inermis* (H.B.K.) Burkart
var. *horrida* (Kunth) Burkart
36. *P. nigra* (Grisebach) Hieronymus
var. *nigra*
var. *ragonesei* Burkart
var. *longispina* Burkart
37. *P. caldenia* Burkart
38. *P. laevigata* (Humboldt & Bonpland ex Willd.) M.C. Johnston
var. *laevigata*
var. *andicola* Burkart
39. *P. flexuosa* DC.
forma *subinermis* Burkart
40. *P. glandulosa* Torrey
var. *glandulosa*
var. *torreyana* (Benson) Johnston
var. *prostrata* Burkart
41. *P. alpataco* R.A. Phillipi
42. *P. alba* Grisebach
var. *alba*
var. *panta* Grisebach
43. *P. velutina* Wooton
44. *P. pugionata* Burkart

RAPD and isozyme markers have been utilized to determine the population structure of Algarobia species (Juárez-Muños *et al.* 2002, Ferreyra *et al.* 2007, Hamza 2010). Based on the phenetic data derived from isozyme and RAPD analysis of 17 populations of 5 species from Algarobia, Ferreyra *et al.* (2007) do not support the division of section Algarobia into series. Biochemical and molecular techniques have also been employed to infer the relationship between different species in the genus (Bessega, Saidman, and Vilardi 2000, Landeras *et al.* 2006). These findings suggest reducing the rank of many species and varieties classified by Burkart (1976) to a lower one.

Many species of section Algarobia are commonly known by the vernacular names ‘mezquite’ or ‘mesquite’ in North America and ‘algarrobo’ in South America. Species of section Strombocarpa are commonly known as screwbeans due to their spirally coiled pods (Pasiiecznik *et al.* 2001). Previous researchers have studied the need for proper identification of *Prosopis* species since many were wrongly identified in the past (Pasiiecznik, Harris, and Smith 2004). This led to the incorrect naming of the species and has fuelled further taxonomic confusion. Also, extensive interspecific hybridisation within the genus means that many populations may not be restricted to a single species (Pasiiecznik *et al.* 2001). Occasional hybridisation between species of the section Algarobia followed by introgression has introduced genetic variation into the populations (Solbrig *et al.* 1977). This genetic variation introduced by outcrossing is more prominent in section Algarobia than in Strombocarpa (Hunziker *et al.* 1975). Introgression is frequent among species of the series Ruscifoliae, Pallidae and Chilenses within section Algarobia (Palacios and Bravo 1981, Vázquez-Garcidueñas *et al.* 2003). Also, host specificity was shown by insects attacking *Prosopis* species from different

sections. Insects attacking plants from section *Algarobia* did not feed on plants from *Strombocarpa* (De Loach 1982). Isoenzyme content also varied between sections in the genus (Solbrig and Bawa 1975).

The high incidences of interspecific hybridisation could be due to few chromosomal differences and lack of incompatibility barriers between closely related species (Hunziker *et al.* 1975, Solbrig *et al.* 1977, Saidman 1990). Genetic and chromosomal barriers restricting hybridisation between species of the same section is limited in the genus (Solbrig *et al.* 1977). Other factors which favour interspecific hybridisation are sympatry, protogyny, partial overlap of flowering times, and little specific discrimination by pollinating insects (Solbrig *et al.* 1977, Naranjo, Poggio, and Enus Zeiger 1984). Many hybrid populations with intermediate morphological characters compared with their parental types have been observed in section *Algarobia* (Solbrig *et al.* 1977, Naranjo, Poggio, and Enus Zeiger 1984, Ferreyra *et al.* 2007). However the pollen viability and fertility was remarkably lower than their pure species which suggests cryptic structural hybridity (Naranjo, Poggio, and Enus Zeiger 1984). Evidence of interspecific hybridisation between *P. caldenia* Burkart and other two species namely, *P. flexuosa* and *P. alpataco* R.A.Philippi from *Algarobia* is reported by Saidman (1990). Although this was the case, no adult morphohybrids were found in the zones of sympatry. This suggest an isolating mechanism operating, either prezygotic, which prevents heterologous pollination or postzygotic, which prevents the development of hybrid seeds or seedlings (Saidman 1990). However, active reproductive barriers prevent the formation of hybrids between sections as seen between species of section *Algarobia* and *Strombocarpa*. Thus, identifying the reproductive barriers between

species should help in resolving their taxonomy (Pasiiecznik *et al.* 2001).

1.3 Natural distribution of the genus

The natural distribution of *Prosopis* species (Figure 1.1) ranges from the arid and semi-arid regions of the Americas, from southwestern United States and Mexico to Central Chile and Argentina, Southwest Asia and Africa (Burkart 1976). The species are predominantly distributed in Americas with the centre of polymorphism in Argentina (Burkart 1976).

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Figure 1.1: Natural distribution of the *Prosopis* and its species (from Pasiiecznik *et al.* 2001)

According to Burkart (1976), the American *Prosopis* species probably divided again and became widely separated into two centers, the Mexican-Texan center and the Argentine-Paraguayan-Chilean one. There is an intermediate region between the two

centers populated by both *P. juliflora* and *P. pallida* and occupies northern South America, southern Central America and the Caribbean (Pasiiecznik *et al.* 2001). The species from each of these three regions are geographically distinct (Pasiiecznik *et al.* 2001). Asian *Prosopis* species are native to the Middle East, extending east to India, north to Georgia and Turkmenistan, and west to Algeria along the north African coast (Pasiiecznik *et al.* 2001). *P. africana* (Guill., Perr. & Rich.) Taubert is native to tropical Africa, extending from Senegal in the west to Sudan and Kenya in the east throughout the Sudano-Guinean ecozone (Pasiiecznik *et al.* 2001).

1.4 *Prosopis* and its uses

The species of this genus, owing to their ecological and economic uses, have been used for food, fuel and forage in the arid and semi-arid regions (Leakey and Last 1980). Species of the section *Algarobia* are of particularly high resource value. They have been widely used for human consumption and were the staple food for many native North Americans in the desert regions of Arizona, California and north-western Mexico (Felger 1977, Felker 1981). The trees are also used for firewood and lumber (Felger 1977). The abundant growth of *Prosopis* in dense populations on soils with relatively low nutrients and water makes them an ideal source of firewood in many arid and semi-arid regions of the world (Perera *et al.* 2005). In India nearly 70% of firewood is provided by *Prosopis* species (Muthana and Arora 1983). The high calorific value of 1700 Jkg⁻¹ means that it is an excellent source of fuel (Ffolliott and Thames 1983). The wood of most *Prosopis* species burns evenly and the high content of carbon and lignin provides good heat of combustion (Goel and Behl 1996). The dark colour and hardness of the wood of these species means that they are particularly favoured in Mexico for wood carvings and making furniture (Felker 1981, Felker and Guevara 2003). The

wood is also used for round wood construction and posts. *P. juliflora* wood is said to add a pleasant taste in food cooked over it due to the presence of particular polycyclic aromatic hydrocarbons (Maga 1986).

Almost all *Prosopis* species are nitrogen fixing through symbiotic relationship with *Rhizobium* thus increasing soil fertility (Felker and Clark 1980, Kohl *et al.* 1981) in arid ecosystem where soil nitrogen is low (Perera *et al.* 2005). The strains of *Rhizobium* identified in *Prosopis* spp. are found to be tolerant to drought, salinity, high temperature and low soil moisture (Miettinen 1989, Diagne 1996). In their study, about 18 rhizobial strains were isolated from root nodules of *Prosopis alba* Grisebach in North Spain (Iglesias *et al.* 2007). Phylogenetic relation of these rhizobial strains to *Sinorhizobium medicae*, *Sinorhizobium meliloti* and *Rhizobium giardinii* were proved from intergenic spacer and 16S rRNA gene sequence analysis (Iglesias *et al.* 2007).

Leaf extracts of *Prosopis* species have shown bio-control properties and are found to be effective in combating weeds, insects, nematodes, pathogenic fungi and viruses (Perera *et al.* 2005). The alkaloid 'juliflorine' extracted from *P. juliflora* leaves are known to cause developmental malformations on larvae, pupae and adults of the common house fly (*Musca domestica*) (Jahan *et al.* 1990). The leaf extracts have also had been found to have adverse effects on fish (Srivasankar, Bhat, and Patil 1991). Fruit and leaf extracts of the species are known to possess antibacterial properties against *Staphylococcus aureus* and *Escherichia coli* (Maldonado 1990). Root and stem extracts of *P. africana* have inhibitory effects on microorganisms (Kolapo *et al.* 2009). Studies in India have shown that viruses destroying mung bean and cow pea can be effectively targeted using

the extracts (Kannan and Doraiswami 1993, Manickam and Narayanasamy 1994). Using leaf and fruit extracts as biopesticides can be beneficial in organic farming and in sustaining biodiversity (Perera *et al.* 2005).

The economic value of *P. pallida* is recognized in the northeast of Brazil where the hardwood is used in mosaics, high quality firewood and charcoal, and the pods for alcohol production and consumption by animals and humans (Silva 1990b). Cocktails made from *Prosopis* pods known as ‘algarobina’ are used in Peru (Bravo, Grados, and Saura-Calixto 1998). *P. tamarugo* F.Philippi, *P. glandulosa* and *P. juliflora* are major sources of livestock food (Pasiiecznik *et al.* 2001). The dried and crushed pods of *P. juliflora* are a major source of livestock feed in many parts of India (Varshney 1996).

Prosopis species play an important role in modifying extreme environmental features and maintaining ecosystem complexity (Mares *et al.* 1977, Beresford-Jones *et al.* 2009). Non-erect *Prosopis* accessions have been used against desertification in Sudan and some other semi-arid zones (Felker 1981). *P. juliflora* is known for its use in the dry areas of Sudan in stabilising moving sands and thereby protecting agricultural lands (Mustafa 1990). *Prosopis alba* is also a shade trees for arid gardens in the southwest USA (Irish 2008).

1.5 *Prosopis juliflora* and *Prosopis pallida*

P. pallida (H. & B. ex. Willd.) H.B.K. and *P. juliflora* (Sw.) DC., two truly tropical species of this genus (Pasiiecznik *et al.* 2001), belong to the section Algarobia. *P. pallida* is from the series Pallidae and *P. juliflora* from series Chilenses of this section

(Burkart 1976). They occupy a wide geographical area with respect to their native ranges (Pasiiecznik *et al.* 2001) and are highly valued in the regions where they are introduced (Esbenshade 1980, Silva 1990b). *P. juliflora* is a valuable multipurpose tree species in desert ecosystems (Singh 1996). In parts of the introduced range, *P. juliflora* can become highly invasive species and may threaten native flora and ecological integrity (Victor, Pillay, and Al-Minji 2007). The habitat of existing species can also be destroyed by this invasion (Kathiresan 2006).

Biochemical and ecological benefits play a great role in the successful adaptation of polyploids to a new environment (Soltis and Soltis 2000). A study on the chromosome data of 640 endangered plant species and 81 invasive species by Pandit, Poccock, and Kunin (2011) related ploidy to invasiveness. Their study which involved *P. juliflora* and *P. cineraria* analysed the influence of plant genomic attributes to their rarity and invasive potential and found that invasive plant species are 20% more likely to be polyploid than diploid.

1.5.1 Problems in identification of *P. juliflora* and *P. pallida*

P. juliflora and *P. pallida* are similar in leaf morphology and tree form, and Pasiiecznik *et al.* (2001) assumed that naturally occurring hybridisation in their overlapping native ranges led to the difficulty in differentiating the two species. The native range of *P. juliflora* extends from the southern Central America from Mexico to Panama and the Caribbean islands to northern South America from Venezuela to northern Peru, while *P. pallida* occupies Peru, Ecuador and Colombia (Burkart 1976). The two species are sympatric in parts of their native and introduced ranges (Trenchard *et al.* 2008). This

overlapping, along with naturally occurring hybridisation has resulted in great variation within and between varieties, races and tree forms, making morphological distinction between the two species very challenging (Pasiiecznik *et al.* 2001). Due to their similarity, Pasiiecznik *et al.* (2001) suggested treating both the species together as the *Prosopis juliflora* - *Prosopis pallida* complex. Three different races are also identified in the complex: the Peruvian-Ecuadorian or 'southern' race, the Central American or 'north-western' race and the Colombian-Caribbean or 'north-eastern' race (Pasiiecznik *et al.* 2001). Burkart (1976) and Johnston (1962) also noted two varieties/races of *P. juliflora*. In India, at least five different forms were noted (Raizada and Chatterji 1954, cited in Pasiiecznik *et al.* 2001). *P. pallida* was also divided into two forms, mainly based on armature (Ferreira 1987).

In his work on Mimosoideae, Bentham (1875) grouped all the plants he identified in the section Algarobia, as varieties or forms of one species, *P. juliflora*. Since then, authors like Johnston (1962) and Burkart (1976) have identified new species and proposed raising the rank of the varieties and forms to species. However many researchers continued assigning the binomial *P. juliflora* to describe 'taxonomically good species' (Pasiiecznik *et al.* 2001). This misnaming certainly led to much confusion in the genus and in the identification of the two species.

In Hawaii, *P. pallida* was wrongly identified as *P. juliflora* and *P. chilensis* before it was correctly identified by Johnston (1962). Since it is mostly confused with the morphologically similar *P. juliflora*, incorrect binomials can cause much confusion in the areas where they are introduced (Pasiiecznik *et al.* 2001). In the USA and Mexico

most of the taxonomic confusion is related to misnaming of species especially *P. glandulosa* and *P. velutina* Wooton which have been referred to as *P. juliflora* (Pasiiecznik *et al.* 2001). Often the varietal name and authority of the introduced species were missed out causing wrong labeling. When *P. juliflora* var. *glandulosa* (Torrey) Cockerell was introduced from USA or Mexico prior to 1970s, the varietal name was gradually omitted to become *P. juliflora*, when the species actually is *P. glandulosa* Torrey (Pasiiecznik *et al.* 2001). The lack of preserved herbarium specimens also contributed to misidentification (Pasiiecznik *et al.* 2001).

Successful attempts were made to distinguish between the two species using foliar characters (Bentham 1875, Benson 1941, Johnston 1962, Burkart 1976, Díaz Celis 1995), ploidy (Hunziker *et al.* 1975, Harris *et al.* 2003, Trenchard *et al.* 2008) and RAPD techniques (Landeras *et al.* 2006, Singh *et al.* 2011). Identification keys for *Prosopis* species in Mexico, Chile and Peru have also been described by Ffolliott and Thames (1983). In their work, Pasiiecznik *et al.* (2004) developed keys for the identification of tropical *P. juliflora* and *P. pallida* based on leaf morphological characters. However, very accurate identification of these tropical species is required for the proper management and genetic improvement, especially in their native ranges and for tackling weediness in the areas where they are introduced (Pasiiecznik *et al.* 2001).

1.5.2 Proposed taxonomic revisions to *P. juliflora* and *P. pallida*

Recent advancements using numerical analysis and molecular markers to resolve the taxonomy and phylogenetic relationship between species have led many authors to

propose a revision in the occurrence, ecology and distribution of *P. juliflora* and *P. pallida* (Landeras *et al.* 2006, Burghardt *et al.* 2010, Palacios *et al.* 2011). However, this thesis accepts Burkart's (1976) taxonomic classification of the genus as the complete one. The proposed revisions to the taxonomy could be taken into account in future research programs involving the two tropical species.

In the classification of Mimosoideae by Bentham (1875), he placed all the species of *Prosopis* sec. Algarobia under the binomial *P. juliflora*. This all-embracing, collective *P. juliflora* concept of Bentham was disregarded by Johnston (1962). Burkart (1976), later differentiated the species based on foliar characters and grouped the 30 species in the section Algarobia. However, the initial classification by Bentham (1875) continued to be followed by many publications. This resulted in the confusion regarding the nomenclature in the American Algarobia section of the genus (Pasiiecznik *et al.* 2001).

The common *Prosopis* in Sudan was identified as a hybrid between *P. chilensis* and *P. glandulosa* with tetraploidy values of $2n=56$ (Bukhari 1997a). In his 2 other published work, Bukhari (1997b & c) recorded tetraploid values ($2n=56$) for *P. chilensis* in 6 seed accessions obtained from Sudan and 2 from Kenya. However, flow cytometry studies on *Prosopis* spp. by Trenchard *et al.* (2008) identified all samples from Sudan as *P. juliflora*, with ploidy values of $2n=4x=56$. Therefore the occurrence of *P. chilensis* in Sudan is a misidentification and one has to accept the common species in that region is *P. juliflora*, as has also been supported by other studies (El Fadl 1997, Pasiiecznik *et al.* 2001). Misidentifications in the regions where they are introduced are common in the case of the *P. juliflora* and *P. pallida*. Samples from Brazil and Senegal, originally

identified as *P. juliflora* (Hunziker *et al.* 1975, Bukhari 1997c), have been shown to be diploid *P. pallida* (Harris *et al.* 2003, Trenchard *et al.* 2008).

In his monograph, Burkart (1976) placed *P. juliflora* and *P. pallida* in two separate series within section Algarobia. Detailed study on the leaf, floral and fruiting characters were undertaken in the classification of the genus *Prosopis* into sections. The uniformity of the fruit and vegetative diversification in the armature were the most important characteristics considered by Burkart (1976) when he placed the 44 species in their respective series. The type species in the largest section Algarobia is *P. juliflora*. Burkart (1976) recognized 3 varieties of *P. juliflora*, vars. *juliflora*, *inermis* and *horrida*.

The taxonomy of *Prosopis* species in northern Peru is still a matter of debate. The occurrence of *P. juliflora* and *P. pallida* and possible sympatry between them in the south of Ecuador and northern Peru is reported (Burkart 1976, Ferreyra 1987, Díaz Celis 1995, Pasiecznik *et al.* 2001). Recent studies on the quantitative leaf characters of *Prosopis* specimens along the coast of Peru and Ecuador dismisses the presence of *P. juliflora* in those regions (Burghardt *et al.* 2010). However, the wide distribution of *P. limensis* (synonym of *P. pallida*) as a distinct species is reported in their study. Mom *et al.* (2002) had also separated them into two distinct species based on morphological attributes. Burghardt *et al.* (2010) identifies only three species, *P. pallida*, *P. limensis* and *P. chilensis* in the Peruvian-Ecuadorian coast. AFLP analysis and morphological character analyses on the *P. pallida* - *P. juliflora* complex by Palacios *et al.* (2011) identifies two distinct species, *P. pallida* and *P. limensis* in the Peruvian-Ecuadorian coast. The occurrence of *P. juliflora* is restricted to the Caribbean Basin of Colombia

and Venezuela. However, in their work, no statistically significant difference in morphological characters was detected between *P. pallida* and *P. limensis* seedlings (Palacios *et al.* 2011). AFLP analysis showed greater similarity between the Peruvian-Ecuadorian *P. pallida* and *P. limensis* than those two with *P. juliflora* (Palacios *et al.* 2011).

Bentham (1875) originally identified the single species, *P. limensis*, from the Peruvian coast. In his monograph, Burkart (1976) brought *P. limensis* into synonymy with *P. pallida* when no consistent difference in their morphological characters was found. Johnston (1962) also noted that *P. limensis* was closely related to *P. pallida*. Burkart and Simpson (1977) described 5 species as native to Peru: *P. pallida*, *P. juliflora*, *P. reptans*, *P. laevigata* and *P. chilensis*. Ffolliott and Thames (1983) described identification key and description for seven species including *P. juliflora* in Mexico and northern Peru, where the two varieties of *P. juliflora* are also present. Few years later, Ferreyra (1987) identified four forms of *P. pallida* in Peru. However, Díaz Celis (1995) describes only two forms of *P. pallida*, forma *pallida* and forma *armata* as the dominant species in Peru. It is accepted that in Peru, *P. juliflora* occur in the northern coastal zone while *P. chilensis* occur in the dry montane zones, mainly in the south of the country (Díaz Celis 1995, Pasiecznik *et al.* 2001). Attempts were also made to distinguish the species native to South America using new identification keys (Vilela and Palacios 1998).

P. juliflora and *P. pallida* can be distinguished using foliar characters (Johnston 1962, Burkart and Simpson 1977, Díaz Celis 1995), although species showing intermediate

characters of both species are difficult to identify especially in the regions where their native ranges overlap (Pasiiecznik, Harris, and Smith 2004). The populations of *P. juliflora* are subject to genetic variation especially in the native ranges and many races have been reported (Pasiiecznik *et al.* 2001). *P. juliflora* var. *inermis* (H.B.K.) Burkart and *P. pallida* forma *pallida* which occur in Peru and Ecuador are thornless (Pasiiecznik, Harris, and Smith 2004). However, their natural range overlaps with thorny *P. juliflora* var. *horrida* (Kunth) Burkart. *P. juliflora* occupying the coastal regions of Central America are very thorny (Pasiiecznik, Harris, and Smith 2004). In Mexico, its natural range overlaps with *P. glandulosa* and *P. laevigata* (Burkart 1976). In Mexico, Palacios (2006) includes *P. vidaliana* as a distinct species occupying the Pacific Coast from Sinaloa, Mexico to Panama. Palacios (2006) restricted the population of *P. juliflora* to a small location on the northern coast of the Yucatan. However, this proposed change has not been widely accepted and hence this thesis follows the accepted classification of *P. juliflora* in Central America as proposed by Johnston (1962), Burkart (1976) and Pasiiecznik *et al.* (2001). Johnston (1962) described keys based on quantitative foliage characters to distinguish the North American *Prosopis*. He noted that the foliar characters were of prime taxonomic value in geographical classification of the North American *Prosopis* species. Johnston (1962) distinguished two races of *P. juliflora*, the southeastern (West Indian) and northwestern (Mexican) races. He even proposed that due to the variation in foliar characters between the two races, the northwestern race might be treated as a separate taxon, *P. vidaliana* A. Náves. However, Burkart (1976) describes *P. juliflora* as good species and accepted *P. vidaliana* as its synonym.

1.5.3 Comparative morphology of *P. juliflora* and *P. pallida*

The tree size and form of *P. juliflora* and *P. pallida* may vary between species, populations and individuals due to environmental factors. They vary from erect forms to shrubby and multi-stemmed ones. The heights of tree forms vary from 12 to 20 m if the environmental conditions are favorable (Burkart 1976). The trunks are however, short and often crooked or twisted with a diameter of up to 65 cm (Pasiecznik *et al.* 2001). The shrub forms attain a height as low as 3 m (Burkart 1976).

P. juliflora has longer leaves and the leaflets are larger and wider than *P. pallida*. *P. juliflora* leaves have 1-4 pairs of pinnae per leaf and there are 6-29 pairs of leaflets on each pinna (Burkart 1976, Ffolliott and Thames 1983, Díaz Celis 1995).

The root systems of *Prosopis* species are extensive and develop soon after germination into a deep root system and a superficial system, which is adaptable to different environmental conditions (Pasiecznik *et al.* 2001). The tap root can reach great depths until it reaches the water table. The seedlings of *P. juliflora* can develop root systems that may reach to a depth of up to 40 cm in eight weeks (Pasiecznik *et al.* 2001) while *P. pallida* tap roots are able to draw water from water tables 20 to 25 m deep (Díaz Celis 1995).

Presence or absence of armature is another distinguishing character between the species. Burkart (1976) divided the genus *Prosopis* into sections based on the presence of thorns or spines or their complete absence. The thorns are 0.5 to 7.5 cm long and 2 to 5 mm in thorn base diameter (Pasiecznik *et al.* 2001). One thornless variety of *P. juliflora* was described by Burkart (1976). *P. juliflora* var. *juliflora* is totally thornless unlike *P.*

juliflora var. *horrida*. Thornless *P. pallida* populations were identified in Hawaii by Esbenshade (1980). However *P. pallida* forma *armata* possesses thorns (Ferreyra 1987).

P. juliflora inflorescences are spike-like racemes, 5-10 cm long solitary or in clusters near leaf axils (Burkart and Simpson 1977). Individual flowers are small, fragrant, densely gathered and pale yellow to yellow white in colour (Burkart and Simpson 1977).

P. pallida inflorescences are similar to *P. juliflora* with spike-like racemes (Burkart 1976). Flowers are small, pale yellow in colour but dense with 200-250 per raceme (Ffolliott and Thames 1983). Inflorescences are also longer in *P. juliflora* than *P. pallida*. Inflorescence and seeds/pods of *P. juliflora* and *P. pallida* are shown in Figures 1.3 and 1.4. The seeds of both species are small, ovoid and hard (Saxena 1998).

The fruit is described as a 'drupaceous loment' with a fleshy mesocarp and endocarp divided into segments containing a single seed (Burkart 1976). The mesocarp is rich in sugar and protein. The pods vary in length from 8 to 40 cm attaining a width of 9-18 mm and thickness of 4 to 10 mm (Burkart 1976, Díaz Celis 1995). The colour also varies with maturity. The immature green pods turn yellow when fully ripe. There are up to 30 seeds per pod (Burkart 1976).

Habits of *P. juliflora* and *P. pallida* are shown in Figures 1.2, 1.3 and 1.4. The presence of glands is also another distinguishing character. In *P. juliflora*, glands are seen at the junction of pinnae and leaflets, but in *P. pallida* they are present only at the junction of pinnae (Díaz Celis 1995).

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(a) *Prosopis juliflora* in Senegal

(b) *Prosopis pallida* in northern Peru

Figure 1.2: *Prosopis juliflora* in Senegal and *Prosopis pallida* in northern Peru (from Pasiiecznik *et al.* 2001)

Figure 1.3: Inflorescence and seeds of *P. juliflora* (from Barbadine 2007 and Felker 2004)

Figure 1.4: Inflorescence, leaves and pods of *P. pallida* (photos taken in Hawaii by Carr 2000 and Starr 2001)

1.5.4 Cytotaxonomy and ploidy

The most comprehensive work on the genus *Prosopis* is by Burkart (1976), where 44 species were described, taxonomically and geographically. A detailed account of the morphological characters of all the species including *P. juliflora* and *P. pallida* is included by Burkart (1976) in his monograph. Johnston (1962) differentiates in detail some North American *Prosopis* and these two species based on their inflorescence, leaf characters and pod structure.

The genus *Prosopis* is predominantly diploid with a basic chromosome number of 14 ($2n = 28$) suggesting it is a primitive genus (Burkart 1976, Burkart and Simpson 1977). *P. juliflora* is the only tetraploid species with a chromosome number $2n=4x=56$ (Trenchard *et al.* 2008). Previous work on ploidy and chromosome number has suggested diploid and tetraploid forms of *P. juliflora* and *P. koelziana* (Hunziker *et al.* 1975, Zaeifi *et al.* 2002). Occurrence of tetraploid *P. chilensis* was reported by Bukhari (1997a), but this is likely to be *P. juliflora* since recent flow cytometry analysis on the ploidy of *Prosopis* has suggested *P. juliflora* to be the only truly tetraploid species (Trenchard *et al.* 2008). Ploidy analysis could be used to help to identify the origin of introduced plants in this genus (Pasiecznik *et al.* 2001). The populations of *P. juliflora* in Haiti and Colombia were found to be truly tetraploid (Hunziker *et al.* 1975). *Prosopis pallida* was found to be entirely diploid with a chromosome number of $2n=2x=28$ (Trenchard *et al.* 2008). Variations in chromosome numbers are also observed in the populations of *P. juliflora* - *P. pallida* complex within the native and introduced ranges but this could be due to misidentification (Pasiecznik *et al.* 2001). The presence of

triploid individuals was observed in five different *Prosopis* seed samples analysed by Harris *et al* (2003). The observed triploid seedlings could be hybrids between tetraploid *P. juliflora* and any diploid *Prosopis* species, but more studies on the hybridisation of these species in their native ranges is required (Harris *et al.* 2003).

Polyploidy was first recorded in *Prosopis* by Atchinson (1951). Isoenzyme studies on *P. juliflora* revealed the species to be fixed in a heterozygous state due to polyploidy (Panetta and Castairs 1989). A similar result was observed by Solbrig and Bawa (1975) when they also observed fixed heterozygosity in *P. juliflora* populations indicating its polyploid nature. However, in their work, *P. pallida* populations showed less genetic variation.

Polyploidy is less frequent in *Prosopis* than in the related genus *Acacia* (Bukhari 1997c). Somatic chromosome size is small (Hunziker *et al.* 1975) and bivalent formation during metaphase 1 suggests possible homology between parental chromosomes (Solbrig *et al.* 1977). Occurrence of polyploidy in *P. juliflora* may be attributed to its frequent hybridisation and subsequent stabilization due to evolution (Pasiiecznik *et al.* 2001). The invasive potential of *P. juliflora* has been linked to its polyploid genome (Pandit, Pocock, and Kunin 2011).

1.5.5 Resource value of the two species

Timber

P. juliflora and *P. pallida* woods are heavy, durable and possess good strength and hardness (Harsh *et al.* 1998). The timber is utilized for construction purposes and the

manufacture of farm equipment (Kanzaria and Varshney 1998). The heartwood of *P. juliflora* and *P. pallida* is extremely durable and preferred for making fence posts that can last many years if properly treated (Esbenshade 1980). They are also used for manufacturing hand tools (Shukla *et al.* 1990) and agricultural implements (Rajput and Tewari 1986, cited in Pasiecznik *et al.* 2001). In the Americas, the timber from these species is mostly utilized in furniture manufacture (Figure 1.5) and parquet flooring (Pasiecznik *et al.* 2001). The pulped wood of *P. juliflora* is used for the production of writing and printing papers, textile fibres, tyre cord or cellophane (Madan and Tandon 1991)

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Figure 1.5: Coffee table made from *P. juliflora* wood (L. N. Harsh from Pasiecznik *et al.* 2001)

Fuel

P. juliflora is highly valued as firewood in many rural regions where wood is an important source of their domestic fuel. This species is preferred in India for fuel due to its even burning without emitting excessive smoke or sparks (Kathiresan 2006). The wood has a high calorific value of $4200 \text{ kcal kg}^{-1}$ (Kanzaria and Varshney 1998). The wood is also used in charcoal production and power generation (Patel 1986, cited in Pasiecznik *et al.* 2001). Gasification of *P. pallida* wood enables the generation of

electricity (Sutaria, Andharia, and Patel 1986, cited in Pasiiecznik *et al.* 2001). Dried and crushed green twigs are mixed with *Prosopis* pod powder and other materials to make briquettes for use in furnaces (Varshney 1996).

Gums, tannins and dyes

The exudate gum from *P. juliflora* and *P. pallida* is used in food, pharmaceutical preparations and chemical industries (Pasiiecznik *et al.* 2001). In Brazil it is used in the manufacture of glue similar to that of gum Arabic from *Acacia senegal* (Mendes 1990). In fact the gum was found to have similar amino acid composition to gum Arabic (Anderson *et al.* 1985). It is also an excellent source of the sugar arabinose (Varshney 1996). The gum of *P. juliflora* is used for sizing cloth, as a paper adhesive, binder in tablet manufacture, in confectionery and also for treating eye infections (Vimal and Tyagi 1986, cited in Pasiiecznik *et al.* 2001). *P. juliflora* gum is valued in the food industry as it is similar to carob gum (*galactomonas* type) (Mendes 1990).

In *P. juliflora* and *P. pallida*, tannins are concentrated in their wood, bark and roots (Kazmi and Singh 1992). Tannins are used for making dye, ink, conserving fibres used in fishing nets, and to reduce the viscosity of drill mud (Pasiiecznik *et al.* 2001). In Mexico, tannins extracted from roots of *Prosopis* species are used to make purple dye for cotton (Díaz Celis 1995). In Brazil, tannins from *P. juliflora* are used in tanneries (Mendes 1990). Tannins are also used as an astringent and to treat burns as they form a protective coating on healing tissue (Rocha 1990).

Medicinal value

P. juliflora and *P. pallida* are valued for their medicinal properties in many rural parts

of the world. Extracts from the species possess bactericidal and fungicidal properties and hence they are used to treat wounds, eye infections, stomach disorders and skin ailments (Pasiiecznik *et al.* 2001). Many alkaloids, flavanoids and tannins have been isolated from both species (Vimal and Tyagi 1986, cited in Pasiiecznik *et al.* 2001, Rocha 1990). Alkaloid extracts from leaves of *P. juliflora* were found to be more effective against gram positive bacteria than commercially available antibiotics (Ahmed *et al.* 1986, Aqeel *et al.* 1989). However, the toxic effects of extracts from *P. juliflora* have also been studied. High doses of julifloricine extracted from *P. juliflora* are found to be lethal in mice and rabbits (Aqeel *et al.* 1991). Syrup extracted from pods is known for its aphrodisiac properties and is also a refreshing drink in parts of Brazil (Rocha 1990). However, the presence of toxic furfural was detected in *P. juliflora* syrup (Rocha 1990). Tea made by infusing *P. juliflora* pods is good for digestive discomfort and skin lesions (Rocha 1990), but excessive consumption may irritate the stomach inducing vomiting and nausea due to the presence of tannins (Rocha 1990). However, further research is warranted to fully exploit their potential uses as medicinal plants.

Food products

P. juliflora pods are acclaimed for their high nutritional value with a crude protein level within the range of 7 to 17% although seed protein content may vary from 34 to 39% making it suitable for human consumption (Pasiiecznik *et al.* 2001). The seed contains about 38% protein, 3% fat and 9% crude fibre (Del Valle *et al.* 1983). The dry ripe pods are ground in mills to obtain flour (Cruz 1990) that can be used for making cakes, bread, biscuits, sweets, jelly, syrup and alcoholic products (Simpson 1977b, Mendes 1990). In northern Peru, a sugary drink called 'algarrobina' prepared from *P. pallida* is

considered a healthy food supplement (Bravo, Grados, and Saura-Calixto 1998, Cruz 1990). *P. juliflora* pods are processed to make powder which is used as a substitute for coffee during drought periods (Rocha 1990). In Brazil, protein enriched flour and protein isolate are produced from *P. juliflora* seeds (Baião *et al.* 1987 cited in Pasiecznik *et al.* 2001). *P. pallida* pods have high concentration of dietary fiber and protein and hence are also used as animal feed (Bravo, Grados, and Saura-Calixto 1994). *P. juliflora* and *P. pallida* flowers are a source of bee forage for the commercial production of honey in many parts of the world. Both species flower twice a year; even during drought periods and have been the main source of pollen and nectar in parts of Mexico, Brazil, Hawaii and India (Silva 1990a, Mendes 1990, Varshney 1996).

Honey from *P. juliflora* is valued for its medicinal properties and higher grade (Varshney 1996). Figure 1.6 shows some of the food products obtained from the species.

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(a) Bread rolls

b) Coffee powder substitute and Algarrobina syrup

Figure 1.6: (a) Bread rolls made using flour obtained from *P. pallida* pods. (b) Coffee powder substitute and Algarrobina syrup produced from *P. pallida* pods (from Pasiecznik *et al.* 2001)

Fodder

P. juliflora and *P. pallida* pods are a highly nutritive animal feed in their native and introduced ranges. The pods from the species can be the main source of forage. They are milled to avoid any nutritional loss before feeding to livestock, since the whole seeds can pass undigested through the animal gut (Pasiiecznik *et al.* 2001). They are stored in special rooms to prevent extensive damage due to fungal infections (Díaz Celis 1995). The pods are easily digestible owing to their low tannin content (Shukla *et al.* 1984, cited in Pasiiecznik *et al.* 2001). However, *P. pallida* pods were reported to have caused ill effects when cattle were fed solely with them (Alder 1949, cited in Pasiiecznik *et al.* 2001). The whole pods were not found to be suitable for livestock feeding due to their high sugar content and indigestibility (Shukla *et al.* 1984). In Brazil, the flour from pods of *P. juliflora* is known to be useful as a substitute for wheat bran for feeding hens (Silva 1990b).

On the contrary, the presences of tannins in the leaves of both species make them unpalatable (Anttila, Alakoski-Johansson, and Johansson 1993). Palatability can also be influenced by the soil type and nutritive status, observed in *P. juliflora* (Sharma 1984). However, palatability can be increased by treating with certain chemicals or by mixing with other feeds (Pasiiecznik *et al.* 2001). The abundance of foliage and nutritiveness promotes the use of *P. pallida* and *P. juliflora* as a choice livestock feed in their native and introduced ranges. *P. pallida* leaves are useful as a dry season fodder in Peru (Díaz Celis 1995).

Other uses of these species

These species can be beneficial to afforestation projects owing to their fast growth, drought resistance and ability to thrive in poor soils (Silva 1990b). In India, the species have been used in the reclamation of degraded grasslands and wastelands (Muthana 1990). *P. juliflora* plantations are known to restore the productivity and fertility of highly sodic soils (Bhojvaid, Timmer, and Singh 1996). Tall *P. juliflora* trees with large canopies encourage the growth of shrubs and grasses under them. It is noted that the plants growing under the shade of *P. juliflora* have higher nitrogen contents than those which do not (Maldonado 1990). These species are also helpful in wildlife conservation by providing highly nutritive pods, shelter from heat and wind and dense thickets which prevents poachers with vehicles from approaching the wildlife (Muthana 1990).

The pods, when used as a sugar source in fungal culture media, promoted fast growth (Bohra, Sharma, and Lodha 1998, Santos and Pereira 1990). *P. juliflora* wood can be pulped for making papers, textile fibres and cellophane (Madan and Tandon 1991). *P. juliflora* leaf exudates contain three plant growth inhibitors suggesting their allelopathic property (Nakano 2010).

1.6 Research, scope, aims and objectives

The present work attempts to utilize a variety of tried and tested methods to be able to distinguish *P. juliflora* and *P. pallida* based on their stomatal anatomy, RAPD profile, microsatellite transferability and chloroplast trnL/F gene sequencing. 11 other species from the genus were also selected in the experiments to establish the findings.

The following were the specific objectives

- To determine if stomatal anatomy and the number of guard cell chloroplasts differ between the two species.
- To develop a protocol for the artificial polyploidisation of *P. juliflora* and *P. pallida* to analyse the impact of ploidy in their genetic relationship.
- To determine if the RAPD marker profiles vary between the two species, thus identifying useful genetic markers to distinguish them.
- To compare the RAPD profiles of *P. juliflora* and *P. pallida* with selected species from sections Algarobia and Strombocarpa to determine the relationship between the selected *Prosopis* species.
- To determine the transferability rate and amplification profile of 6 microsatellite primer pairs in all the *Prosopis* species selected for this study.
- To determine if there are any sequence difference in the chloroplast DNA intron region between *P. juliflora* and *P. pallida*.
- To identify if the cpDNA intron region could be informative in analyzing the relationship between the two tropical species and other selected species in the genus

The experiments conducted, results obtained and discussion are outlined in the chapters 2 to 4. Chapter 2 describes the work undertaken to differentiate the two species using cytological methods such as analysing leaf cell characters. Polyploidisation experiments are also described. Chapter 3 and 4 describes molecular methods employed to differentiate the two species. Their relationship with other selected species from section Algarobia and Strombocarpa is also considered in this study. Chapter 6 is an overall discussion of the results in relation to the literature to address the above aim.

2. DIFFERENTIATION OF *PROSOPIS JULIFLORA* AND *PROSOPIS PALLIDA* BASED ON STOMATAL CHARACTERS AND THEIR POLYPLOIDISATION BY COLCHICINE

2.1 Introduction

2.1.1 Differentiation of *P. juliflora* and *P. pallida* using stomatal characters

Prosopis pallida (H. & B. ex. Willd.) H.B.K. and *P. juliflora* (Sw.) DC. are the only two *Prosopis* species which are identified as truly tropical apart from *Prosopis africana*, which is native to tropical Africa (Pasiiecznik, Harris and Smith 2004). They share similar morphology and tree form and have been acknowledged both as a valuable resource and invasive weeds (Pasiiecznik *et al.* 2001). *P. pallida* is less invasive than *P. juliflora* and could be more widely utilised as a resource (Pasiiecznik *et al.* 2001). Often misidentification and lack of knowledge of the species where introduced have hampered efforts for their proper management, genetic improvement and utilisation (Pasiiecznik, Harris and Smith 2004). *P. juliflora* and *P. pallida* are particularly difficult to identify and differentiate due to their habitat similarity and possible hybridisation in their overlapping native ranges (Pasiiecznik *et al.* 2001). Sympatry exists in the populations of *P. juliflora* and *P. pallida* in their native range of Ecuador and Peru and interspecific hybrids are reported (Burkart 1976). This overlapping along with naturally occurring hybridisation has resulted in great variation within and between varieties, races and tree forms, making morphological distinction between the two species very challenging (Burkart 1976). Taxonomic confusion has also resulted due to misidentification of the two species (Pasiiecznik *et al.* 2001). *P. pallida* was wrongly identified as *P. juliflora* and *P. chilensis* in Hawaii before its correct identification by Johnston (1962). *P.*

juliflora has often been mistakenly identified as other *Prosopis* species and this has fuelled confusion in its native range and particularly in regions where they are introduced. Also, the lack of common and accurately identified reference herbarium specimen has led to false conclusions (Pasiecznik *et al.* 2001).

Morphological taxonomy is as important as molecular taxonomy in resolving the phylogenetic relationship between species (Taia 2005). Many plant species whose taxonomical position is still questionable have benefited from stomatal character analysis to determine the relationships among their different taxa (Hayat *et al.* 2010). Stomata have played an important role in speciation and evolutionary change. Even two clades of a species can be separated on the basis of stomatal distribution and structure (Hetherington and Woodward 2003). Early taxonomical classifications of species were entirely based on morphological characters. However due to advances in molecular methods utilising DNA sequences in the reconstruction of phylogeny, morphological characters utilised in such studies are less frequent. Scotland, Olmstead, and Bennett (2003) argues that the best way forward is the precise and critical anatomical studies of fewer morphological characters along with molecular data in clarifying the phylogeny of a taxon.

Successful attempts have been made to distinguish between *P. juliflora* and *P. pallida* and between these and other species in the genus using foliar characters (Burkart 1976, Johnston 1962, Díaz Celis 1995, Harris *et al.* 2003, Palacios *et al.* 2011) and ploidy (Hunziker *et al.* 1975, Burkart and Simpson 1977, Harris *et al.* 2003, Trenchard *et al.* 2008). Recent analyses on molecular markers also suggest that *Prosopis* species can be

differentiated at the DNA level (Ramírez *et al.* 1999, Landeras *et al.* 2006, Palacios *et al.* 2011, Sherry *et al.* 2011, Singh *et al.* 2011). Following morphological and ploidy studies on *Prosopis* species, keys have been developed for their identification and differentiation (Harris *et al.* 2003, Pasiecznik, Harris, and Smith 2004). However, attempts to differentiate these species in the field by looking at the morphological characters can sometimes be challenging due to varying forms and hybrids with intermediate characters (Pasiecznik, Harris, and Smith 2004). Hence it would be useful to develop and assign further keys using stomatal characters to ensure accurate identification for researchers.

2.1.2 Polyploidisation of *P. juliflora* and *P. pallida* using colchicine

Changes in ploidy have had a huge impact in the evolution of plant species (Stebbins 1971). It is estimated that about 70% of the angiosperms have undergone one or more polyploidisation events in their evolutionary history (Masterson 1994) whereas in gymnosperms it is less than 1% (Khoshoo 1959). Natural polyploidy has led to the evolution of many cultivated plants (Davis and Heywood 1967) and has thus encouraged plant breeders to artificially induce polyploidy to enhance elite characters in many crop species (Weimarck 1973, Lavania, Srivastava, and Sybenga 1991). Artificial polyploidy induction has been favoured for improving biochemical qualities such as increased alkaloid and secondary metabolite contents (Bhatt and Heble 1987, Milo *et al.* 1987) and also for producing disease resistant varieties (Hamill, Smith, and Dodd 1992).

Colchicine, a natural alkaloid extracted from wild meadow saffron, *Colchicum autumnale* L. (Eigsti and Dustin, 1955, Emsweller, 1988), is effective in inducing

polyploidy in many plant and animal species (Blakeslee and Avery 1937, Edwards 1954). Colchicine affects the microtubules in the late prophase of mitosis, disrupts the nuclear envelope and attaches to chromatin during late interphase, thus preventing microtubule polymerization (Blakeslee and Avery 1937, Eigsti 1938). The toxic effects of colchicine and the undesirable mutagenic activity caused some authors to prefer oryzalin as an alternate ploidy induction chemical. Oryzalin was found to be more effective than colchicine in inducing polyploidy in *Lilium* and *Nerium* species (van Tuyl, Meijer and van Diën 1992).

Conventional methods of determining chromosome number in plants include microscopic chromosome counting and Feulgen scanning microspectrophotometry (Price *et al.* 1980, Price 1988). For the past few years, flow cytometry has also been used as a reliable method in the detection of DNA ploidy and the determination of ploidy levels in different histogenic layers in plants (Bunn *et al.* 1980, Costich *et al.* 1993, Horii *et al.* 1998, Lysak and Dolezel 1998, De Schepper *et al.* 2001, Emshwiller 2002). This technique is accurate in analysing ploidy and determining the relation between plant DNA content and chromosome number (Eaton *et al.* 2004). The flow cytometry protocol developed by Galbraith *et al.* (1983) for the analysis of intact nuclei of plant tissue has provided a rapid method of estimating plant DNA content. Chromosome counting to establish ploidy levels is often time consuming and laborious and is not suitable for detecting mixoploids in leaf tissues, where there are few dividing cells (Uhlik 1981). Flow cytometry is far less laborious than traditional chromosome counting and it is accurate, convenient, low cost and rapid (Dolezel 1997, Galbraith 1990).

In the genus *Prosopis*, the rate of occurrence of polyploidy was reported to be very low (Hunziker *et al.* 1975, Trenchard *et al.* 2008). Understanding the ploidy within species would help in understanding their evolutionary history and polymorphisms. It is known that polyploids adapt better to varying ecological circumstances and possess increased heterozygosity (Stebbins 1950). Moreover, polyploid populations are dynamic and susceptible to evolutionary change (Soltis and Soltis 1995). Multiple polyploidisation events generate a diverse array of polyploid genotypes and this creates more genetic and morphological complexity (Soltis and Soltis 1999). Thus several independent events of polyploidisation in *P. juliflora* may explain its adaptability and invasive ability in many countries where it is introduced.

Comparison of the stomatal characters of polyploidised *P. juliflora* and *P. pallida* with their controls may provide answers regarding their taxonomic status. Understanding the species evolutionary processes and their different phenotypes are extremely important considering the fact that there are still complications involving the taxonomy of the genus. Gathering data on their ploidy levels and the ways ploidy affected the species could help clear some confusion surrounding them. There have been no reports on the artificial polyploidisation of *Prosopis*, hence induction of tetraploids and octoploids is of interest.

2.2 Materials and methods

2.2.1 Stomatal character measurements of *P. juliflora* and *P. pallida*

2.2.1.1 Plant materials

Fresh leaves taken from different accessions of *P. juliflora* and *P. pallida* established in the greenhouse of Coventry University were used for the stomatal character studies. Fourteen accessions of *P. juliflora* and nine accessions of *P. pallida* were used for the analysis. The ploidy of all the greenhouse accessions was already known and confirmed by Flow Cytometry (Plant Cytometry Services, Schijndel, the Netherlands). All the *P. juliflora* accessions were tetraploid and the *P. pallida* accessions in this study were diploid (Harris *et al.* 2003, Trenchard *et al.* 2008). Since previous research by Harris *et al.* 2003 and Trenchard *et al.* 2008 were carried out to differentiate *P. juliflora* and *P. pallida* based on foliar characters and ploidy, stomatal characters were considered in the present study to differentiate the species. Five different quantitative variables were chosen to distinguish between *P. juliflora* and *P. pallida* anatomically. They were stomatal length, stomatal density, stomatal index, epidermal cell density and guard cell chloroplast number. The samples of *P. juliflora* and *P. pallida* analysed along with their descriptive code and collection site/country of origin are given in Table 2.1.

Table 2.1: Samples of *P. juliflora* and *P. pallida* with their descriptive codes and collection site/origin if known

<i>Prosopis juliflora</i>	
Sample	Collection site/Origin if known
Ethiopia 08	Ethiopia, Afar, Dirk Kebele
Ethiopia 12	Ethiopia, Afar, Entiadoyta
Ethiopia 13	Ethiopia, Afar, Edelafafee
Niger 1	Niger, Niamey, Grand Hotel
Niger 2	Niger, Mardi, Soumarana
Srilanka 1	Sri Lanka, Hambantota
Srilanka 2	Sri Lanka, Hambantota
Srilanka 3	Sri Lanka, Hambantota
UAE 2	United Arab Emirates
UAE 3	United Arab Emirates
Kakuma 1-2	Kenya, Kakuma
Sudan 13	Sudan, Ed Debbra
Antigua 06	Antigua, Fort Road
Galapagos 01	Ecuador, Galapagos Islands
<i>Prosopis pallida</i>	
Sample	Collection site/Origin if known
Australia 1	Australia, Queensland
Australia 2	Australia, Queensland
Australia 5	Australia, Queensland
Australia 6	Australia, Queensland
Australia 7	Australia, Queensland
Australia 10	Australia, Queensland
Mombasa 5	Kenya, Mombasa
Brazil 1	Brazil, Rio Grande del Norte, Natal
Hawaii 1	Hawaii, E. Maui, Lipoa, Kihei

2.2.1.2 Measuring stomatal index

The term stomatal index (SI) was introduced by Salisbury (1927) to relate the number of stomata per unit leaf area (S) to the number of epidermal cells plus stomatal guard cells per unit leaf area (E). Thus $SI = (S/E+S) \times 100$. The method followed for measuring the stomatal index is as follows. Four or five fresh leaves from each accession were selected and a thin film of quick drying nail varnish (Revlon, UK) was applied to the abaxial surface of fresh leaves and left to dry. When moderately dry it was peeled off with a tweezer so that some of the epidermal layer came off with the nail varnish. The 'peel' was then placed on a glass slide mounted with a drop of water, and a coverglass was carefully placed on top ensuring there were no air bubbles trapped in the specimen.

The slides were viewed through a light microscope (Kyowa MEDILUX 12, Japan). An eyepiece micrometer was used for all the observations of stomatal length. The light microscope was calibrated at 40x and 100x objective magnifications using the calibration slide by coinciding with the eyepiece micrometer and the area of the field of view was calculated for each magnification. Leaflets from 4 or 5 leaves were measured and for each character, measurements from 25 different fields of view were recorded. The number of stomata was counted at 400x total magnification while stomatal length and the number of epidermal cells were counted at 1000x total magnification.

2.2.1.3 Measuring chloroplast number in each stoma

Epidermal peel was obtained using nail varnish and also with a razor blade. A thin section was retrieved from the abaxial surface of the leaf and placed on a glass slide with a drop of iodine stain (Jensen 1962). The iodine stain was prepared by dissolving 2g potassium iodide in 100 ml of distilled water, then adding 0.2 g of iodine. The mounted specimens were covered with a cover slip and kept in the dark for 15 min before viewing under the microscope at 400x total magnification.

2.2.1.4 Statistical analysis

Data obtained from all the stomatal and chloroplast measurements were entered onto Microsoft Excel spreadsheet. Mean values were taken for all the characters and standard deviation of the mean was calculated. Standard error was calculated using the formula $SE = SD/\sqrt{n}$, where n = number of measurements for each character and SD = standard deviation.

Student's t-test

Student's t-test was performed using the mean value calculated for each parameter. The t-test determines if the means of two parameters are statistically different from each other.

Multivariate analysis

Multivariate analysis is a collective term to define all the techniques used to analyse two or more random and interrelated variables simultaneously to measure the degree of relation among them (Hair *et al.* 2006). In a multivariate analysis the related variables

considered simultaneously are of equal importance. Since there were five different pairs of variables in stomatal characters measured between *P. juliflora* and *P. pallida*, multivariate methods seemed to be the appropriate method of analysis. The aim of differentiating the species based on stomatal characters meant that the multivariate method chosen should look for any pattern, differences or similarities in the data (Everitt and Dunn 2001). All the characters analysed here were quantitative but both qualitative and quantitative data can be analysed using this technique.

Detrended correspondence analysis was chosen as the main ordination technique for this data as it reduces the data into a manageable number of variables and it allows for a visual examination of any structure or pattern in the data (Everitt and Dunn 2001). Ordination arranges the species according to their similarity and describes the characters which influence the relationship between them (Kent and Cocker 1992). Hill and Gauch (1980) developed the detrended correspondence analysis (DCA) to overcome the limitations of correspondence analysis (CA) or reciprocal averaging (RA) such as the 'arch effect' and compression of gradient along the first axis. The main advantages against RA are simultaneous ordination of both the samples and species and handling of large data sets accurately. However, extreme outliers and discontinuities in the data can present limitations to DCA (Hill and Gauch 1980).

DECORANA and TWINSpan

DECORANA (Hill 1979a, Hill and Gauch 1980) and TWINSpan (Hill 1979b) are two multivariate analyses programs developed at the Institute for Terrestrial Ecology (now a part of Centre for Ecology and Hydrology). DECORANA and TWINSpan are

FORTRAN based programs which are widely used in the comparison of communities from species abundance and in the exploration of ecological data (Dytham 2003).

DECORANA is widely used for the ordination of multivariate species data, correspondence analysis and detrended correspondence analysis (Hill 1979a). The main axis of variation within the samples is calculated by dividing the first axis into many segments and recalculating the second axis scores within each segment to generate a mean value of zero (Kent and Coker 1992). The compression of points at the end of the first axis is removed by rescaling the segments in the first axis. DECORANA analysis produces four axes which summarise the association of stomatal characters with the samples analysed. The four axes summarise variation in descending order of importance. Only axis 1 and axis 2 are considered in plotting the two-dimensional ordination graph.

TWINSpan (Two-way indicator species analysis) is widely used for numerical classification of vegetation data (Kent and Coker 1992). It carries out sample and character classification hierarchically (Hill 1979b). TWINSpan splits the data into groups based on detrended correspondence analysis ordination of the samples. Each stomatal character analysed here are weighted and the values are rescaled until they converge on a single value. Hence the sample score is the weighted mean character scores.

TWINSpan is a divisive classification technique. It works by making repeated dichotomies until the samples are placed in a hierarchy ranging from the total collection

of samples to each individual sample. The dichotomy is characterised by a group of similar samples on one side and another group of similar samples on the other side. The two main groups can again be divided based on the data size and number of levels in the classification. The two groups are assigned as positive and negative and thus do not refer to any presence/absence data but shows dichotomy. The samples which occur exclusively on either side of the dichotomy form a positive indicator or negative indicator; the perfect positive indicator score being +1 and perfect negative indicator score being -1. Finally the stomatal characters used to classify the samples are also assigned positive and negative sides. The whole analysis generates a stomatal character by sample two-way table. This shows the division of original sample into positive and negative groups indicated by ones and zeros (Kent and Coker 1992).

2.2.2 Polyploidisation of *P. juliflora* and *P. pallida*

The germinated seeds of *P. juliflora* and *P. pallida* were selected for polyploidisation experiments since it is essential that the colchicine ($C_{22}H_{25}O_6N$) solution affects the growing shoot tips so that the emerging shoots would be polyploid. All the seeds for the present study were obtained from the Henry Doubleday Research Association (HDRA), UK and DANIDA Forest Seed Centre (DFSC), Denmark. Germinated seeds were treated with different concentrations of colchicine for about two weeks to induce polyploidy.

2.2.2.1 Material selection and preparation

Fifty seeds of each species were selected. The seeds were decontaminated by soaking in 1g l^{-1} of Sodium dichloro iso cyanurate (SDIC) for 10 min and washing thoroughly with sterile distilled water before scarifying the testas mechanically for better imbibition. The

scarified seeds were then sown in Petri dishes under aseptic conditions and incubated overnight at 37°C. After the incubation period the seeds were transferred to new Petri dishes lined with filter paper dampened with sterile distilled water. The Petri dishes were sealed with parafilm and germinated for 5 to 8 days at day-night temperature of 20 ± 2 °C. Uniform seedlings 4cm long with fully expanded cotyledons were sown in pots of compost and transferred to greenhouse at a temperature of 22 ± 2 °C to be colchicine treated.

2.2.2.2 Colchicine treatment of shoot tips

Polyploidy was induced in *P. juliflora* and *P. pallida* seedlings using the drop method described by Atwood (1944) for clover plants. Colchicine solutions of 0.05%, 0.1%, 0.2% and 0.3% were applied to the emerging shoot buds of the 4cm long potted seedlings using a pipette so that a single drop is collected between the cotyledons. The solution was applied three times per day for duration of up to 2 weeks. The seedlings used as control were treated with distilled water for the same period. All the plants were allowed to grow normally. The youngest leaves were harvested for ploidy screening by flow cytometry after 3-4 months.

2.2.2.3 Ploidy determination

Fluorescence intensity of the stained nuclei was analysed using flow cytometry. It is an accurate method for the identification of ploidy levels of plant species. The analysis was done on fresh leaves where at least three replicates of each sample were provided. The samples were sealed in labelled self-seal polybags lined with moist filter paper and sent to Plant Cytometry Services, Schijndel, the Netherlands for ploidy analysis. A modified

method of Arumuganathan and Earle (1991) was adapted for ploidy analysis. Approximately 100 mg of fresh leaves were co-chopped with an internal standard in 1 ml of ice cold nuclei isolation buffer. Iceberg lettuce (*Lactuca sativa* var. *capitata*) was used as an internal standard (2 C= 5.30 pg). The nucleic suspension is then filtered through a nylon filter to isolate the nuclei and then stained with DAPI (4', 6-diamidino-2-phenylindole). After staining, the suspension is passed through the flow cytometer (Partec PAS II), which measures the amount of fluorescence produced. The signals are converted by a computer connected to the flow cytometer into a graph plotting the intensity of emitted fluorescence against the number of cells emitting it at a given time. Ploidy level of the plant sample was calculated by comparing the mean peak ratio of fluorescent intensity of the standard and unknown sample. Histograms of DNA intensity produced by flow cytometry are directly related to the DNA ploidy level.

2.2.2.4 Measurement of stomatal characters

The leaf epidermal characters such as stomatal length, stomatal density, epidermal cell density, stomatal index and chloroplast number of the polyploidised plants produced from colchicine treatments were examined. Since there was insufficient tetraploid material of *P. pallida*, it was excluded from analyses. The methodology for measuring stomatal characters and chloroplast number is given in section 2.2.1.2 and 2.2.1.3

2.3 Results

2.3.1 Differentiation of *P. juliflora* and *P. pallida* using stomatal characters

A total of 23 accessions from both *P. juliflora* and *P. pallida* were analysed for their stomatal characters. The characters measured were stomatal length, stomatal density, epidermal cell density, stomatal index and chloroplast number. The photomicrograph showing leaf anatomical features of both the species at 40x and 60x magnifications are shown in Figure 2.1. The structure of stomata, epidermal cells and stained chloroplasts are clearly visible. The shape of epidermal cells and sizes of chloroplast in both the species exhibited characteristic differences.

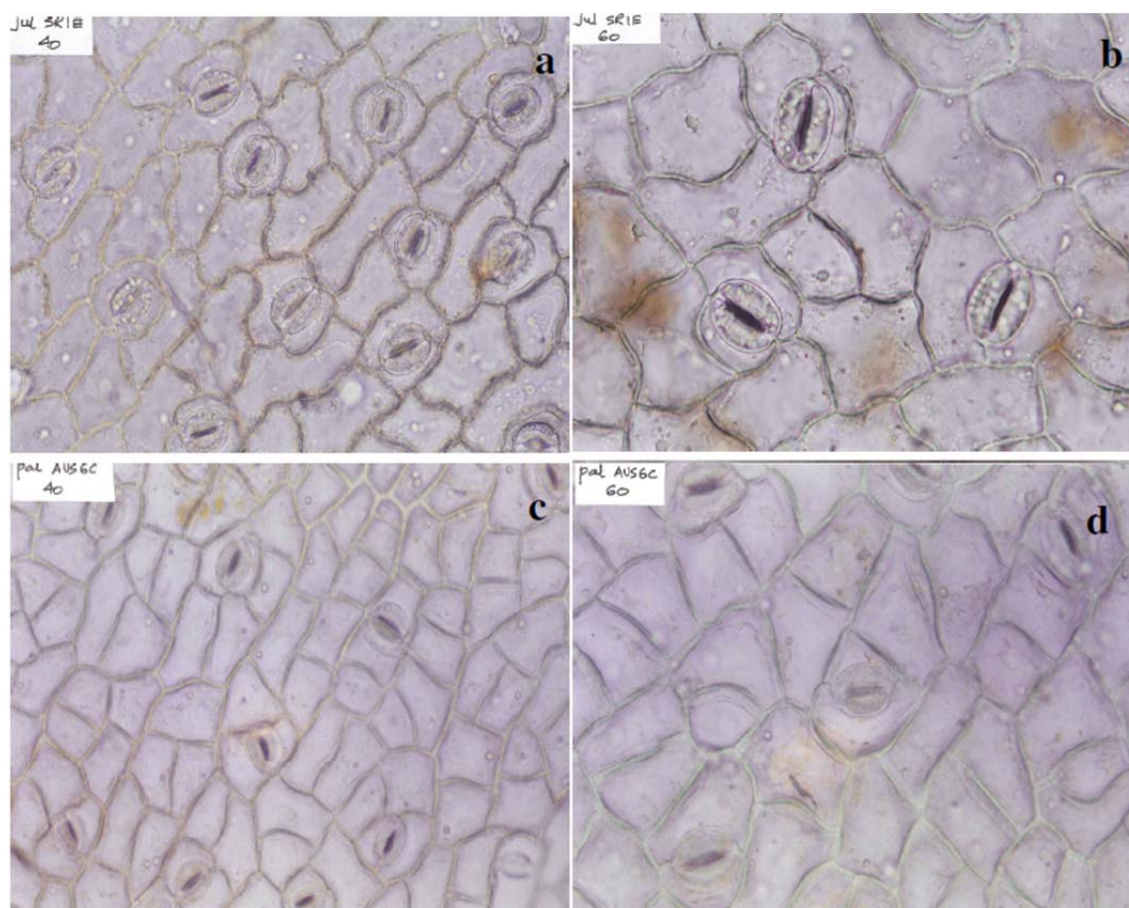


Figure 2.1: Stomatal structure seen on the epidermal peel of *P. juliflora* (a, b) and *P. pallida* (c, d) at 40x and 60x magnifications respectively

The guard cell chloroplasts stained with iodine are clearly countable in both species (Figure 2.2, a-d). The number of chloroplasts was slightly higher in the tetraploid *P. juliflora* than in the diploid *P. pallida*.

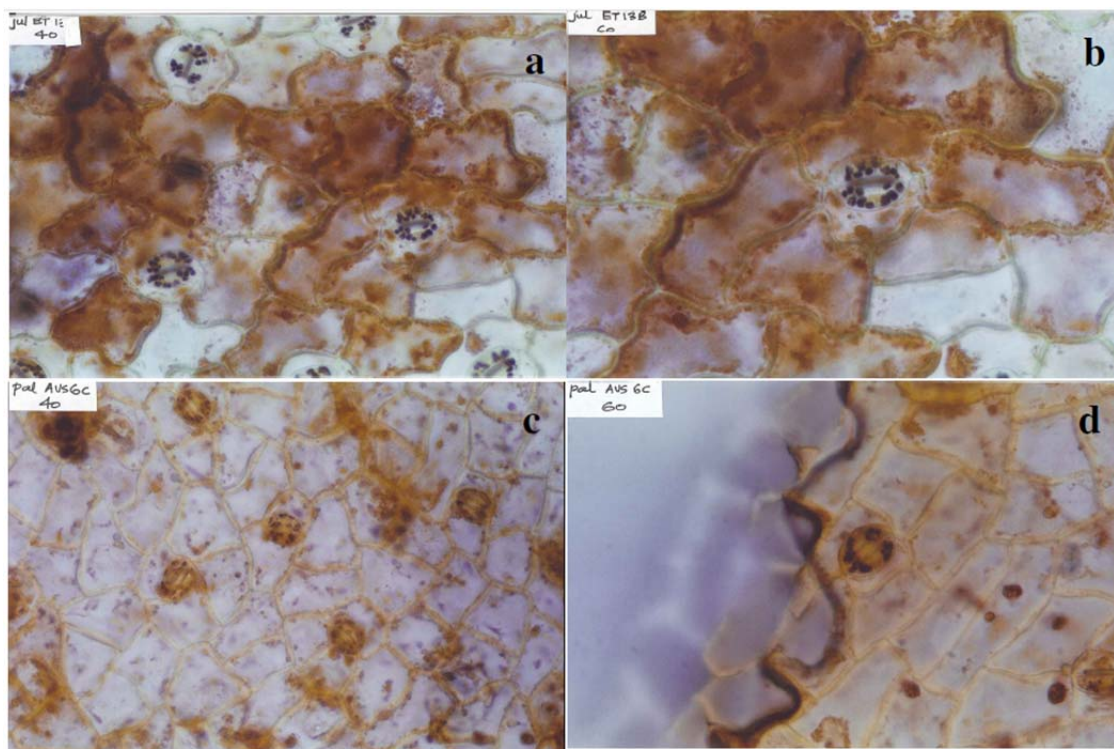


Figure 2.2: Stomatal guard cell chloroplasts in tetraploid *P. juliflora* (a, b) and diploid *P. pallida* (c, d) taken at 40x and 60x magnifications respectively

2.3.1.1 Quantitative data analysis

The stomatal characters of *P. juliflora* and *P. pallida* were analysed to identify additional means to effectively differentiate the two species. Based on those characters, dichotomous keys were developed to identify the species correctly. The results were effective in determining; (a) the range of data for each character for each species, (b) the relationship between the stomatal characters, and (c) if the data ranges between two species overlap. All analysis was carried out using Microsoft Excel. The results of the quantitative data analysis is given in Table 2.2

Table 2.2: Quantitative analysis of stomatal characters in *P. juliflora* and *P. pallida*

Anatomical characters	Species	Maximum value (mean)	Minimum value (mean)	Standard deviation	Standard error	Mean±SE
Stomatal length(µm)	<i>P.juliflora</i>	29.01	24.82	1.81	0.36	26.11 ± 0.36
	<i>P.pallida</i>	25.86	20.19	1.44	0.29	22.13 ± 0.29
Stomatal density(mm ²)	<i>P.juliflora</i>	152.19	59.96	11.52	2.30	94.08 ± 2.30
	<i>P.pallida</i>	158.34	87.89	12.78	2.55	124.77 ± 2.55
Epidermal cell density (mm ²)	<i>P.juliflora</i>	1496.85	787.40	94.36	18.87	1075.20 ± 18.87
	<i>P.pallida</i>	1972.44	1412.59	144.58	28.92	1700.09 ± 28.92
Stomatal index (%)	<i>P.juliflora</i>	11.71	5.69	1.74	0.47	8.11 ± 0.47
	<i>P.pallida</i>	7.99	5.52	0.88	0.29	6.89 ± 0.29
Chloroplast number	<i>P.juliflora</i>	19.96	13.80	1.92	0.38	16.93 ± 0.38
	<i>P.pallida</i>	15.72	11.80	1.47	0.29	13.59 ± 0.29

2.3.1.2 Comparison of stomatal characters using scatter graphs

Scatter graphs show the association between any two stomatal characters in both species, and were generated using Microsoft Excel and shown in Figures 2.3 and 2.4. The variables considered are stomatal length, stomatal density, epidermal cell density, stomatal index and chloroplast number. The pairs of stomatal characters which separated the two species into two clusters are illustrated in Figure 2.3. The pairs of characters which overlapped between the two species and hence less effective in separating them are illustrated in Figure 2.4.

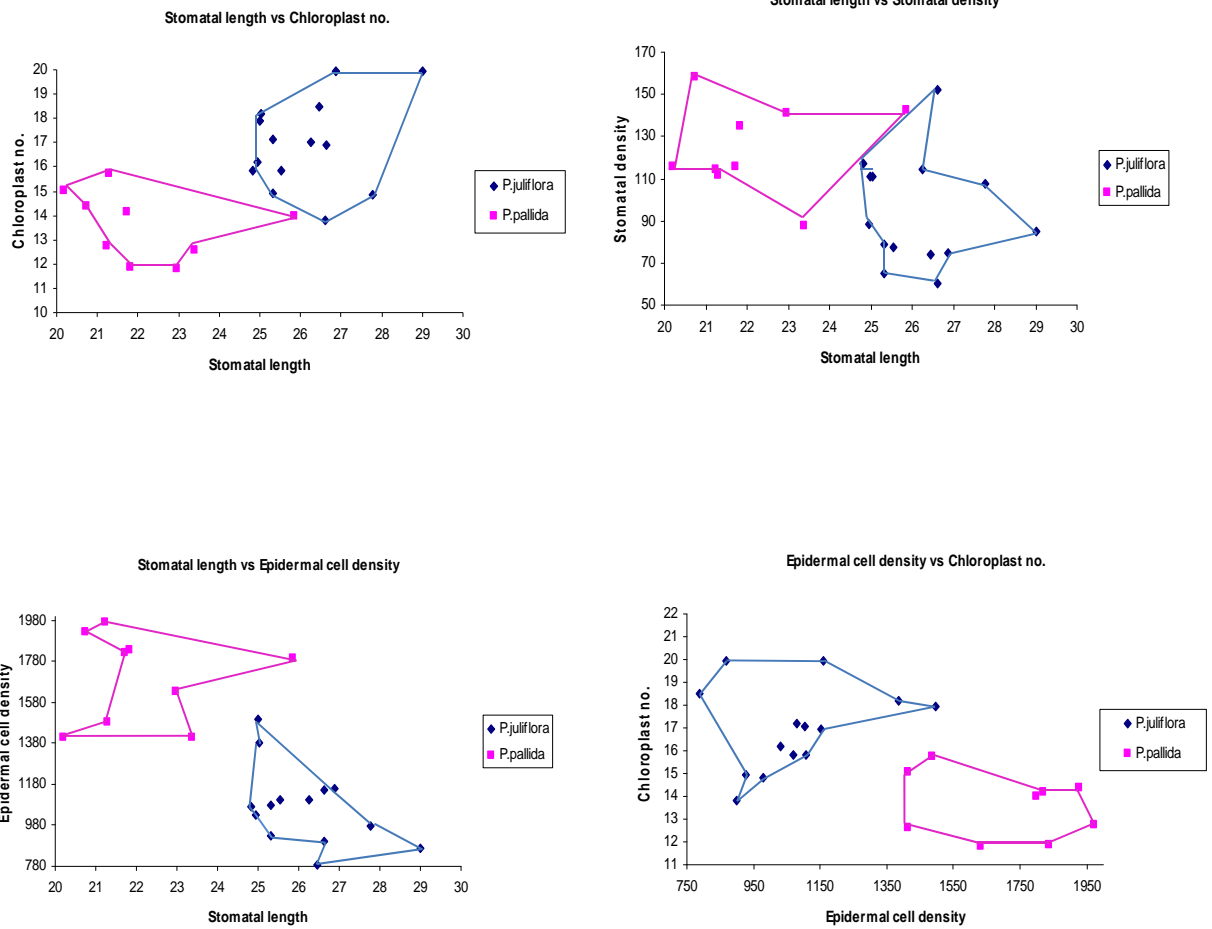


Figure 2.3: Scatter graphs showing the pairs of stomatal characters which separates *P. juliflora* (blue dots) and *P. pallida* (pink dots) into two clusters

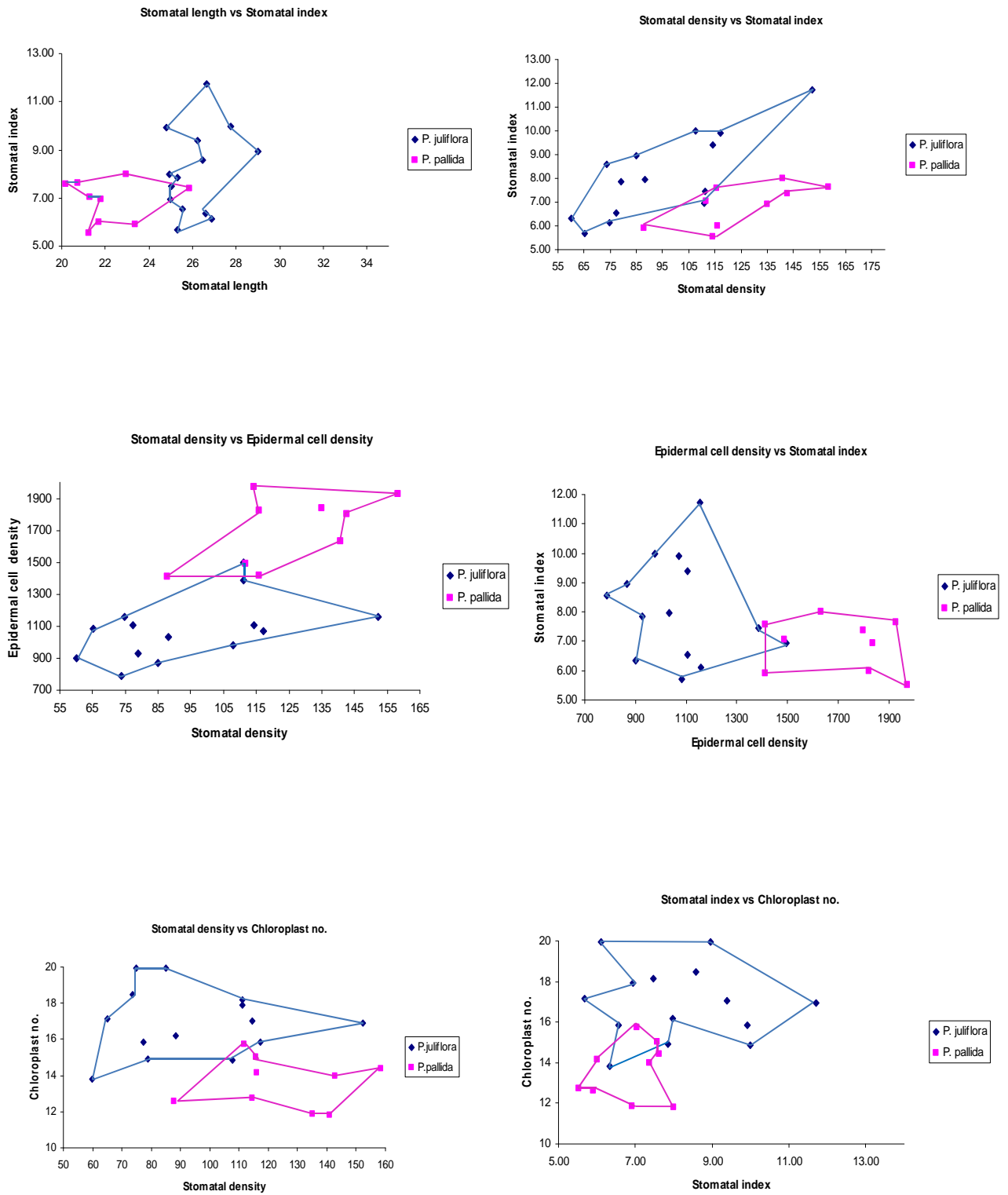


Figure 2.4: Scatter graphs showing the pairs of overlapping stomatal characters between *P. juliflora* (blue dots) and *P. pallida* (pink dots)

2.3.1.3 Analysis using Student's t-test

The statistical significance of the means of all variables was calculated using the Student's t-test. The degrees of freedom for the samples were 21. The threshold of significance was set at 0.001. Variances of the two samples were assumed to be equal. The t-test result is summarised in Table 2.3. The mean value for stomatal length, epidermal cell density and chloroplast number differed significantly between *P. juliflora* and *P. pallida* but mean value for stomatal density and stomatal index did not differ significantly.

Table 2.3: Comparison of means using Students t-test

	<i>Prosopis juliflora</i> Mean \pm SD	<i>Prosopis pallida</i> Mean \pm SD	P-value	T-stat	Significance
Stomatal length (μm)	26.11 \pm 1.81	22.13 \pm 1.44	< 0.001	6.5	yes
Stomatal density (mm^2)	94.08 \pm 11.52	124.77 \pm 12.78	0.007	-2.99	no
Epidermal cell density (mm^2)	1075.20 \pm 94.36	1700.09 \pm 144.58	< 0.001	-7.22	yes
Stomatal index	8.11 \pm 1.74	6.89 \pm 0.88	0.067	1.93	no
Chloroplast number	16.93 \pm 1.92	13.59 \pm 1.47	< 0.001	4.63	yes

2.3.1.4 Multivariate analysis using DECORANA and TWINSpan

Out of the total 23 samples analysed, 14 were *P. juliflora* and 9 were *P. pallida*. The multidimensional data was analysed using the multivariate analysis tools DECORANA and TWINSpan.

DECORANA analysis

Ordination of the data was carried out using the DECORANA computer package. DECORANA eigen values show the degree of variation in the data as explained by each axis in the analysis. The eigen value for the first two axis is 0.140 and 0.021. Since the eigen values for the 3rd and 4th axis are lower than the first axis they are not considered to be of any significance here. The values for axis 1 and 2 accounted for 42.76% and 6.41% of the total variance in the data for the four axes. DECORANA values obtained for each characters and the eigen values associated with each axis is shown in the Table 2.4

Table 2.4: The eigen values and scores for each character along the four axes

Stomatal Characters	Axis 1	Axis 2	Axis 3	Axis 4
	Eigen value = 0.140	Eigen value = 0.021	Eigen value = 0.005	Eigen value = 0.001
Stomatal length	-66	52	157	153
Stomatal density	148	97	26	51
Epidermal cell density	179	-53	59	52
Stomatal index	34	144	-6	-102
Chloroplast no.	-16	-85	-107	-44
Percentage variance of 4 axes	42.76	6.414	1.527	0.305

The character scores of both the species from axis 1 versus axis 2 were plotted in Figure 2.5 below. There is a clear separation in the ordination values observed for the species which indicates that these two species can be separated by their stomatal characters. This also substantiates the results from quantitative data analysis.

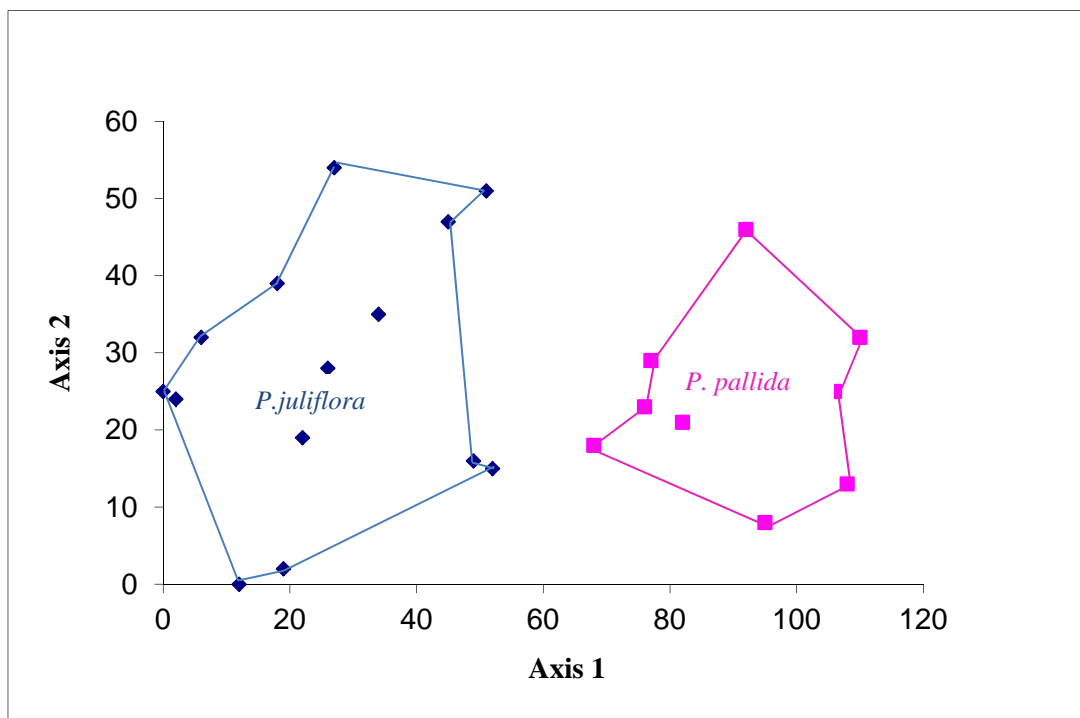


Figure 2.5: Ordination plot from DECORANA for the character scores for *P. juliflora* and *P. pallida* along axis 1 and axis 2.

TWINSpan analysis

Classification of the the two species in terms of their stomatal characters were achieved by TWINSpan analysis. It divided the samples into groups. Of the 23 samples, all the 14 *P. juliflora* samples fell into the group A while all 9 samples of *P. pallida* were in group B. The results obtained in DECORANA and TWINSpan analysis were used to develop identification keys for *P. juliflora* and *P. pallida*. The dendrogram produced by TWINSpan analysis is shown in the Figure 2.6 below.

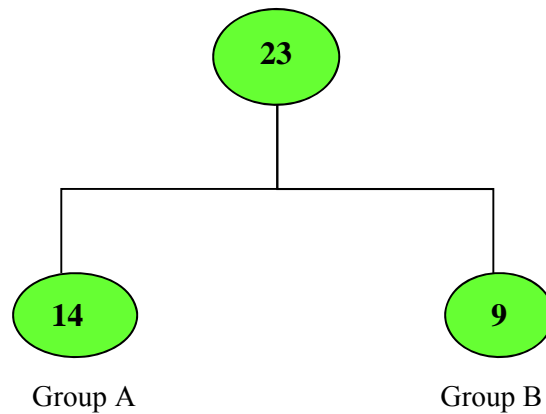


Figure 2.6: TWINSpan dendrogram showing the groups obtained in the first level of dichotomous division.

2.3.1.5 Development of a dichotomous key

Based on the evidence from exploratory data analysis, a dichotomous key was developed to differentiate the two species using stomatal characters. Stomatal length and epidermal cell density were the two most discriminatory characters identified by TWINSpan, hence these characters were selected to provide the distinction between *P. juliflora* and *P. pallida*.

Dichotomous key differentiating *P. juliflora* and *P. pallida*

1. Stomatal length $\leq 23\mu\text{m}$*P. pallida*
 Stomatal length $> 23\mu\text{m}$2
2. Epidermal cell density $\geq 26.9\mu\text{m}$*P. pallida*
 Epidermal cell density $< 26.9\mu\text{m}$*P. juliflora*

2.3.2 Polyploidisation of *P. juliflora* and *P. pallida*

P. juliflora and *P. pallida* shoot tips were treated with colchicine at concentrations of 0.05, 0.1, 0.2 and 0.3% for upto two weeks (Figure 2.7 c & d). Seedlings which were treated with colchicine for more than a week either failed to survive or showed stunted growth and were eventually discarded. This appears to affirm observations that prolonged treatment using colchicine can have adverse effect on plant tissue and leads to high mortality rates (Ajalin, Kobza, and Dolezel 2002). In the present study, 55% of all the colchicine treated seedlings survived, and were allowed to grow normally in the greenhouse before screening by flow cytometry using DAPI after approximately 8 weeks (Figure 2.7 e & f). Only half of the survived seedlings were selected for initial flow cytometric analysis. Slower rates of growth of colchicine treated plants were observed with both the species compared with their controls although the growth index was not measured. The reduced growth rate might be due to the reduced metabolic activities induced by colchicine (Goswami *et al.* 1996). There were no observable difference in the morphology between treated and control plants of either *P. juliflora* or *P. pallida*. Hence no leaf morphological character analysis was carried out on the polyploidised samples. Flow cytometric analysis was carried out on all the treated plants as the initial screening method for polyploidisation.

In general, out of the 19 *P. juliflora* samples sent for flow cytometry analysis, only 2 were identified as octoploids and 4 were identified as chimaeras. The rest were not affected with colchicine treatment and remained tetraploids. Out of the total 20 *P. pallida* samples, none were identified as 100% tetraploids, but 3 were chimaeras. The rest were unaffected by colchicine treatment and remained diploids. The fluctuations of

the results suggest more experimental replications should be carried out so that the effect of colchicine or concentrations and duration of treatments on survival rate of shoot tips can be confirmed. Stomatal measurements were carried out on the octoploid *P. juliflora* plants to identify any stomatal character differences. No stomatal measurements were carried out on the *P. pallida* chimaeras as there were not sufficient samples that showed any marked ploidy changes.



Figure 2.7: Colchicine treated *P. juliflora* and *P. pallida* seedlings at different stages of growth (a-f)

2.3.2.1 Ploidy levels of colchicine treated *P. juliflora* and *P. pallida*

Prosopis pallida diploid controls showed a mean peak ratio of 0.21 (Figure 2.8a), whereas the tetraploid controls of *P. juliflora* showed a mean peak ratio of 0.40 (Figure 2.8b), which is approximately double the ratio of diploid control. *P. pallida* chimaeras (2x-4x) possessed some percentage of tetraploid nuclei and they showed mean peak ratio with both the diploid and tetraploid values (Figure 2.9c). *P. juliflora* chimaeras (4x-8x) possessed some percentage of nuclei with octoploid complement of DNA (Figure 2.9d). Octoploid *P. juliflora* plants showed a mean peak ratio of 0.79, which is approximately double the ratio of tetraploid plants. The ploidy levels among the diploid, tetraploid and octoploid plants show arithmetic patterns of increase.

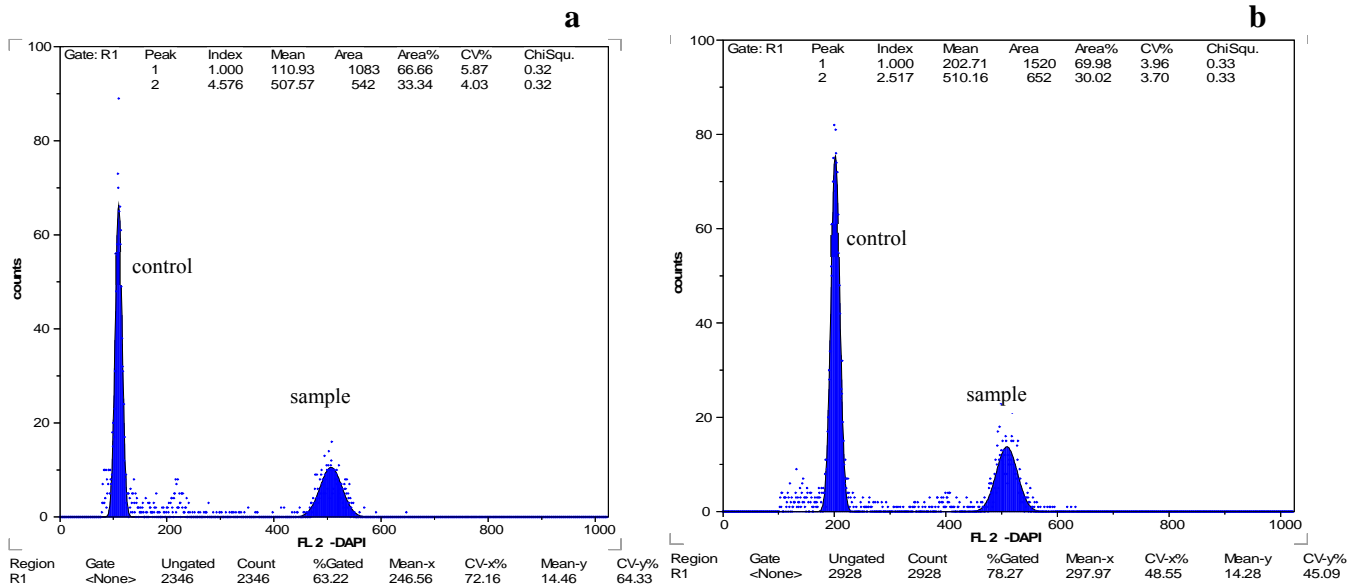


Figure 2.8: Representative flow cytometric histograms of nuclei isolated from leaves of control samples (a) diploid *P. pallida* and (b) tetraploid *P. juliflora*

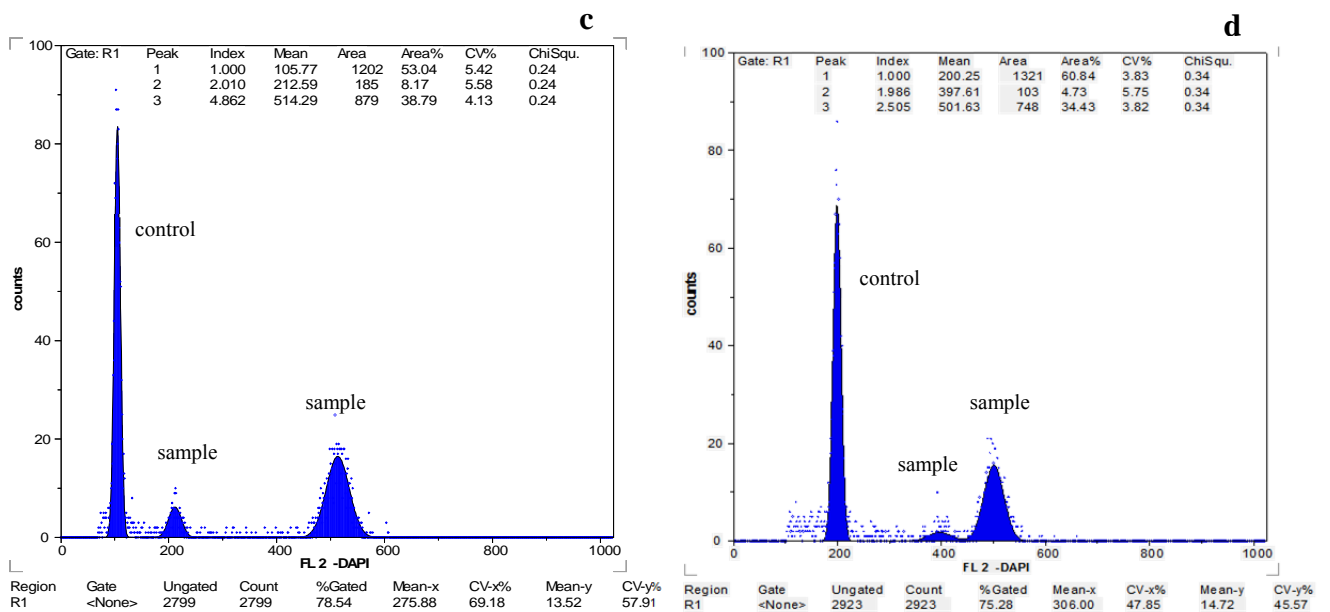


Figure 2.9: Representative flow cytometric histograms of nuclei isolated from leaves of mixoploid samples (c) *P. pallida* and (d) *P. juliflora*

2.3.2.2 Stomatal character analysis

Stomatal measurements were carried out on two octoploid *P. juliflora* plants where the ploidy level was confirmed by flow cytometry. Since there were no successful tetraploid *P. pallida* samples, it was excluded from the stomata analysis. Stomatal measurements were done to identify any significant attributes which might differentiate between the polyploidised and control samples. The characters considered for this analysis were stomatal length, stomatal density, epidermal cell density, stomatal index and chloroplast number. A summary of the descriptive statistics for the treated and control groups of *Prosopis juliflora* are provided below (Table 2.5).

Table 2.5: Quantitative data analysis of the colchicine treated octoploid and control tetraploid plants of *P. juliflora*

Stomatal characters	<i>P. juliflora</i>	Maximum value (mean)	Minimum value (mean)	Standard deviation	Mean \pm SE
Stomatal length (μm)	Treated octoploid	34.92	33.84	2.39	34.38 \pm 0.48
	Control tetraploid	26.88	25.31	1.89	26.10 \pm 0.36
Stomatal density (mm^2)	Treated octoploid	81.06	69.06	14.97	75.06 \pm 3.00
	Control tetraploid	74.89	65.14	7.58	70.02 \pm 1.52
Epidermal cell density (mm^2)	Treated octoploid	897.39	607.83	94.36	752.61 \pm 18.87
	Control tetraploid	1159.84	1081.10	90.45	1120.47 \pm 18.09
Stomatal index	Treated octoploid	10.24	8.45	2.1	9.35 \pm 0.42
	Control tetraploid	6.12	5.69	0.75	5.91 \pm 0.15
Chloroplast number	Treated octoploid	22.24	19.52	1.99	20.88 \pm 0.40
	Control tetraploid	19.96	17.16	1.68	18.56 \pm 0.36

In tetraploid and octoploid *P. juliflora*, length of the stomata proved to be significantly different at $P \leq 0.05$ (Table 2.6). The average stomatal length of the tetraploid control was 26.10 μm whereas in octoploid it was 34.38 μm .

Table 2.6: Comparison of means of the treated and control plants using Student's T test

<i>Prosopis juliflora</i>	Treated group Mean \pm SD	Control group Mean \pm SD	P-value	T-stat	Significance
Stomatal length (μm)	34.38 \pm 2.39	26.10 \pm 1.89	0.013	8.695	yes
Stomatal density (mm^2)	75.06 \pm 14.97	70.02 \pm 7.58	0.581	0.653	no
Epidermal cell density (mm^2)	752.61 \pm 94.36	1120.47 \pm 90.45	0.134	-2.452	no
Stomatal index	9.35 \pm 2.10	5.91 \pm 0.75	0.065	3.737	no
Chloroplast number	20.88 \pm 1.99	18.56 \pm 1.68	0.357	1.189	no

There were no marked differences in the number of chloroplasts in guard cells of tetraploid and octoploid *P. juliflora*. Other stomatal characters analysed in this study did not differ significantly between the two groups. However, this preliminary study has identified stomatal length as a reliable factor in distinguishing *Prosopis* species of different ploidy levels.

This study has also confirmed that polyploidisation can be achieved using colchicine in *Prosopis* species, but more detailed work could be carried out in the future with regard to the efficiency of other ploidy inducing chemical such as oryzalin. Other options such as different methods of treatment and duration of treatment could also be considered.

2.4 Discussion and conclusions

2.4.1 Differentiation of *P. juliflora* and *P. pallida* based on stomatal characters

P. juliflora and *P. pallida* are considered to be of high value both economically and ecologically in tropical arid and semi-arid zones though *P. juliflora* has often become weedy (Harris *et al.* 2003). These two species are difficult to distinguish because of their similarity in leaf morphology and habit. They are tropical species sharing similar environments and genetic affinities (Pasiiecznik *et al.* 2001). The assumed frequent interspecific hybridisation and formation of hybrids and varieties (Ferreyra 1987) in their overlapping ranges has made it difficult to distinguish them taxonomically (Pasiiecznik *et al.* 2001).

Morphological markers are often the primary factor in determining the relationship between species by identifying their ploidy levels (Ajalin, Kobza, and Dolezel 2002). The length of the guard cells in stomata is a good indicator of the level of ploidy in many plant species (Speckmann, Post, and Dijkstra 1965, Cohen and Yao 1996), thus enabling the identification of different cytotypes in mixed or sympatric populations (Joachimciak and Grabowska-Joachimciak 2000). Guard cell length was useful to identify even closely related species with different ploidy levels (Joachimciak and Grabowska-Joachimciak 2000). In the present study, stomatal length was higher for the tetraploid *P. juliflora* than diploid *P. pallida* which supports previous studies in other genera. Similarly the frequency of stomata can also provide accurate measure of the ploidy in plants (Przywara, Pandey, and Sanders 1988). In the present study, stomatal frequency (stomatal density) is higher in diploid *P. pallida* than in tetraploid *P. juliflora*. Presence

of larger epidermal cells could contribute to the reduction in stomatal frequency at higher ploidy levels (Mishra 1997). A similar result was noticed in the ploidy studies in *Acacia* species by Beck, Fossey, and Mathura (2003). Epidermal cell density also followed a similar trend. As the ploidy increased in *P. juliflora*, the epidermal cell density values also decreased. The t-test compared the means between the two species and found that mean values of stomatal length and epidermal cell density differed significantly among them.

Chloroplast counts helps in the rapid screening of populations with varying ploidy levels and it can be done at any stage of growth of the plant (Bingham 1968). Study in the genus *Gossypium* have shown that chloroplast number is hugely influenced by the ploidy, and chloroplast counting could be utilised as a rapid method in screening for polyploids (Krishnaswami and Andal 1978). The values for chloroplast number were slightly higher with *P. juliflora* as it had larger guard cells. But no large differences between the values were noticed between the species. The guard cell chloroplast number correlates with the chromosome number in certain plant species, thus showing an increase in number with the increase in ploidy (Soloveva 1990, Beck, Dunlop, and Fossey 2003a). Analysis using t-test showed significant difference in chloroplast number in the tetraploid *P. juliflora* compared with the diploid *P. pallida*. Work on haploid cotton has also revealed that chloroplast number is significant in differentiating haploids from tetraploids (Chaudhari and Barrow 1975). Stomatal index may not be a good indicator of ploidy levels (Mishra 1997), however, but stomatal index did not fluctuate with varying ploidy levels in the above study. This result is comparable to that

observed in *Coffea* where stomatal index was found to be least indicative of any change in ploidy levels (Mishra 1997).

Differentiation of the two species using leaf morphology and ploidy has been described by Harris *et al.* (2003). Molecular studies were also employed to separate the two species (Landeras *et al.* 2006). Foliar characters were also utilised to develop keys for the identification of these tropical *Prosopis* species (Pasicznik, Harris, and Smith 2004). All the above studies are supported by the results achieved in this study, where stomatal characters were found to differentiate the two species. Despite their morphological similarities the two species were clearly separated into two groups on TWINSpan based on their stomatal characters. All the diploid *P. pallida* samples formed one group and the tetraploid *P. juliflora* samples in other. Two of the stomatal characters, the stomatal length and epidermal cell density have been successfully used to produce a dichotomous key to differentiate between *P. juliflora* and *P. pallida* species. Using DECORANA and TWINSpan analysis, all the samples were separated into two groups. All *P. juliflora* were placed in one group while all *P. pallida* in the other.

2.4.1.1 Conclusions

The dichotomous key developed was successfully used to separate *P. juliflora* and *P. pallida* samples analysed in this study. Further collection of samples from their native and introduced ranges would provide conclusive evidence to the effectiveness of developed keys in screening large populations of the species. Stomatal characters are shown to be influenced by genetic and environmental factors (Salisbury 1927, Fernandez and Muzica 1973, Kharazian 2007) and such factors should be taken into

account while developing keys using large samples of the two species. Correct identification of these tropical species is indispensable for their management, and especially controlling weediness.

2.4.2 Polyploidisation of *P. juliflora* and *P. pallida* using colchicine

It is noted that increased colchicine levels often have adverse effect on the survival rate in plant species (Brennan 1996). In this study, only 55% of treated plants survived and prolonged treatment using colchicine increased the mortality rate. The germination percentage also declined with increases in colchicine concentration (Blakesley *et al.* 2002). Slow growth rate was observed among treated samples of *P. juliflora* and *P. pallida*, which could be due to a reduced rate of cell division (Stebbins 1984) and a slower rate of metabolic activities in response to treatment (Goswami *et al.* 1996). However, Wright (1976) noted that the slow growth and growth abnormalities in colchicine treated plants were indicators of successful treatment.

Changes in the morphological characters are indicators of the changes in level of ploidy (Ajalin, Kobza, and Dolezel 2002), but no significant morphological changes were observed in treated *P. juliflora* and *P. palida*. Since very few treated plants were completely polyploidised, no foliar character analysis was undertaken. The different concentrations of colchicine also did not bring about observable morphological changes in both the species, which relates to the work by Blakesley *et al.* (2002). In *Viola*, however, for leaf apex treatment with colchicine, the optimum level of the chemical was found at its lowest concentration and for the shortest treatment period (Ajalin, Kobza, and Dolezel 2002).

Often large numbers of mixoploids are derived from artificial polyploidy induction methods using chemical like colchicine (Wan, Petolino, and Widholm 1989). Mixoploid plants with both diploid and tetraploid levels in treated *P. pallida* and with tetraploid and octoploid levels in treated *P. juliflora* plants suggest that ploidy has not happened in all the treated tissues. The production of mixoploid plants during colchicine treatment is common as seen in other studies (Huang *et al.* 2010, Nimura *et al.* 2006) and they have been successfully used in breeding programmes (Koutoulis *et al.* 2005) resulting in agronomically stable cultivars (Van Harten 2002).

A positive proportional relationship was found between ploidy and stomatal length on polyploidised *P. juliflora* plants. Only stomatal length was significant in distinguishing tetraploid and octoploid plants. This result supports findings by Tan and Dunn (1973) in *Bromus* species where no reliable difference in stomatal length was observed among different ploidy levels. Smith *et al.* (2004) also found it to be a suitable character in distinguishing *in vitro* colchicine treated plants. However stomatal character was not helpful in the successful screening between diploids and tetraploids in alfalfa populations (Bingham 1968).

Many previous authors have commented on the significance of chloroplast number as a reliable character in distinguishing polyploidised plants (Nicholson 1981, Ho *et al.* 1990, Ewald *et al.* 2009, Tunbridge, Sears, and Elle 2011). In this study, no significant difference was observed in the range of chloroplast number between tetraploid and octoploid *P. juliflora*. This may be due to the deleterious effects of high gene dose

beyond the tetraploid level (Pradeep and Jambhale 2000). Variations in the number of chloroplasts are observed within a single plant or between plants of same line (Beck, Fossey, and Mathura 2003).

2.4.2.1 Conclusions

The artificial induction of polyploidy in *P. juliflora* and *P. pallida* are of particular interest as there are no previous reports of colchicine polyploidisation in *Prosopis* species. This study has proved that colchicine can be used to induce polyploidy in the genus. Oryzalin has been successful in the induction of ploidy in other species (Geoffriau *et al.* 1997, Kermani *et al.* 2003, Dhooghe *et al.* 2009). Polyploidisation studies in the future involving *Prosopis* species could compare the effectiveness of oryzalin in inducing tetraploid and octoploid plants. Comparing polyploidised *P. juliflora* and *P. pallida* with already established cytotypes in natural environments will help in evaluating the role of polyploidy in their adaptation and speciation. Experimental studies which compare the genetic and phenotypical characters of induced polyploids against established natural polyploids in *Prosopis* are warranted. Perhaps such studies could explore the role of polyploidy in contributing to the invasiveness of *P. juliflora* species where it is introduced. This would help in the genetic improvement and proper management of the species.

3. MOLECULAR DIFFERENTIATION BETWEEN *PROSOPIS JULIFLORA*, *PROSOPIS PALLIDA* AND SELECTED SPECIES OF *PROSOPIS* ON THE BASIS OF RAPD AND MICROSATELLITE TRANSFERABILITY STUDIES

3.1 Introduction

Genetic differentiation among plant species can be assessed by several methods although the choice of a particular method is largely dependent on the plant material studied and the questions considered. With the isolation of restriction enzymes and the polymerase chain reaction (PCR), naturally occurring genetic polymorphisms can now be assessed at the DNA level (Saiki *et al.* 1988, Glaubitz and Moran 2000). The invention of PCR based molecular markers has played an important role in the field of genetics. This has rectified the limitations associated with cytological or morphological markers in assessing genetic relationships among many taxa. DNA markers have found applications in a wide variety of species including humans (Botstein *et al.* 1980), other animals (Bishop, Hawkins, and Keeler 1995) and plants (Rivin *et al.* 1983), and are unaffected by physiological or environmental factors (Bowditch *et al.* 1993).

Molecular markers have been useful owing to their technical simplicity and an ability to generate and score large number of markers (Rieseberg and Ellstrand 1993, Dawson *et al.* 1996). They have found application in assessing gene flow levels among species, biodiversity studies and phylogenetic differentiation of hybridizing taxa and their close relatives (Rieseberg and Ellstrand 1993, Noel, Machon, and Porcher 2007) and also are utilised in the identification and characterisation of hybrids (Rieseberg, Soltis, and Soltis 1988, Avise 1994).

Genetic diversity in natural populations has been analysed by three major DNA based techniques, namely RFLP (Restriction Fragment Length Polymorphism) (Grodzicker *et al.* 1975, Botstein *et al.* 1980), RAPD (Random Amplified Polymorphic DNA) (Williams *et al.* 1990) and AFLP (Amplification Fragment Length Polymorphism) (Vos *et al.* 1995). RFLP is the most commonly use molecular marker. It is frequently used in the genomic mapping and genetic variation studies of a number of crop species (Tanksley *et al.* 1989, Zhang *et al.* 1992). However, the extraction of a sufficient amount of DNA for RFLP analysis is time consuming, laborious and expensive. AFLP is increasingly used in genetic diversity studies across a number of species, thanks to its high reproducibility and resolution compared with other DNA markers (Akerman *et al.* 1996, Muluvi *et al.* 1999, Mariette *et al.* 2001). The Random Amplified Polymorphic DNA (RAPD) marker can detect polymorphisms between even closely related genotypes (Williams *et al.* 1990). Moreover, the arbitrary primer sequences can be used for different species without prior knowledge of the host DNA sequence. They have been applied in a variety of studies like genetic fingerprinting (Multani and Lyon 1995), detecting DNA polymorphism (Venkatachalam, Sreedhar, and Bhagyalakshmi 2008), genotype identification and genetic diversity (Fernandez, Figueiras, and Benito 2002, Sen, Skaria, and Abdul Muneer 2010), germplasm characterisation (Korbin, Kuras, and Zurawicz 2002), molecular ecological studies (Hadrys, Balick, and Schierwater 1992), genetic mapping (Levin, Crittenden, and Dodgson 1993) and understanding the phylogenetic relationship among species (Stammers *et al.* 1995, Sehgal *et al.* 2009). RAPD primers are inexpensive and relatively simple to use. However, the technology has its limitations as most of the polymorphic markers revealed are dominant markers

(Williams *et al.* 1990); the co-migration of these bands at different loci and reproducibility of markers among various laboratories are to be considered (Black 1993).

Microsatellites (Litt and Luty 1989) or Simple Sequence Repeats (SSR) are tandem repeats of short sequences, 1-6 base pairs long. They are co-dominant PCR markers (Tautz 1989) which are randomly distributed and occur as widely dispersed repetitive elements in all eukaryotic genomes (Tautz and Renz 1984). These repetitive sequences were first detected in humans (Weber and May 1989) and later found to be frequent and widely distributed in plants (Morgante and Olivieri 1993). Microsatellites are highly variable and are more applicable in genetic linkage mapping studies than the use of dominant markers like RAPDs and AFLPs. Microsatellites are used in the construction of genetic linkage maps which can give information about inter and intraspecific genome organisation (Isagi and Suhandono 1997, Streiff *et al.* 1998). Microsatellites fall into the category of highly polymorphic VNTR (Variable Number of Tandem Repeats) markers that have high evolutionary rate (Nakamura *et al.* 1987). Microsatellites can be applied in many areas of genetics due to their high reproducibility, multi-allelic nature, high resolution and PCR based amplification. Cross-species amplification of microsatellites is highly valuable in the case of taxa with low microsatellite frequencies or where isolation is difficult (Primmer and Merila 2002). Co-dominant markers like microsatellites are more highly polymorphic than dominant ones, they are found to be more informative in the number of recognisable alleles per locus and thus differentiating all the genotypes (Hardy *et al.* 2003, Bessega *et al.* 2011).

RAPD and isozymes have been utilised in the determination of population structure of *Prosopis* species, especially those of section *Algarobia* (Juárez-Muños 2002, Ferreyra *et al.* 2007, Hamza 2010). Enzyme and molecular marker studies have concluded that section *Algarobia* is not a natural group (Bessega, Saidman, and Vilardi 2005, Burghardt and Espert 2007). Although isozyme analysis proved to be useful, the enzymatic expression is restricted to specific developmental stages or plant tissue influenced by the environment (Solbrig and Bawa 1975, Whitmore and Bragg 1979, Saidman and Vilardi 1987). However, RAPD markers have shown clear advantages over isozymes in detecting genetic variability between species within the genus (Goswami and Ranade 1999, Bessega *et al.* 2000, Ferreyra *et al.* 2007). A number of studies in recent years have utilised DNA markers such as RAPD, AFLP and microsatellites in assessing the genetic relation between *Prosopis* species (Bessega, Saidman, and Vilardi 2005, Mottura *et al.* 2005, Landeras *et al.* 2006, Bessega *et al.* 2011, Palacios *et al.* 2011).

Genetically divergent but morphologically cryptic genotypes can be easily identified by molecular markers (Pfenninger and Schwenk 2007). *P. juliflora* and *P. pallida* are similar in leaf morphology and tree form, and Pasiecznik *et al.* (2001) assumed that naturally occurring hybridisation in their overlapping native ranges led to the difficulty in differentiating the two species. Molecular studies have been useful in differentiating the two species and have reported them to be closely related (Landeras *et al.* 2006). Later studies (Harris *et al.* 2003, Trenchard *et al.* 2008), confirmed that that *P. juliflora* was the only known naturally occurring tetraploid species in the genus, though the

origin of *P. juliflora* remains unresolved. Following the complexity in the taxonomy on the two species, an integrated approach which utilises both morphological and molecular markers would be extremely useful in differentiating them as shown by recent studies (Palacios *et al.* 2011). Hence RAPD analysis was used in conjunction with stomatal and epidermal morphology to differentiate the two species. Stomatal character analyses on accessions of *P. juliflora* and *P. pallida* (Chapter 2) have shown that there is a clear separation between the two species. Even though this may be the case, it is important that through molecular analyses, accurate knowledge of the two species is obtained for various conservation projects involving them. Due to the morphological continuities among these species, there have been reports of misidentification, particularly involving *P. juliflora* (Pasiecznik *et al.* 2001) in their introduced ranges.

In Chapter 2, stomatal characters were used to differentiate the two species. RAPD and microsatellite primer pairs were also utilised to assess the genetic relationship and cross species amplification profile of *P. juliflora* and *P. pallida*. Their relationships to selected *Prosopis* species from section Algarobia and section Strombocarpa were also analysed.

Through RAPD analysis, the genetic relationship and clustering pattern of six species from section Algarobia in addition to *P. juliflora* and *P. pallida* were analysed, namely *P. alba* Grisebach, *P. chilensis* (Molina) Stuntz emend. Burkart, *P. glandulosa* Torrey, *P. laevigata* (Humb. & Bonpl. ex Willd.) M. C. Johnston, *P. articulata* S. Watson and *P. velutina* Wooton. From section Strombocarpa, *P. tamarugo* F. Philippi was included.

Except for *P. pallida* and *P. articulata* from the series Pallidae, all the other Algarobia species selected are from series Chilenses.

A total of six microsatellite primer pairs designed for *P. chilensis* and *P. flexuosa* by Mottura *et al.* (2005) were also screened for possible transferability and amplification. Three more species, *P. nigra* (Grisebach) Hieronymus, *P. caldenia* Burkart and *P. affinis* Sprengel, all from section Algarobia were also selected for the microsatellite transferability studies. They were excluded from RAPD analysis since this study did not focus on differentiating *Prosopis* species between sections. However, analysing the microsatellite rate in other species from Algarobia would help to identify sequence similarity in DNA sequences flanking microsatellites. Also the identity of *P. affinis* from Peru obtained from DFSC is disputed. The seeds obtained for that species are highly likely to be *P. pallida* as supported by other researches (Pasicznik *et al.* 2001, Palacios *et al.* 2011). The high rate of transfer of markers among related species would suggest homology in the DNA sequences in SSR flanking regions (Primmer and Merila 2002). These SSR flanking sequences are conserved and could be used to examine evolutionary relationships (Rossetto, McNally, and Henry 2002). Polymorphic DNA analyses were noted to be helpful in fully exploiting these species especially in the areas where they are introduced. Molecular differentiation of the two species would allow for their accurate identification, better management and genetic improvement.

3.2 Materials and methods

3.2.1 *Prosopis* accessions

Eleven out of the twelve *Prosopis* species selected for RAPD and microsatellite cross species amplification study were from section *Algarobia*. The only other selected species from Strombocarpa, *P. tamarugo*, is from the series Cavenicarpae of that section. For the RAPD analysis, only nine species representing the two sections were selected. Eight of them were from the two series, Pallidae and Chilenses of the section *Algarobia*. The seeds for all the eleven species of *Prosopis*, except for *P. juliflora*, analysed in this study were obtained from the Henry Doubleday Research Association (HDRA), UK and Danida Forest Seed Centre (DFSC), Denmark. These were collected from a single tree or from several trees in a population either from their natural or introduced ranges. *Prosopis tamarugo* seeds from Strombocarpa were selected from its native range. Seeds of the *Algarobia* species *P. chilensis*, *P. pallida*, *P. laevigata*, *P. glandulosa*, *P. caldenia* and *P. nigra* were also selected from their native ranges (Burkart 1976, Johnston 1962). Seeds for *P. alba*, *P. articulata* and *P. velutina* were collected from the USA.

The seeds for *P. juliflora* were obtained from the introduced range in the Galapagos Islands and seedlings were established at the Coventry University greenhouse. The identity of the *P. juliflora* samples from Galapagos was initially established using leaf morphology before its ploidy confirmation by flow cytometry (Trenchard *et al.* 2008). *Prosopis juliflora* populations in the Galapagos Islands might have become naturalised through numerous introductions (Wiggins and Porter 1971). For the RAPD and

microsatellite study, the selected species from Danida Forest Seed Centre (DFSC) were identified by collectors by comparing the species morphology with botanical descriptions (Lars Schmidt, DFSC, Denmark, pers. comm. 2006). The identity of species obtained from HDRA was determined by Dr Peter Felker based on morphology. Seeds of a putative *P. chilensis* × *P. pallida* from Cape Verde were also obtained from HDRA and are included in the present study. The hybrid was putatively identified by the collector Nick Pasiecznik based on the assumed identity of mother tree and that all surrounding flowering trees were of a single different species (Nick Pasiecznik pers. comm.). Genomic DNA was extracted from leaves in the case of *P. juliflora* and from the cotyledons of the other species. A list of the species and sources are given in Table 3.1

Table 3.1: List of the *Prosopis* species used giving section, series and species with their accession number, country of origin and source

Section	Series	<i>Prosopis</i> species	Accession number	Provenance /Country	Collector/source (if known)
Strombocarpa	Cavenicarpae	<i>P. tamarugo</i>	HDRA 562	1019/Chile	P. Felker
Algarobia	Pallidae	<i>P. pallida</i>	DAN 1490/85	San Jacinto de Cachche/Peru	DFSC
Algarobia	Pallidae	<i>P. affinis</i>	DAN 1653/86	Codigo C1/Peru	DFSC
Algarobia	Pallidae	<i>P. articulata</i>	HDRA 349	0593/USA	P. Felker
-----	-----	<i>P. chilensis</i> x <i>P. pallida</i> hybrid?	HDRA 813	Monte Vaca/Cape Verde	N. Pasiecznik
Algarobia	Chilenses	<i>P. chilensis</i>	HDRA 314.4	Chacabuco Region V, Chile	University of Chile/HDRA
Algarobia	Chilenses	<i>P. juliflora</i>	Gal 01	Ecuador, Galapagos Islands	Sourced from Coventry University
Algarobia	Chilenses	<i>P. nigra</i>	HDRA 568	1135/Argentina	P. Felker
Algarobia	Chilenses	<i>P. caldenia</i>	HDRA 652	AH542'90/Argentina	University of Cordoba
Algarobia	Chilenses	<i>P. laevigata</i>	HDRA 810	OFI78/93/1/Mexico	J. Hawkins
Algarobia	Chilenses	<i>P. glandulosa</i> var. <i>torreyana</i>	DAN 1211/83	Concepcion Del Oro, Mexico	DFSC
Algarobia	Chilenses	<i>P. alba</i>	HDRA 905.5	0591/USA	P. Felker
Algarobia	Chilenses	<i>P. velutina</i>	HDRA 545	0860/USA	P. Felker

3.2.2 DNA extraction

Bulked DNA samples were prepared for extraction as described by Michelmore, Paran, and Kesseli (1991). Ten to fifteen seeds from each species were randomly selected for RAPD analysis. The seeds were first rinsed in ordinary tap water for 1 or 2 min and then in de-ionised water for at least 2 min. The seeds were then soaked in water containing two drops of non-ionic surfactant Nonidet P-40 and shaken vigorously for a few minutes, before washing thoroughly with sterile distilled water until all traces of surfactant were removed. The seeds were soaked for 10 min in 1 g l⁻¹ of sodium dichloro iso cyanurate (SDIC) and washed thoroughly with sterile distilled water before scarifying the testas mechanically to improve imbibition. The scarified seeds were then sown in Petri dishes under aseptic conditions and incubated overnight at 37°C, before transferring to new Petri dishes lined with filter paper dampened with sterile distilled water. The Petri dishes were sealed with parafilm and germinated for 5 to 8 days at room temperature with a 12 h photoperiod and 80% relative humidity. When the cotyledons were fully expanded they were harvested for DNA extraction.

Total cellular DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, UK) following the manufacturer's instructions. For each *Prosopis* species, cotyledons from ten seedlings were bulked prior to extraction, and about 30 µg of total cellular DNA was obtained. DNA concentrations were measured using NanoDrop® ND-1000 UV-Vis spectrophotometer and the concentrations of total samples ranged from 20-90 ng µl⁻¹.

3.2.3 Randomly Amplified Polymorphic DNA (RAPD) analysis

3.2.3.1 Polymerase chain reaction

A total of 63 arbitrary primers were used and all were 10-mers custom synthesised by Invitrogen, UK. Of these primers only those which gave consistently good polymorphism and reproducibility were chosen for further experiments. List of the 25 selected primers and their sequences are shown in Table 3.2

Table 3.2: List of primers and their sequence used for phylogenetic study of 17 species of *Prosopis*

Primers	Sequence
A08	5' ACG CAC AAC C 3'
B11	5' GTA GAC CCG T 3'
B12	5' CCT TGA CGC A 3'
E20	5' ATC GGT GAC C 3'
L01	5' GGC ATG ACC T 3'
L03	5' CCA GCA GCT T 3'
L09A	5' AGC AGG TGG A 3'
N03	5' GGT ACT CCC C 3'
N12	5' CAC AGA CAC C 3'
N14	5' TCG TGC GGG T 3'
R05	5' TGC GCC CTT C 3'
R07	5' GGT GAC GCA G 3'
R08	5' GTC CAC ACG G 3'
R18	5' CCA CAG CAG T 3'
R23	5' AGC CAG GCT G 3'
R25	5' GGG TGC AGT T 3'
R27	5' TCG CTG CGG A 3'
R29	5' GGG GGA GAT G 3'
R30	5' CTG TGT GCT C 3'
R32	5' AAC GGC GGT C 3'
R35	5' TGA TGC CGC T 3'
R37	5' TCA GCA CAG G 3'
R39	5' ACC ACG CCT T 3'
S07	5' TCC GAT GCT G 3'
S11	5' AGT CGG GTG G 3'

Polymerase chain reactions (PCR) for the generation of RAPD markers were performed with Go Taq Green master mix (Promega, UK), using 40-120 ng of genomic DNA as the template and 0.8 μ M primer in a final volume of 25 μ l. The mixture was then mixed gently, with two drops of mineral oil dropped on top to prevent evaporation during the amplification cycles. Amplification was performed on a Peltier Thermal Cycler-200. For each PCR, the following sequence was used: initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 42°C for 1 min and extension at 72°C for 1 min. The final primer extension was done at 72 °C for 5 min.

Experiments were duplicated at different times with the same DNA and primers to ensure the reproducibility. Two different controls, one without the template DNA and the other without the primer, were routinely used in all experiments to rule out the possibility of any contamination.

3.2.3.2 Visualisation of amplified DNA

The amplification products were separated by electrophoresis using a 1.5% agarose gel stained with Ethidium Bromide, EtBr (0.5 μ g ml⁻¹ for 30 min). It was visualised under ultraviolet light on a BIORAD gel documentation system. Images of the gels were copied and printed for the comparison of banding patterns.

3.2.3.3 Statistical analysis of data

Similarity index measurement

The different RAPD bands from the images were scored manually based on their presence (1) or absence (0) for each template–primer combination. Very faint bands

were not scored. The binary data matrix was analysed using Dice's coefficient similarity to generate similarity coefficients between all the possible pairs of ten accessions of *Prosopis* including the hybrid. Similarity coefficients/indices measure the degree of relatedness between species and can be applied to both qualitative (presence or absence data) and quantitative data (Kent and Coker 1992). Dice coefficient (D) after Dice (1945), calculates similarity as $S_{ij} = \frac{2a}{2a+b+c}$ where S_{ij} is the similarity between two individuals i and j , a is the number of bands present in both i and j , b is the number of bands present in i and absent in j and c is number of bands present in j and absent in i . Dice's index is particularly useful for presence/absence data and varies from 0 (no similarity) to 1 (complete similarity). Hence the complementary genetic distance between accessions can be estimated by subtracting the similarity indices from 1 (Manly 2005).

Cluster analysis method

The main aim of this analysis was to find the relationships between different *Prosopis* species according to their similarities. Closely related species are grouped in the same cluster and those dissimilar are grouped in separate clusters. This method increases the homogeneity between species in a cluster (Hair *et al.* 2006). A hierarchical clustering method clusters the species in several steps. Thus different clusters are sequentially combined so that the numbers of clusters get reduced until a single cluster is achieved (Everitt and Dunn 2001). The hierarchical cluster method produces a dendrogram which summarises the genetic relationships between species (Lessig 1972).

Several hierarchical clustering methods are available for phylogenetic analysis. For the present analysis, Average Linkage Clustering Algorithm (ALCA) or the Group Average

method by Sokal and Michener (1958) was used. This is an agglomerative hierarchical clustering technique (Everitt and Dunn 2001). The similarity index calculated from Dice's coefficient was clustered with the Group Average method, where the dissimilarity between the clusters is determined by average distance between all possible clusters. The quality of this cluster analysis approach was tested by calculating the Cophenetic Correlation Coefficient (CCC). The CCC finds the distortion between the similarity matrix and the dendrogram (Romesberg 1984). A CCC lower than 0.7 indicate that there has been distortion in the clustering process. This would prove the inadequacy of the clustering method to solve the data presented in the original similarity matrix. A CCC value of 0.9 shows a sufficiently good correlation between the data matrix and dendrogram (Mantel 1967, Romesberg 2004).

3.2.4 Microsatellite marker amplification

3.2.4.1 Microsatellite primer pairs

Six microsatellite loci developed for *P. chilensis* and *P. flexuosa* by Mottura *et al.* (2005) were used for the present amplification study. The reproducibility of amplification products were tested with two different DNA extractions of each species. The extractions were carried out on different days so that chances of cross contamination are avoided. PCR reactions on separate days confirmed the robustness of all the microsatellite markers. These primer pairs are highly variable producing well-scorable and reproducible bands. Their sequence details and specific annealing temperature used in the PCR is summarised in Table 3.3

Table 3.3: Sequences and annealing temperature of six microsatellite loci (Mottura *et al.* 2005)

Locus name	Primer sequences (5'-3')	Annealing temperature (T_a)
Mo05	F:AATTCTGCAGTCTCTTCGCC R:GATCCCTCGTGA CTCTCAG	64°C
Mo07	F:GAAGCTCCCTCACATTTTGC R:CTATTTGCGCAACACACAGC	59°C
Mo08	F:TATCCTAAACGCCGGGCTAC R:TCCCATTTCATGCATACTTAAACC	59°C
Mo09	F:ATTCCTCCCTCACATTTTGC R:CATTATGCCAGCCTTTGTTG	59°C
Mo13	F:TTGATTAGAGTTGCATGTGGATG R:TGCAGTCCCAAGTGT CAGAG	58°C
Mo16	F:CATTGCCCCAATATCACTCC R:GGGTCCATCCAGAGTAGTGG	60°C

3.2.4.2 DNA amplification

PCRs were performed with 12.5 µl of 2xGo Taq Green master mix (Promega, UK), 40-120 ng of genomic DNA as the template and 0.6 µM primer in a final volume of 25 µl. The mixture was then mixed gently with two drops of mineral oil placed on top to prevent evaporation during the amplification cycles. The amplification was performed using a Peltier Thermal Cycler-200. The cycling profile is as follows

94°C	-	5 min (initial denaturation)	} 30 cycles
94°C	-	45 s (denaturation)	
58-64°C (primer specific)	-	45 s (annealing)	
72°C	-	45 s (extension)	

3.2.4.3 Resolution of amplification products

The amplified microsatellite markers were resolved by Polyacrylamide Gel Electrophoresis (PAGE) for better separation of bands. PAGE was performed in 6% polyacrylamide gels without urea. The gel was prepared by diluting 30% acrylamide (29% w/v acrylamide-1% w/v bis-acrylamide) with 5x TBE (0.45 M Tris-Borate, 0.01 M EDTA, pH 8.3 in Molecular Biology Grade Water), polymerised with 0.1% TEMED (N, N, N', N'-tetramethylethylenamide) and 0.1% w/v ammonium per sulphate (freshly prepared 10% solution). The gel was run in 1x TBE at a constant voltage of 150 V for 1 h.

3.2.4.4 Analysis of amplified products

After electrophoresis the gels were stained with ethidium bromide, EtBr ($0.5 \mu\text{gml}^{-1}$) for 15 min. It was visualised under ultraviolet light on a BIORAD gel documentation system. Images of the gels were copied and printed for marker analysis. The molecular sizes of amplified products were estimated using the 50 bp DNA step ladder (Promega, UK). The size of the reference standard ranged from 800 to 50 bp. Percentage of transferability of the microsatellite primer pairs were calculated using the formula, Percentage transferability = (No. of species in which amplification occurred / total no. of species) \times 100

3.3 Results

3.3.1 RAPD analysis of *Prosopis* species

3.3.1.1 Primer selection

Randomly amplified polymorphic DNA (RAPD) patterns of nine *Prosopis* species and a hybrid were successfully analysed for their identification and differentiation. Of the 63 arbitrary primers, 25 produced scorable, distinguishable bands and they were selected for subsequent experiments, whereas those which produced less significant or faint bands were excluded. The 25 oligonucleotide primers yielded different polymorphic banding patterns that were unique to each primer and distinguishable over all species analysed. A total of 968 bands were generated by the primers in all the species analysed. The size of the observed markers ranged from 1500 bp to 140 bp.

3.3.1.2 RAPD markers

The *Prosopis* species selected for the study are mostly from the section Algarobia with the exception of *P. tamarugo* from section Strombocarpa. Few markers of taxonomic interest were amplified by the RAPD primers. The primer A08 generated three molecular markers of approximately 1000 bp, 800 bp and 290 bp which are present in all the species of the section Algarobia, but absent in *P. tamarugo*. Similarly a fragment of approximate size 350 bp is amplified in all the species except *P. tamarugo* using primer R18. The RAPD profile of primers A08 and R18 are shown in Figure 3.1a & b. The amplified markers and their size in base pairs can also be seen (arrows shown).

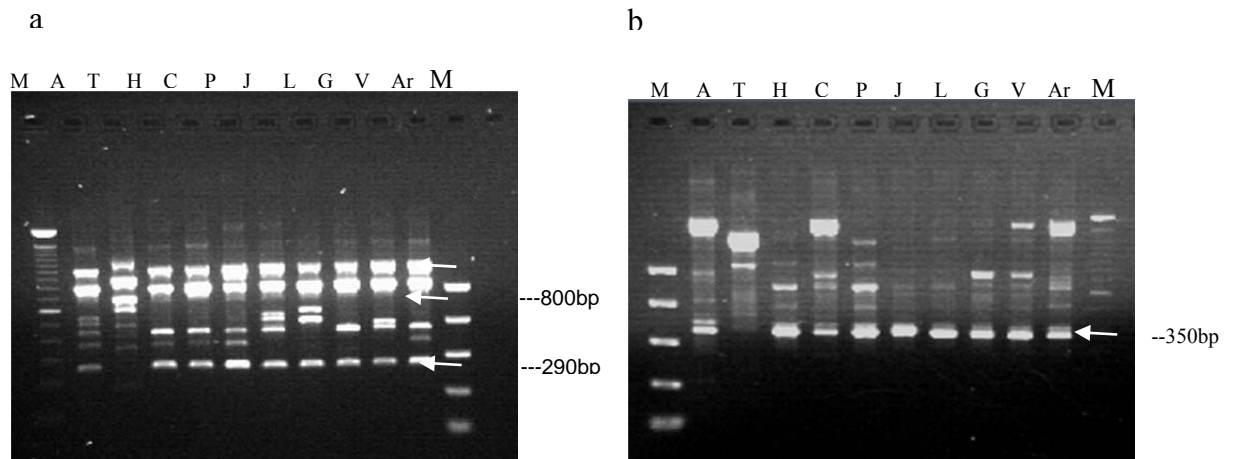


Figure 3.1: (a) RAPD profile of primer A08 and (b) primer R18

Lane names– M, Molecular size marker, A- *P. alba*, T- *P. tamarugo*, H- *P. pallida* × *P. chilensis*?, C- *P. chilensis*, P- *P. pallida*, J- *P. juliflora*, L- *P. laevigata*, G- *P. glandulosa* var. *torreyana*, V- *P. velutina*, Ar- *P. articulata*

A few markers of interest are amplified in the North American species of *Prosopis*. The primer R37 generates a band of size 300 bp (Figure 3.2 a) which is commonly present in the Central American *P. juliflora* and North American species. When the primer S07 was used, another marker of approximate size 550 bp (Figure 3.2 b) was amplified in all the North American species and *P. juliflora*. This primer also amplifies a fragment of approximate size 1100 bp in all the species of section Algarobia (Figure 3.2 b). Interestingly, primer R07 also yields a marker of size 1400 bp (Figure 3.3 a) which is present in the three North American Algarobia species.

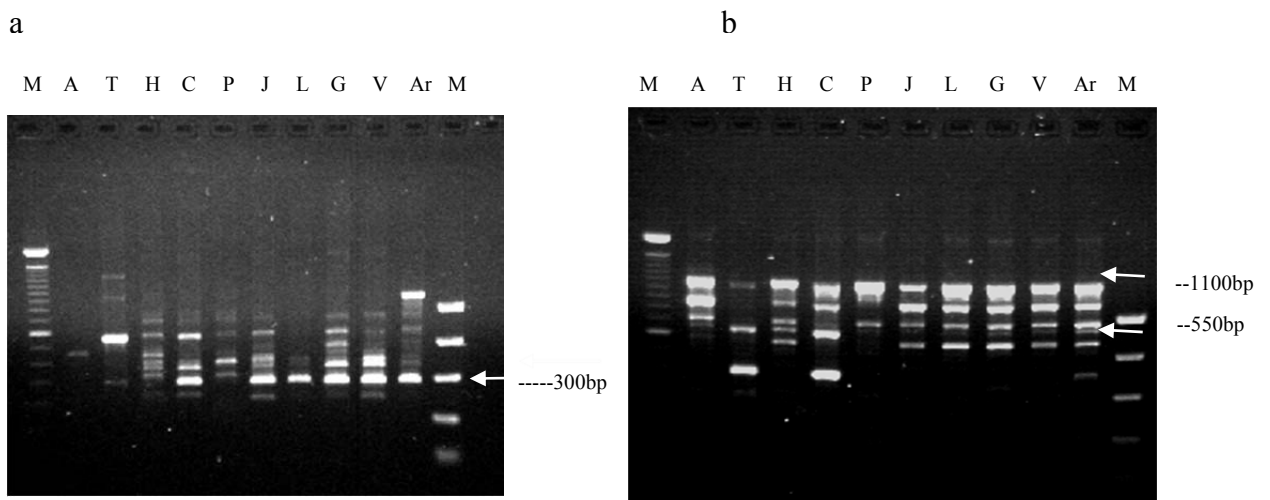


Figure 3.2 (a): RAPD profile of primer R37 and (b): S07

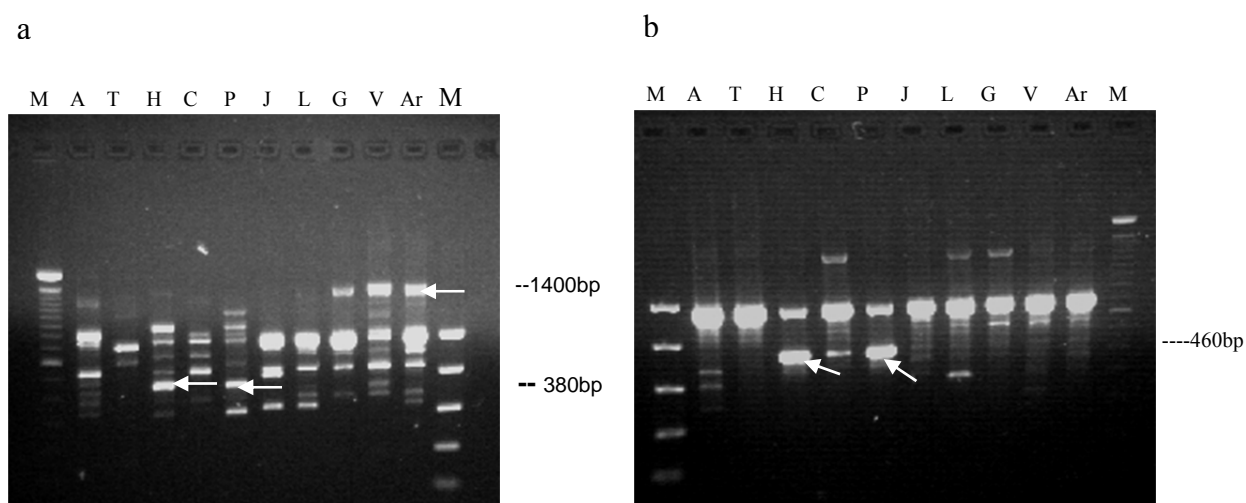


Figure 3.3: (a): RAPD profile of primer R07 and b: S11

Few unique banding profiles were generated in *P. pallida* and the putative *P. pallida* × *P. chilensis* hybrid. A molecular marker of approximately 380 bp is seen in the profile produced by primer R07 which is present in *P. pallida* and putative hybrid (Figure 3.3 a). The primer S11 also yields a 460 bp marker identifying *P. pallida* and the putative hybrid (Figure 3.3 b). RAPD primer N14 amplified fragments of size 1300 bp approximately in *P. pallida* and the hybrid alone (Figure 3.4 a). This fragment is not present in *P. chilensis*. The marker of size 1500 bp (arrow shown) generated by primer R08 (Figure 3.4 b) is only present in the hybrid and *P. pallida*, one of the putative parent of the putative hybrid.

RAPD primer N03 amplified fragments of size 710 bp and 550 bp approximately in *P. tamarugo* (Figure 3.5 a), which are not present in any *Algarobia* species studied. A 900 bp marker can also be seen amplified in *P. pallida* and the hybrid which is absent in other *Prosopis* species (Figure 3.5 a). When amplified with primer R05 there is a

distinctive band of approximately 900 bp in size (arrow shown) present in *P.juliflora* which cannot be seen in any other species (Figure 3.5 b).

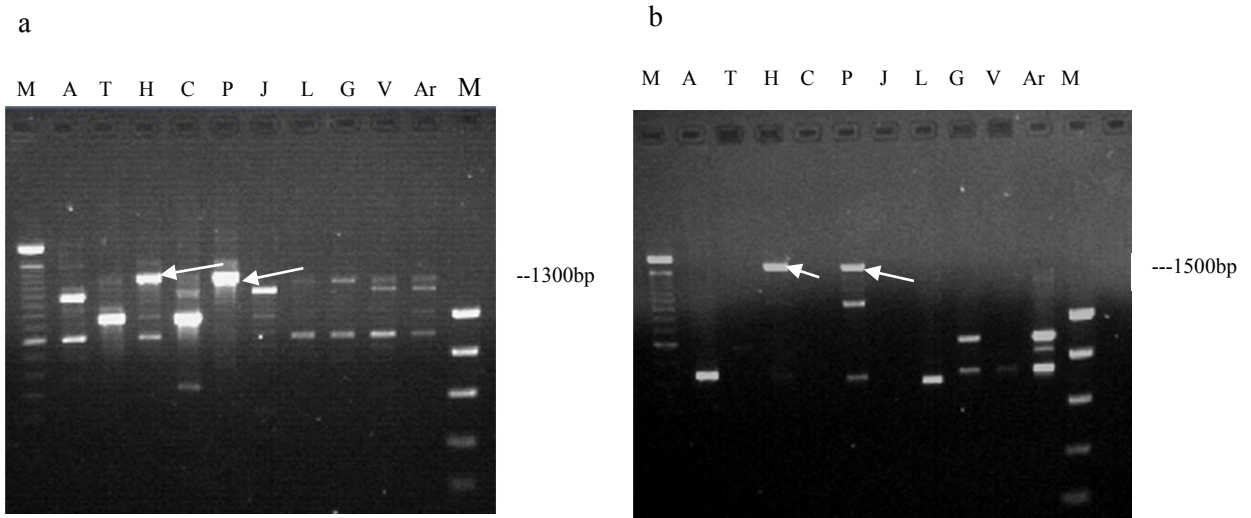


Figure 3.4 (a): RAPD profile of primer N14 and (b): R08

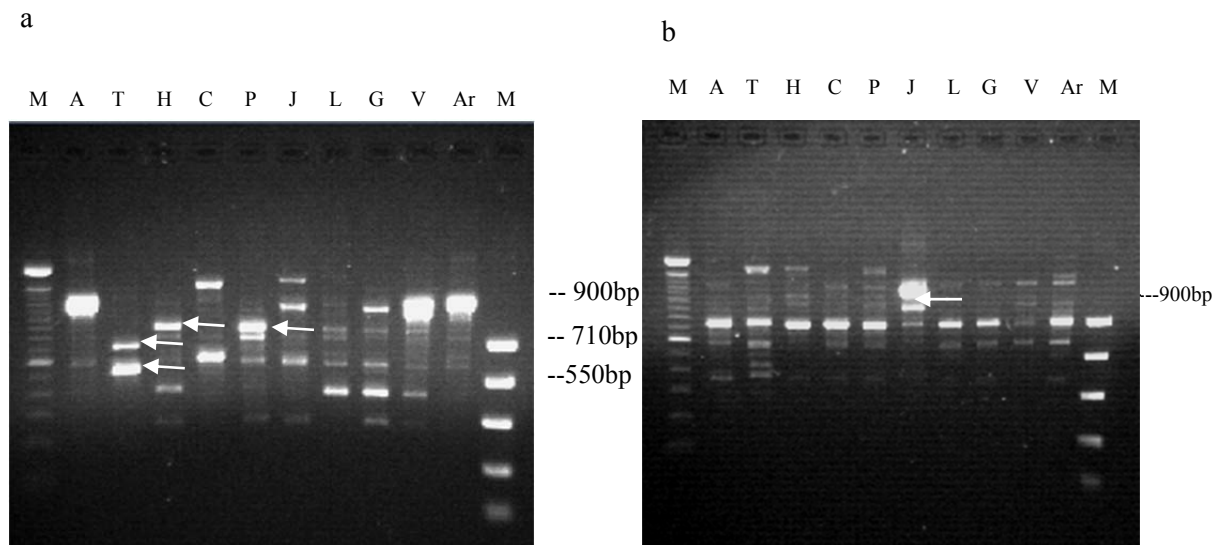


Figure 3.5 (a): RAPD profile of primer N03 and b: R05

Lane names– M, Molecular size marker, A- *P. alba*, T- *P. tamarugo*, H- *P. pallida* × *P. chilensis*?, C- *P. chilensis*, P- *P. pallida*, J- *P. juliflora*, L- *P. laevigata*, G- *P. glandulosa* var. *torreyana*, V- *P. velutina*, Ar- *P. articulata*

The RAPD profile variation among different accessions of *P. juliflora* were analysed to determine intraspecific variation within the species. RAPD profiles of primers N12 (Figure 3.6) and A01 (Figure 3.7) shown below clearly shows that there is very low intraspecific variability within *P. juliflora*.

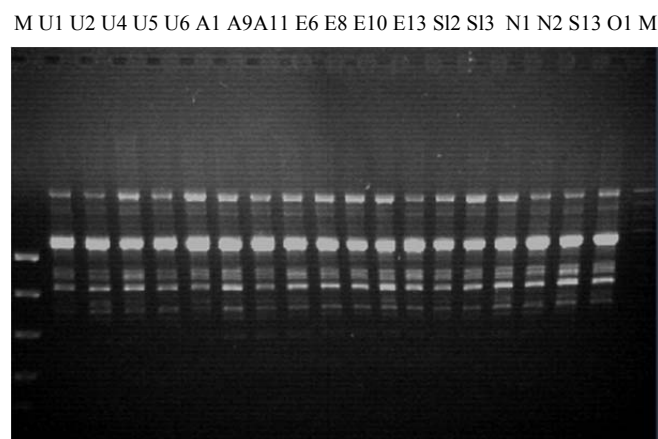


Figure 3.6: RAPD profile of primer N12

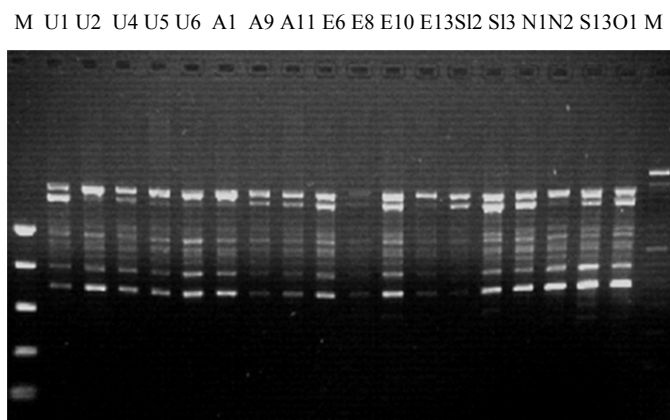


Figure 3.7: RAPD profile of primer A01

Lane names– M, Molecular size marker, U1-U6 (*P. juliflora* acc. from UAE), A1-A11 (acc. from Antigua), E6-E13 (acc. from Ethiopia), S12-S13 (acc. from Srilanka, N1-N2 (acc. from Niger), S13 (acc. from Sudan), O1 (acc. from Oman)

3.3.2 Cluster analysis

The main aim of the cluster analysis was to find the genetic relationship between the tested species of *Prosopis* belonging to section Algarobia. *P. tamarugo* from section Strombocarpa was also selected for comparison between the two sections. The relationship between tetraploid *P. juliflora* and diploid *P. pallida* was analysed closely since the origin of *P. juliflora* has yet to be identified. The group average dendrogram shown in Figure 3.8 identifies four major clusters.

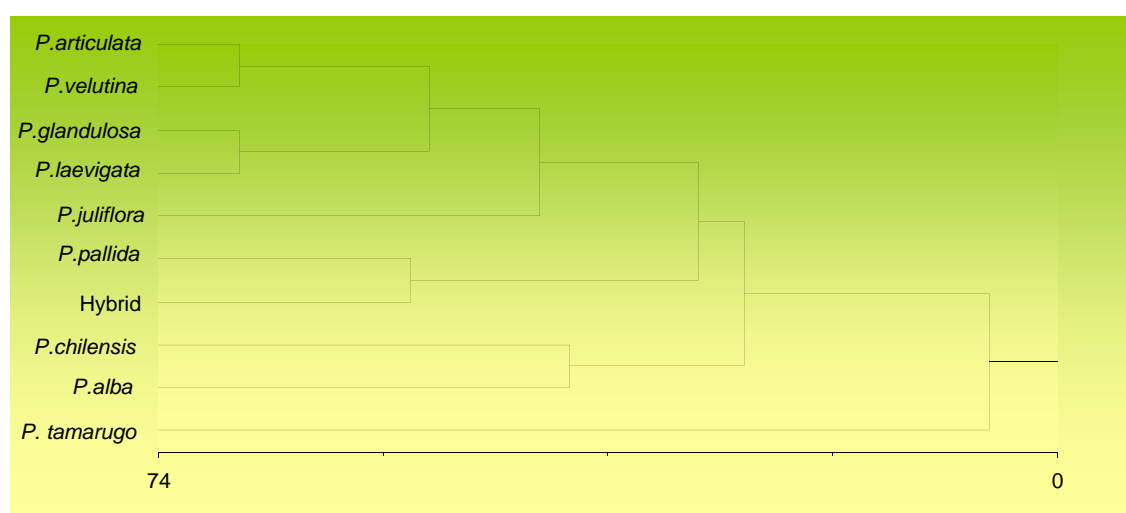


Figure 3.8: A group average dendrogram of 12 *Prosopis* species based on their RAPD profiles.

P. glandulosa and *P. laevigata* with *P. articulata* and *P. velutina* forms the two major clusters. The third major cluster involves *P. pallida* and the putative *P. pallida* × *P. chilensis* hybrid. The fourth cluster involves *P. chilensis* and *P. alba*. Two clusters formed by *P. articulata*/*P. velutina* and *P. glandulosa*/*P. laevigata* show the highest overall similarity with a value of 0.67 in each cluster. These North American species showed a clustering tendency that followed their geographical proximity. *P. juliflora* forms a cluster with four North American species with a similarity index value of 0.425.

P. tamarugo did not cluster with any other species and remained separate. The CCC (Cophenetic Correlation Coefficient) (Sokal and Rohlf 1962) shows a value of 0.938. This indicates a higher correlation and lesser distortion between the actual input data matrix and cluster method.

3.3.3 Similarity index

Dice's similarity indices for all the primers are given in the Table 3.4, ranging from 0 to 0.67.

Table 3.4: Similarity index based on RAPD data between 10 *Prosopis* species

	<i>P. alba</i>	<i>P. tamarugo</i>	Hybrid	<i>P. chilensis</i>	<i>P. pallida</i>	<i>P. juliflora</i>	<i>P. laevigata</i>	<i>P. glandulosa</i>	<i>P. velutina</i>	<i>P. articulata</i>
<i>alba</i>	-									
<i>P. tamarugo</i>	0.03	-								
Hybrid	0.30	0.03	-							
<i>P. chilensis</i>	0.40	0.13	0.30	-						
<i>P. pallida</i>	0.27	0.02	0.53	0.23	-					
<i>P. juliflora</i>	0.18	0.09	0.22	0.18	0.27	-				
<i>P. laevigata</i>	0.17	0.08	0.37	0.21	0.26	0.43	-			
<i>P. glandulosa</i>	0.16	0.09	0.42	0.24	0.27	0.44	0.67	-		
<i>P. velutina</i>	0.32	0.03	0.38	0.30	0.17	0.46	0.53	0.60	-	
<i>P. articulata</i>	0.39	0.00	0.33	0.35	0.26	0.37	0.44	0.49	0.67	-

The lowest genetic similarity was between *P. tamarugo* from section *Strombocarpa* and *P. articulata* from section *Algarobia*, and *P. tamarugo* is clearly distinct from all the other species. The similarity index of *P. tamarugo* with other species falls below 0.2 which demarcate it from the rest of the species of section *Algarobia*.

The highest similarity was shown between the North American species, *P. articulata*, *P. velutina*, *P. glandulosa* and *P. laevigata* with an index of 0.67 between them. Similarly, the index between all the North American species selected in the present study is relatively high showing a strong relation reflected in their geographical proximity. The distance between *P. pallida* and *P. juliflora* is only 0.27 compared with 0.46 between *P. juliflora* and *P. velutina*, and *P. juliflora* appears to be more closely related to the North American species than to the South American *P. pallida* or *P. chilensis*. The putative hybrid shows close relation to *P. pallida* than to *P. chilensis* with similarity indices of 0.53 and 0.30, respectively. This data suggests that *P. pallida* is a putative parent of the putative hybrid.

3.3.4 Cross species amplification of microsatellite markers

Six microsatellite markers developed by Mottura *et al.* (2005) in *P. chilensis* and *P. flexuosa* were selected for cross species amplification in the genus. These markers were successful in the cross amplification of most of the species belonging to section Algarobia analysed in this study, but none of the primer pairs produced amplification in *Prosopis tamarugo* from section Strombocarpa. A summary of the species tested for cross amplification and their amplification pattern is shown in the Table 3.5. The amplification product size ranged from approximately 150 bp to 250 bp, agreeing with previously published results (Mottura *et al.* 2005). The levels of polymorphism among populations were not assessed in this study. Distinctive non-specific bands with high molecular weight than expected were observed with the primer pairs Mo08, Mo09 and Mo16. The percentage transferability of the six primer pairs are shown in Table 3.5. Four out of the six primer pairs showed more than 80% transferability across the 12 *Prosopis* species from two sections of the genus. Of all the primer pairs, Mo08, Mo09

and Mo16 were the most successful in amplifying 12 out of the 13 accessions studied, including the putative *P. pallida* × *P. chilensis* hybrid. The least transferable was the primer pair Mo07.

Table 3.5: Cross-species amplification in 12 *Prosopis* species (one sample per species) using six microsatellite primer pairs developed for *P. chilensis* and *P. flexuosa* (Mottura *et al.* 2005). ‘1’ indicates the presence of fragments in the expected size range, ‘0’ indicates no amplification or unclear bands.

<i>Prosopis</i> species	Microsatellite primer pairs					
	Mo05	Mo07	Mo08	Mo09	Mo13	Mo16
<i>P. alba</i>	0	1	1	1	1	1
<i>P. tamarugo</i>	0	0	0	0	0	0
Hybrid	1	0	1	1	0	1
<i>P. chilensis</i>	1	1	1	1	1	1
<i>P. pallida</i>	1	0	1	1	0	1
<i>P. juliflora</i>	1	0	1	1	1	1
<i>P. laevigata</i>	1	1	1	1	1	1
<i>P. glandulosa</i> var. <i>torreyana</i>	1	1	1	1	1	1
<i>P. velutina</i>	1	1	1	1	1	1
<i>P. articulata</i>	1	1	1	1	1	1
<i>P. caldenia</i>	1	1	1	1	1	1
<i>P. affinis</i>	1	0	1	1	0	1
<i>P. nigra</i>	1	1	1	1	1	1
Percentage transferability	84.62%	61.54%	92.31%	92.31%	69.23%	92.31%

The primer pair Mo05 with an expected allele size of 218 bp produced bands within the expected size range in most the species studied (Figure 3.9). Mo05 failed to produce

amplification in *P. alba* and *P. tamarugo*. Stutter bands could also be seen in some of the species. The stutter bands, which are DNA fragments that are one or several repeats shorter than the actual allele, are by-products of PCR amplification (Weber and May 1989). The primer pairs Mo07 and Mo13 failed to give amplification in some of the species. The expected sizes of their alleles are 197 bp and 228 bp respectively. Mo07 produced bands at approximately 200 bp in eight out of the 12 *Prosopis* species studied here (Figure 3.10).

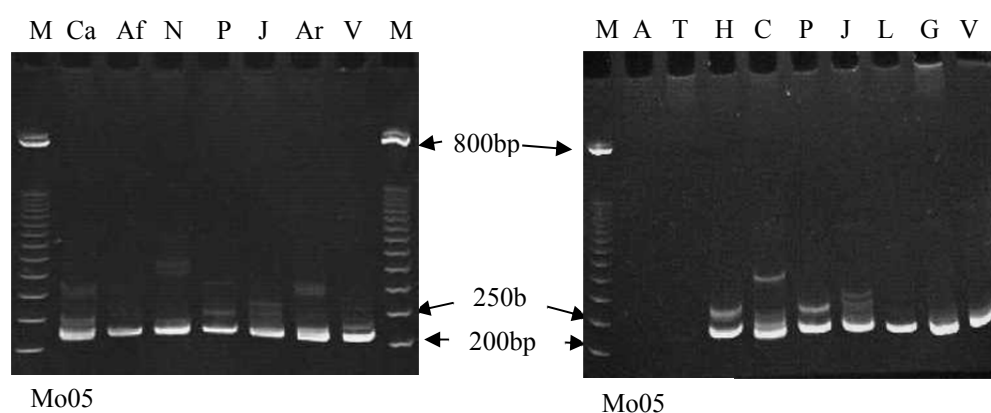


Figure 3.9: Amplification profile of primer pair Mo05

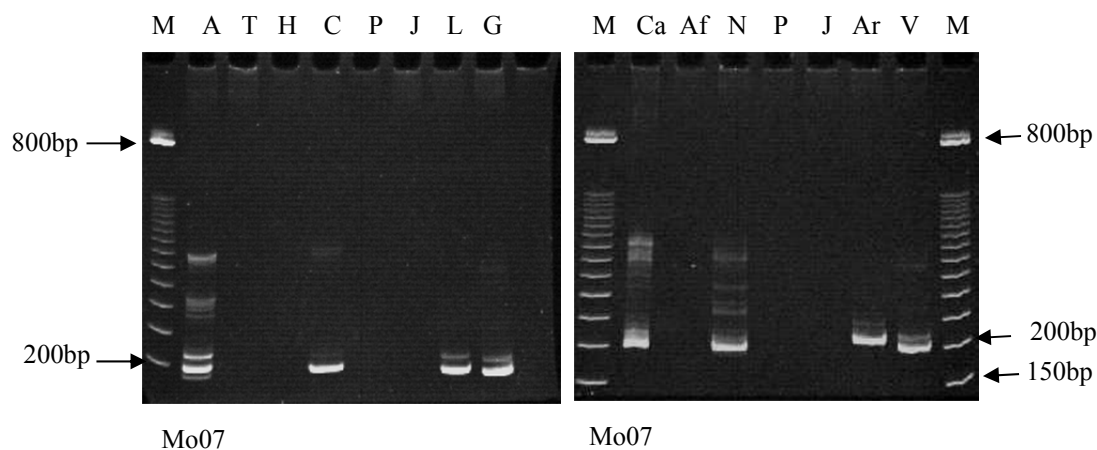


Figure 3.10: Amplification profile of primer pair Mo07

Lane names- M, molecular size marker; A, *Prosopis alba* ; T, *P. tamarugo*; H, putative *P. pallida* × *P. chilensis* hybrid; C, *P. chilensis*; P, *P. pallida*; J, *P. juliflora*; G, *P. glandulosa*; L, *P. laevigata*; Ca, *P. caldenia*; Af, *P. affinis*; N, *P. nigra*; Ar, *P. articulata*; V, *P. velutina*.

The primer pair Mo08 amplified bands in the expected allele size of 218 bp. It failed to produce any amplification in *P. tamarugo*. Although distinct bands are seen amplified in most of the species, presence of multiple bands and stutter bands are evident (Figure 3.11).

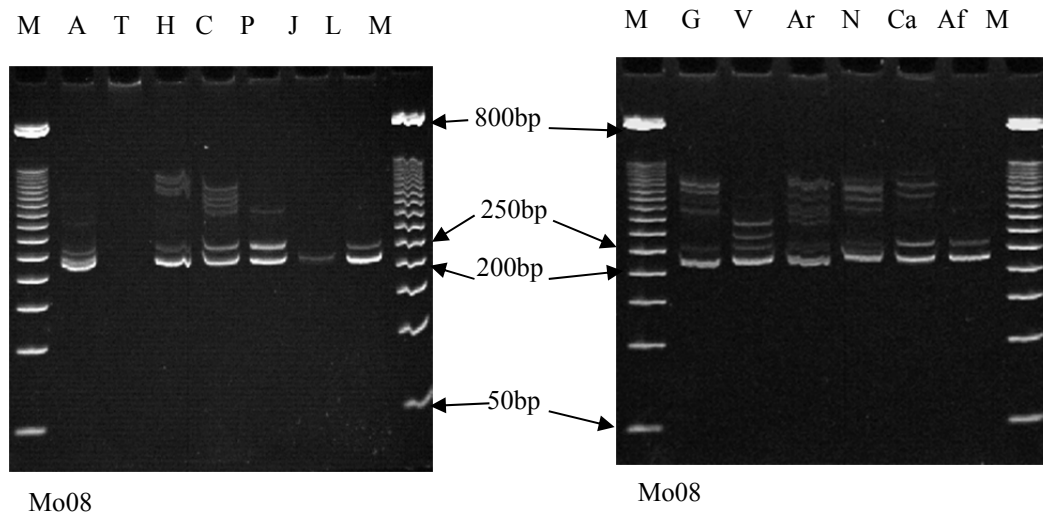


Figure 3.11: Amplification profile of primer pair Mo08

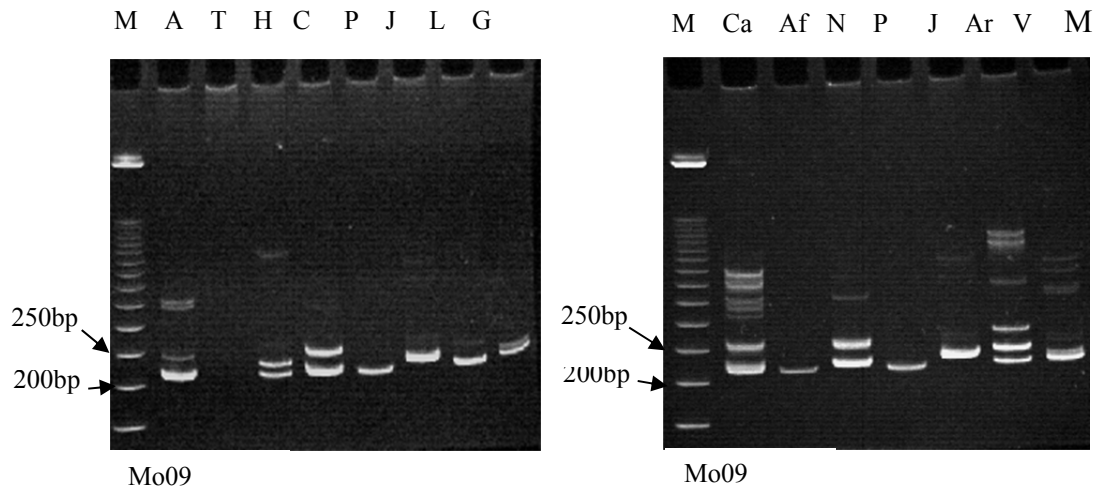


Figure 3.12: Amplification profile of primer pair Mo09

Lane names- M, molecular size marker; A, *Prosopis alba*; T, *P. tamarugo*; H, putative *P. pallida* × *P. chilensis* hybrid; C, *P. chilensis*; P, *P. pallida*; J, *P. juliflora*; L, *P. laevigata*; G, *P. glandulosa*; Ca, *P. caldenia*; Af, *P. affinis*; N, *P. nigra*; Ar, *P. articulata*; V, *P. velutina*

The primer pair Mo09 and Mo16 also failed to produce any amplification in *P. tamarugo*. Their expected allele sizes are 233 bp and 157 bp respectively. Both the primer pairs shows amplification pattern in the expected size range. A number of multiple and stutter bands are also seen in their amplification profile (Figure 3.12 & Figure 3.13).

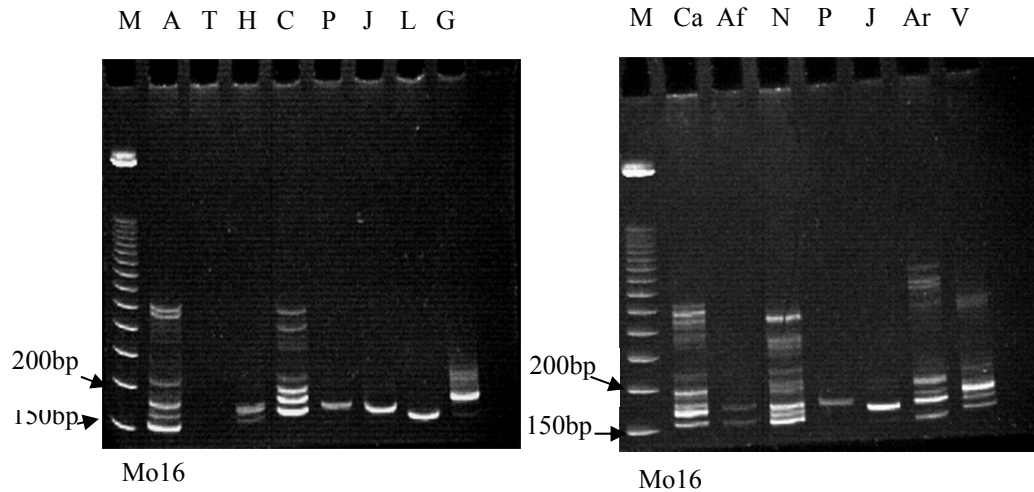


Figure 3.13: Amplification profile of primer pair Mo16

An annealing temperature gradient of 50-60.4°C was applied to measure any significant difference in the banding pattern or stuttering. This experiment in *P. alba* has shown that the annealing temperature did not have an effect on the amplification products (Figure 3.14).

50 50.7 51.6 52.7 54 55.4 56.8 58.1 59.2 60.4
°C °C °C °C °C °C °C °C °C °C

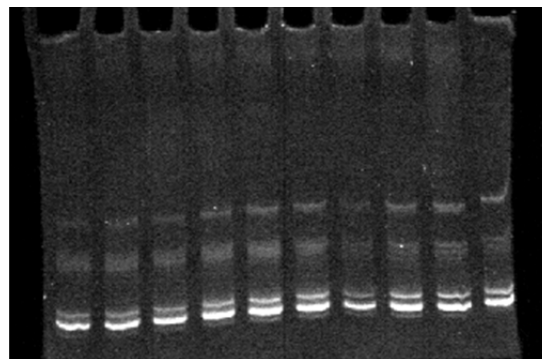


Figure 3.14: Amplification profile of *P. alba* with microsatellite primer Mo07 observed by varying annealing temperature

3.4 Discussion and conclusions

RAPD markers can be used to detect polymorphisms between genotypes. These are 10-mer arbitrary oligonucleotide primers which can amplify segments of genomic DNA in a variety of species. RAPD markers are dominant and are genotype specific detecting even a single nucleotide difference (Williams *et al.* 1990) and have been successfully used for differentiating species within a genus (Thomas *et al.* 2001). In the present RAPD analysis, bulked DNA samples were used, as bulking prior to extraction for RAPD analysis ensures that only the most frequent alleles are favoured during amplification (Michelmore, Paran, and Kesseli 1991). Thus the random arbitrary primers target the frequently occurring template regions in the bulked samples, preventing the amplification of rare sequences.

The present work aimed to identify similarities between the tetraploid *P. juliflora* and diploid *P. pallida* and analyse the genetic relationship between other species in the section Algarobia. Due to their overlapping geographic range and morphological similarities, *Prosopis juliflora* and *P. pallida* have been grouped as a complex (Pasiiecznik *et al.* 2001). Works on ploidy by Harris *et al.* (2003) and Trenchard *et al.* (2008) showed that the two species can be distinguished on the basis of their morphology and ploidy. RAPD markers were also useful in differentiating the two species (Landeras *et al.* 2006). Results obtained here suggest that *P. juliflora* is closer to the four North American species identified in this study than to *P. pallida*.

Landeras *et al.* (2006) identified RAPD markers which are specific to the *P. juliflora* - *P. pallida* complex. These markers could identify the genetic similarity between those species. In the present study three RAPD primers A08, R18 and S07 yielded markers

which were present in all species from Algarobia but absent in *P. tamarugo* from section Strombocarpa. Ramírez *et al.* (1999) described a similar identifiable marker using the primer S07. The group average clustering method separated *P. tamarugo* from the rest of the *Prosopis* species studied. Section Strombocarpa and section Algarobia are considered to be highly differentiated in terms of speciation process and adaptive strategies (Saidman *et al.* 1996). Studies involving pollen grain morphology (Caccavari de Filice 1972) and foliar architecture (Martínez 1984) and seed protein electrophoresis (Burghardt and Palacios 1997) have clearly differentiated species of both sections. Moreover, morphological and biochemical data involving seven species from Strombocarpa suggest that it constitutes a natural taxon (Burghardt and Espert 2007) unlike Algarobia. Isozyme and amino acid analysis also show that the genetic and morphological differences are high among species in section Strombocarpa compared to Algarobia (Carman, Dossaji, and Mabry 1974, Saidman *et al.* 1996). Hence suggestions have been made to treat the two sections as subgenera (Hunziker *et al.* 1986, Saidman *et al.* 1996).

RAPD studies have been useful in the identification of interspecific hybrid formation in the genus (Vega and Hernandez 2005). The putative *P. chilensis* × *P. pallida* hybrid and *Prosopis pallida* shared some common bands when amplified with RAPD primers. The RAPD primer S11 yielded similar size markers in *P. pallida* and the hybrid. A less significant marker can also be seen in *P. chilensis*. In their study using RAPD markers, Landeras *et al.* (2006) identified a similar marker using this primer in all *P. pallida* accessions. These findings suggest that the hybrid is closely related to *P. pallida*. The faint band present in *P. chilensis* cannot be ruled out suggesting that the hybrid may be an interspecific hybrid between the two species as postulated by the

collector. However, there were not many characteristic bands with other RAPD primers to conclusively identify the mother tree. The oligonucleotide primer S11 is found to be reproducible and hence can be used as a species specific marker for identification of *P. pallida* samples.

The putative mother tree, which fruited in abundance, was identified as *P. chilensis* by Nick Pasiecznik (HDRA, UK). In the vicinity of the mother tree, the only other species found flowering was *P. pallida* (Nick Pasiecznik, pers. comm.). Since *Prosopis* species are self-infertile, hybridisation between the two species was assumed (Nick Pasiecznik, pers. comm.). *P. chilensis* occurs naturally in the arid regions of southern Peru, Bolivia, northern and central Chile and northwestern Argentina (Johnston 1962, Burkart 1976, National Academy of Sciences 1979). Although the specimen of *P. chilensis* obtained in this study is from Chile, the species is subject to high genetic variability even within populations (Verzino *et al.* 2003). This variation which results in intermediate morphotypes would be the result of intra specific hybridisation which is common in *Prosopis* species. It could be safely concluded that *P. pallida* is one of the parents of the hybrid, though the status of *P. chilensis* could not be confirmed. In their study using RAPD markers, Landeras *et al.* (2006) suggested this possibility of *P. chilensis* as the other putative parent but their results were also not conclusive. In the group average cluster analysis in this study, *P. pallida* and the hybrid formed a cluster with a similarity index of 0.582. In the group average dendrogram, *P. chilensis* forms a cluster with *P. alba* and their clustering agrees with the result obtained by Ramírez *et al.* (1999).

The highest similarity coefficients in the present study is shown between the North American species, *P. glandulosa* var. *torreyana*, *P. laevigata*, *P. velutina* and *P. articulata*, which range from 0.44 to 0.67. This agrees with the findings of Bessega *et al.* (2000) where isozyme and RAPD studies on populations of *P. laevigata* and *P. glandulosa* proved high genetic similarity between them. Solbrig and Bawa (1975), using isoenzymes found that *P. velutina*, *P. glandulosa* var. *glandulosa* and *P. laevigata* were closely related. The *P. juliflora* sample from the Galapagos Islands shows great affinity to the North American species. The clustering pattern clearly shows that the genetic similarity between *P. juliflora* and the North American species analysed here is higher than the similarity expected with *P. pallida*.

Two of the North American species selected here, *P. glandulosa* and *P. laevigata*, are from their native ranges. The outcrossing nature of *Prosopis* species could contribute genetic variability among them. The similarity coefficients between *P. juliflora* and North American species ranges from 0.37 to 0.67, which is slightly less than the values previously reported (Juárez-Muños, Carrillo-Castaneda, and Rubluo 2006). Their study also suggests that *P. laevigata* and *P. glandulosa* are closely related with a similarity index of 0.79. However, the similarity index values between *P. glandulosa* and *P. velutina* is considerably less than the value of 0.60 obtained in this study. Enzyme marker studies on *Prosopis* by Bessega *et al.* (2005), suggest that *P. velutina* and *P. glandulosa* are so genetically distinct that they would have originated from two independent founder events.

Present work on RAPD markers proved to be helpful in clarifying the identity of the *Prosopis* species selected in this study. The origin of tetraploid *P. juliflora* still

remains unanswered but this study has attempted to solve some of the questions regarding its relation to *P. pallida*. It is not known whether *P. juliflora* is an allotetraploid involving *P. pallida* or an auto tetraploid. The *P. juliflora* sample from the Galapagos Islands could possess some variation with respect to the ones from the rest of the native range, although the intraspecific variation shown by *P. juliflora* accessions for two RAPD primers were very low. It should also be noted that the *P. juliflora* from Galapagos could be more related the same species from Pacific coastal region from Sinaloa, Mexico to Panama, which Palacios (2006) proposed to be raised to a species rank, as *P. vidaliana* A. Náves. The evidence provided seems valid enough to conclude that the two species are genetically dissimilar. The differentiation of *P. pallida* and *P. juliflora* based on ploidy and morphology (Harris *et al.* 2003, Trenchard *et al.* 2008) corresponds with the RAPD data obtained in the present study, although more accessions of each species from their native ranges would give a more conclusive result. Recent study by Palacios *et al.* (2011) utilising the morphological characters of the seedlings along with AFLP analysis of accessions of *P. juliflora* and *P. pallida* has shown differentiation between the two species (Palacios *et al.* 2011). In their work, the occurrence of *P. juliflora* along the coasts of Ecuador and Peru are disregarded, although a clear distinction of *P. juliflora* in the Caribbean basin, Colombia, and Venezuela from the Peruvian-Ecuadorian *P. pallida* was obtained (Palacios *et al.* 2011).

The clustering pattern of the *Prosopis* species selected in this work does not agree with the morphological criteria proposed by Burkart (1976), since species of the same series are not clustered together. There seems to be incongruence between the morphological and molecular data in the case of *Prosopis* species analysed here, and

similar inconsistency was also observed in previous studies (Saidman and Vilardi 1987, Bessega, Saidman and Vilardi 2005, Bessega, Vilardi, and Saidman 2006, Bessega *et al.* 2011).

A total of six previously published microsatellite primer pairs developed in *P. chilensis* and *P. flexuosa* were tested for transferability in 12 *Prosopis* species. Except for *P. tamarugo*, all the other species are from section Algarobia of the genus. All the six microsatellite primer pairs were transferable in most of the Algarobia species, but failed to produce a single amplification in *P. tamarugo*. Mottura *et al.* (2005) tested these six primer pairs for cross-species amplification in seven species of *Prosopis* from the two sections Algarobia and Strombocarpa. Nine additional species including *P. tamarugo* was included in the present transferability study as compared to Mottura *et al.* (2005). In their study, at least three primer pairs gave amplification in all the species tested, but the rate of amplification was comparatively less in Strombocarpa than in Algarobia. Based on the present evidence on microsatellite transferability it cannot be concluded that these markers could be used to differentiate between different sections of the genus. However, the failure of amplification could be due to the lack of any conserved primer binding sites in the DNA for amplification to occur (Weising *et al.* 2005). Any intraspecific sequence variation in the primer flanking sites also could interfere with primer binding (Angers and Bernatchez 1997, Colson and Goldstein 1999). In a study by Butcher *et al.* (2000), the microsatellite primers developed in *A. mangium* failed to show any amplification in species from a different subgenus but produced high rates of amplification in species of the same section. Transferability of SSR markers were also observed among distant taxa (Yasodha *et al.* 2005).

In some species, an intense band can be seen with relatively less intense ones above it. Although there are stutter bands or multiple bands present in each locus, the microsatellite markers were consistent in amplification under the same PCR conditions and all of the bands are scorable. The non-specific bands observed in some of the species at the microsatellite loci is likely due to the primer mismatch positions from the *Prosopis* species DNA analysed here. Formation of stutter bands or multiple bands is common while amplifying SSR markers between species, but this can be reduced by changing the annealing temperature (Rossetto 2001). However, change in annealing temperature did not vary the banding profile observed with *P. alba* in the present study. These bands could interfere with the targeted locus and completely or partially prevent their amplification (Primmer, Moller, and Ellegren 1996). Cloning experiments have shown that these non-specific or stutter bands have one or few repeat units missing (Luty *et al.* 1990).

The high percentage of transferability of primers across *Prosopis* species indicates high level of sequence similarity in the DNA sequences flanking microsatellites. Four out of the six primers gave a transferability percentage of more than 80% and showed robustness in subsequent amplification reactions. The higher cross amplification rate of microsatellites observed in this study would help in the genetic evaluation of a broad range of species in this genus and would provide information regarding their mating system and gene flow.

To conclude, RAPD and microsatellite transferability studies in these *Prosopis* species proved helpful in clearly demarcating the species belonging to two different

sections, Algarobia and Strombocarpa. Perhaps analysis of more species from Strombocarpa could confirm the separation. Moreover the relationship between different species of section Algarobia has also been identified through this study. The *P. juliflora* and *P. pallida* samples analysed in this study did not seem to be genetically related. This preliminary study suggests that within section Algarobia the grouping of series seems artificial which agrees with many other studies, though the fact that seeds of few of the *Prosopis* species were not collected from their native range should also be considered while interpreting the results, and that *Prosopis* species are susceptible to genetic variation due to their protogynous nature, out breeding and environmental factors. Molecular marker analysis of a wide range of species from their native range and the many sections could help to resolve the taxonomic confusion surrounding this genus and would help in the proper exploitation of economically important species.

4. PHYLOGENETIC RELATIONSHIP BETWEEN *PROSOPIS JULIFLORA*, *PROSOPIS PALLIDA* AND SELECTED SPECIES OF *PROSOPIS* INFERRED FROM CHLOROPLAST TRNL-TRNF INTERGENIC SPACER SEQUENCES

4.1 Introduction

Chloroplast DNA (cpDNA) is widely conserved among vascular plants (Palmer and Stern 1986) and this conservative evolution of the chloroplast genome has its advantages in a phylogenetic study. When inferring phylogenies of plant species, cpDNA sequencing has been the most common approach (Clegg and Zurawski 1992) and a large subunit of the ribulose-1, 5-bisphosphate carboxylase/oxygenase (*rbcl*) gene in the cpDNA has been widely used in such studies (Freshwater *et al.* 1994, Doyle *et al.* 1997, Zhang, Fritsch and Cruz 2009). The *rbcl* gene is particularly useful in resolving the phylogeny of higher taxonomic levels (family and above) (Zurawski, Clegg, and Brown 1984, Levin *et al.* 2003). However, at intrafamilial level, rapidly evolving genes such as the non-coding *trnL* (UAA) intron and the intergenic spacer between the *trnL* (UAA) 3' exon and the *trnF* (GAA) gene have proven more suitable than the *rbcl* gene (Clegg 1993, Gielly and Taberlet 1994). These genes have long been used to resolve the phylogenetic status of taxonomically complex species because of their fast rate of evolution and conserved flanking primer sites (Soltis and Soltis 1998). The intergenic spacer region *trnL-trnF* has also been informative in the Mimosoideae subfamily (Leguminosae) (Murphy, Udovicic, and Ladiges 2000, Catalano *et al.* 2008). Taberlet *et al.* (1991) first suggested the use of universal primers for the amplification of non-coding *trn* intron sequences. In their studies they showed that these primers can be successfully used for the amplification within a

broad taxonomic range of plants from algae to bryophytes, vascular cryptograms, gymnosperms and angiosperms. Phylogenetic tools have resulted in resolving the taxonomy of many disputed species over the last decade (Harrison, Kwan, and Johnston 2005, Luckow *et al.* 2005). Since molecular data are not affected by environment or plant developmental stages unlike morphological data, phylogenetic studies based on them have been reliable in differentiating species with confused taxonomy (Baldwin *et al.* 1995, Amundsen and Warnke 2012). Ploidy difference among species or their geographical distribution also does not seem to affect chloroplast based phylogeny (Bukhari, Koivu, and Tigerstedt 1999).

There are several advantages in using cpDNA regions, particularly the non-coding regions. The double stranded cpDNA can be easily amplified in wide taxonomic range of plants, the smaller size of the non-coding region enables the sequencing of the whole region using only the amplification primers and these regions can be successfully used in conjunction with other fast evolving coding or non-coding regions to deduce the phylogenetic relationship at intrageneric level (Gielly and Taberlet, 1994). RAPD markers are dominant, nuclear DNA based markers which are widely used in genetic diversity studies (Esselman *et al.* 2000, Rasul, Hiramatsu, and Okubo 2007). These PCR based markers have high frequency of polymorphism and no prior sequence information is required. With RAPD, it is possible to screen large number of taxa very rapidly thus enabling rapid comparison among species (Friesen *et al.* 1999). However, it can also miss most of the small amount of divergence as can be seen from the sequence comparison of trnL-trnF intergenic spacer region. In this case, cpDNA sequencing has the advantage of finding more sequence variation between species. The mode of inheritance in these two markers i.e., the trnL-trnF intergenic

spacer marker and RAPD marker are different and thus have different evolutionary histories (Palmer 1985). Nuclear genome is inherited in the Mendelian fashion while the chloroplast genome is mostly maternally inherited (Palmer 1985).

Non-coding cpDNA regions are beneficial in low-level systematic studies since they evolve faster than coding regions and have higher no. of variable characters than coding regions (Gielly and Taberlet 1994, Asmussen and Chase 2001, Shaw *et al.* 2005). Due to the near universal nature of the primers, these conserved regions of the tRNA gene have become most widely used non-coding cpDNA sequences in plant systematics (Taberlet *et al.* 1991, Soltis and Soltis 2000b, Bellstedt *et al.* 2001, Makarevich *et al.* 2003, Shaw *et al.* 2005). Chloroplast DNA sequencing allows to detect sequence polymorphisms between species and are advantageous for detecting small DNA changes such as single base substitutions, deletion, insertions at various positions in the non-coding DNA fragments (Hamilton, Braverman, and Soria-Hernanz 2003).

Molecular and numerical taxonomic studies in the past have resulted in a better understanding of the relationships between species in the genus *Prosopis* (Saidman *et al.* 1996, Ramírez *et al.* 1999). Phylogenetic relationships within the genus have long been an area of debate and a taxonomic revision of the genus has been suggested by many authors (Ramírez *et al.* 1999, Landeras *et al.* 2006, and Burghardt and Espert 2007). *P. juliflora* and *P. pallida* are useful tree resources in arid and semi arid regions but are similar morphologically and were considered to hybridise easily contributing to their difficulty in identification and proper management (Pasicznik *et al.* 2001). Detailed studies on the phylogenetic relationship with other species is

required to develop conservation and management strategies for these economically important *Prosopis* species, since chloroplast DNA analyses have enabled the advancement of strategies for the conservation of tree species (Newton *et al.* 1999). However, the current taxonomy remains largely unclear and the morphological similarity between *P. juliflora* and *P. pallida* has also resulted in adding to the confusion in their identification (Pasiiecznik *et al.* 2001). Hence it is important to clarify the relationship between these two species and also their relation with other species in the genus. In the present study the utility of a non-coding intergenic spacer in chloroplast DNA (cpDNA) in inferring the phylogenetic relationships among *P. juliflora* and *P. pallida* and selected other *Prosopis* species was explored. Eight of the species were from the series Chilenses and Pallidae of section Algarobia. The species from series Chilenses in the present study are *P. juliflora*, *P. chilensis*, *P. laevigata*, *P. glandulosa*, *P. alba* and *P. velutina*. The other species *P. articulata* and *P. pallida* are from the series Pallidae. The only species from the section Strombocarpa, *P. tamarugo*, is from the series Cavanicarpae. Section Strombocarpa consists of nine species each with distinguishable morphological and biochemical characteristics (Burkart 1976, Saidman *et al.* 1996). Interspecific hybridisation is not common in this section and isozymic studies have shown intraspecific variation is low compared to section Algarobia (Saidman *et al.* 1996). A putative *P. chilensis* × *P. pallida* hybrid was also analysed in this study. Landeras *et al.* (2006), in their study using RAPD markers, identified *P. pallida* as one of the parents of the above hybrid although the position of *P. chilensis* is ambiguous. Chapter 3 analysed in detail the relationship between the putative hybrid and its supposed parents.

The main objective was to determine whether a difference in the cpDNA intron region existed in *P. juliflora* and *P. pallida*, thus identifying potential informative regions of chloroplast DNA for phylogenetic studies. The present work also contributes to a preliminary assessment of relationships of selected *Prosopis* at a species level. This study also assesses if *P. pallida* and *P. chilensis* are the parents of putative *P. pallida*×*P. chilensis* hybrid.

4.2 Materials and methods

4.2.1 Plant materials

The details of *Prosopis* species selected for chloroplast DNA sequencing are given in section 3.2 of Chapter 3 (Page 69). *P. affinis*, *P. caldenia* and *P. nigra* were selected only for microsatellite transferability studies. Refer to the list of the species and sources given in Table 3.1 (Chapter 3, page 77).

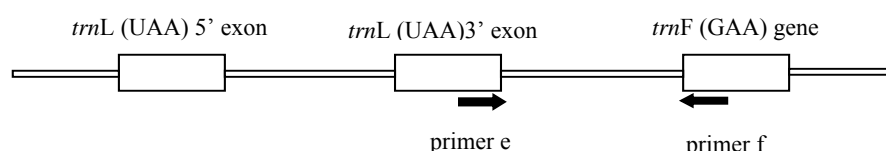
4.2.2 DNA extraction, amplification and sequencing

Refer to section 3.2.2 of Chapter 3 (Page 78) for DNA extraction methodology.

4.2.3 PCR amplification

All the nine accessions of *Prosopis* were used for the amplification of the trnL-trnF intergenic spacer region in the cpDNA. Amplification was achieved using the forward and reverse universal primers, trnL-trnF e (5'-GGTTCAAGTCCCTCTATCCC-3') and trnL-trnF f (5'-ATTTGAACTGGTGACACGAG-3') (Figure 4.1), described in Taberlet *et al.* (1991). Polymerase chain reactions (PCR) were performed with GoTaq Green master mix which consists of GoTaq DNA polymerase supplied in a green GoTaq reaction buffer (pH 8.5), 400 µM of each dNTP, and 3mM MgCl₂ (Promega, UK). 40-120 ng of genomic DNA were used as the template and 0.6 µM of forward and reverse primer in a final volume of 50 µl. The mixture was then mixed gently and two drops of mineral oil was dropped on top to prevent evaporation during the amplification cycles. The amplification was performed on a thermal cycler (Eppendorf mastercycler, UK). For each PCR, the following sequence was used: initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C

for 1 min, annealing at 42°C for 1 min and extension at 72°C for 1 min. The final primer extension was done at 72°C for 5 min. The PCR products were cleaned using QIAquick PCR purification kit (Qiagen, UK) to remove excess template, primer-dimers and oligonucleotides. The purified products were then directly used for sequencing reactions.



primer e (5'-GGTCAAGTCCCTCTATCCC-3')
 primer f (5'-ATTTGAACTGGTGACACGAG-3')

Figure 4.1: Position of the amplified sequences and the primers used

4.2.4 Sequencing of the trnL-trnF region

The purified PCR products were visualised with agarose gel electrophoresis and the approximate band lengths were determined. For PCR products of length 100-200 bp, 3 ng of template was used and for products with length 200-500 bp, 10 ng of template was used for the cycle sequencing, and 3.2 pmols of either the forward or reverse primer were used in a total reaction volume of 10 μ l. Sequencing reactions were carried out with the BigDye Terminator cycle sequencing kit (Applied Biosystems, UK). The PCR conditions for cycle sequencing consisted of 25 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec and extension at 60°C for 4 min. After the purification of templates, they were analysed using the ABI PRISM 3700 DNA Analyser (Applied Biosystems, UK).

4.2.5 Outgroup selection

Outgroup selected for the phylogeny reconstruction should be multiple taxa of a closely related sister group of the ingroup selected (A-Rong *et al.* 2010). An outgroup roots the tree (Maddison, Donoghue, and Maddison 1984) and the topology of the ingroup tree (species tree) can be influenced by the outgroup (Milinkovitch and Lyons-Weiler 1998). Rooting the phylogenetic tree with an outgroup taxa examines the direction in which the trait change occurred when no direct evidence of ancestor-descendant relationship is found (Harrison and Langdale 2006). *Piptadenia floribunda* Kleinhoonte was selected as the outgroup taxa to root the tree. Both *Prosopis* and *Piptadenia* belong to the subfamily Mimosoideae of the tribe Mimoseae. Similarity search using BLAST software (Altschul *et al.* 1990) identified the *Piptadenia floribunda* used in this study to have trnL-trnF sequence homology with other database sequences (E-value 0.0).

4.2.6 Pairwise analysis using Dotlet

The intergenic spacer region trnL-trnF sequence of *P. pallida* and the putative *P. pallida*×*P. chilensis* hybrid was compared using the program DOTLET (Junier and Pagni 2000). The pairwise similarity of the two sequences can be analysed from the resulting graphic representation or Dotplot. Only these two samples were analysed using Dotplot to deduce the percentage of similarity.

4.2.7 Multiple sequence analysis

Nine species of *Prosopis* and the putative *P. pallida*×*P. chilensis* hybrid were included in this analysis and the majority of the species represented the section Algarobia of the genus. The trnL-trnF intergenic spacer sequence for *Piptadenia*

floribunda was downloaded from Genbank and used for multiple sequence alignment along with other *Prosopis* species for comparison.

Multiple sequence alignment was built using ClustalW (Higgins *et al.* 1994) and edited using Jalview (Clamp *et al.* 2004, Waterhouse *et al.* 2009). Gap open and gap extension penalties were set at the default values of 15 and 6.66 respectively. The multiple alignment output was shaded according to their level of conservation using Boxshade. The ClustalW multiple alignment was edited for building the phylogenetic tree using Jalview so that only the regions which were unambiguous were selected for the phylogenetic tree construction. The poorly conserved N-terminus and C-terminus and also the internal gap rich regions were removed to improve the suitability of the alignment.

4.2.8 Phylogenetic tree

A rooted phylogenetic tree for the species of *Prosopis* in the present study was constructed using the phylogeny programs in the PHYLIP Phylogeny Inference Package (version 3.6, Felsenstein 1989). The distance matrix of the nucleotide was computed using the Phylip program DNAdist. A table of similarity between the nucleotide sequences was also computed. The distance between two species is proportional to the total branch length between them. The Kimura (1980) model of nucleotide substitution is used. The Kimura '2-parameter' model allows for a difference between transition and transversion rates. Each distance that is calculated is an estimate of the divergence time between the two species.

A bootstrap (Felsenstein 1985) was performed before the distance analysis by choosing the bootstrap option in the Phylip DNAdist program. To estimate the support for individual clades, bootstrapping using 1000 replicates was performed. This assesses the reliability or quality of each branch in the consensus tree. The Neighbor joining method was then used to estimate the phylogeny of the species from the distance matrix. The CONSENSE program generated the rooted consensus phylogenetic tree based on the majority rule method. Finally the tree was viewed with the phylogenetic tree printing software, Phylodendron (Gilbert 1999).

4.3 Results

4.3.1 Sequence analysis

Total length for the trnL/F sequence ranged from 162 to 472 bp within *Prosopis* species and 475 bp in the outgroup *Piptadenia floribunda*. Dotlet program comparison of *P. pallida* and the hybrid trnL/F sequences by diagonal plot method revealed that above 70% of the residues are identical. This result proves that they are closely related. The trnL-F intron region in all the species was aligned using ClustalW. Many gaps were introduced to make the multiple alignment more meaningful. Boxshade was used to generate a shaded display of the alignment (Figure 4.2). Various kinds of shading applied to the alignment helps in its meaningful interpretation. The consensus sequences are also shown in the alignment output. Twenty-two indels (insertions/deletions) occur in the trnL/trnF intergenic spacer region analysed in this study. The majority of the indel events are insertions or deletions of duplicated or transformed adjacent sequences (Table 4.1). A 318 bp deletion was observed in the *P. pallida* and hybrid trnL/F region but not in other species.

4.3.2 Phylogenetic analyses

The trnL-trnF sequences from all the nine *Prosopis* species and the hybrid were aligned using the Clustal W software. This allowed suitable phylogenetic analyses of the species. A Neighbor joining phylogenetic tree was constructed using the PHYLIP phylogeny package (Felsenstein 1989). *Piptadenia floribunda* is treated as the out group and the tree was rooted with this species. The phenogram shows the genetic relation between the species of *Prosopis* considered in this study (Figure 4.3). In this phenogram, the relationships among the species are well resolved. It is quite evident

that the putative *P. pallida*×*P. chilensis* hybrid and *P. pallida* share very similar sequences but *P. chilensis* is grouped with the North American species, *P. laevigata* and *P. velutina*. In fact, the distance between them and *P. juliflora* and *P. articulata* are the same. *P. glandulosa* forms a cluster with *P. pallida* and the putative hybrid. However all these species can be grouped into a single clade. *P. alba* belonging to the series Chilenses does not group with any other species. The phenogram does not resolve the division of Algarobia into series. *P. tamarugo* from Strombocarpa is also found to be distantly related to the other *Prosopis* species analysed in this study.

```

chil/1-470 1 --- CAAGCCGNTTT ACTCTCTA TTATTTATCCTATATCCTCTTTTTTTTTT AGTGGT
velu/1-468 1 --- CAGGCCGTT --- ACTCTCTA TTATTTATCCTATATCCTCTTTTTTTTTT AGTGGT
juli/1-472 1 --- CAAGCCGNTTT ACTCTCTA TTATTTATCCTATATCCTCTTTTTTTTTT AGTGGT
arti/1-471 1 --- CAAGCCCGTTT ACTCTCTA TTATTTATCCTATATCCTCTTTTTTTTTT AGTGGT
glan/1-470 1 --- CANGCC-GTTT ACTCTCTA TTATTTATCCTATATCCTCTTTTTTTTTT AGTGGT
alba/1-468 1 --- CAAGCC-GTTT ACTCTCTA TTATTTATCCTATATCCTCTTTTTTTTTT AGTGGT
laev/1-465 1 --- CAAGCCGTT --- ACTCTCTA TTATTTATCCTATA CCTCTTTTTTTTTT AGTGGT
tama/1-464 1 --- CAAGCC-GTTT ACTCTCTA TTATTTATCCTATATCCTCTTTTTTTTTT AGTGGT
Pipt/1-475 1 CAAAAGGCCCGTTT ACTCTCTA TTATTTATCCTATATCCTCTTTTTTTTTT AGTGGT
hybr/1-162 1 -----
pall/1-164 1 -----
consensus 1 caagcc ttt actctcta ttatztatcctatatacctctTTTTTTTTT agtggT

chil/1-470 55 TCCAAATTCGTTATGTTTCTTATTCACTATTCTTTTCACAAACGGATCTGAGTGGAATT
velu/1-468 53 TCCAAATTCGTTATGTTTCTTATTCACTATTCTTTTCACAAACGGATCTGAGTGGAATT
juli/1-472 56 TCCAAATTCGTTATGTTTCTTATTCACTATTCTTTTCACAAACGGATCTGAGTGGAATT
arti/1-471 56 TCCAAATTCGTTATGTTTCTTATTCACTATTCTTTTCACAAACGGATCTGAGTGGAATT
glan/1-470 55 TCCAAATTCGTTATGTTTCTTATTCACTATTCTTTTCACAAACGGATCTGAGTGGAATT
alba/1-468 54 TCCAAATTCGTTATGTTTCTTATTCACTATTCTTTTCACAAACGGATCTGAGTGGAATT
laev/1-465 52 TCCAAATTCGTTATGTTTCTTATTCACTATTCTTTTCACAAACGGATCTGAGTGGAATT
tama/1-464 55 TCCAAATTCGTTATGTTTCTTATTCACTATTCTTTTCACAAACGGATCTGAGTGGAATT
Pipt/1-475 59 TCCAAATTCGTTATGTTTCTTATTCACTATTCTTTTCACAAACGGATCTGAGTGGAATT
hybr/1-162 1 -----
pall/1-164 1 -----
consensus 61 tccaaattcgttatgtttcttattcattctattctttcaciaaacggatctgagtggaatt

chil/1-470 115 TTGCTTTTTCTTATCACAA ---- GTGTGGAATATGTAATGGAATATATATGATAGACG
velu/1-468 113 TTGCTTTTTCTTATCACAA ---- GTGTGGAATATGTAATGGAATATATATGATAGACG
juli/1-472 116 TTGCTTTTTCTTATCACAA ---- GTGTGGAATATGTAATGGAATATATATGATAGACG
arti/1-471 116 TTGCTTTTTCTTATCACAA ---- GTGTGGAATATGTAATGGAATATATATGATAGACG
glan/1-470 115 TTGCTTTTTCTTATCACAA ---- GTGTGGAATATGTAATGGAATATATATGATAGACG
alba/1-468 114 TTGCTTTTTCTTATCACAA ---- GTGTGGAATATGTAATGGAATATATATGATAGACG
laev/1-465 112 TTGCTTTTTCTTATCACAA ---- GTGTGGAATATGTAATGGAATATATATGATAGACG
tama/1-464 115 TTCTTTTTCTTATCACAA ---- GTGTGGAATATGTA ---- ATATATATGA ---- CG
Pipt/1-475 119 TTCTTTTTCTTATCACAA CACAA GTGTGGAATATGTAATGGAATATATATGATAGACG
hybr/1-162 1 -----
pall/1-164 1 -----
consensus 121 ttgctTTTTcttatcaciaa gtgttggaaatgtaatggaatataatgatagacg

chil/1-470 170 TAAAATTTGGTTTCTATATGATAAACGTACAAATGAGCATCTTATCTTTGAGCAAGGAAT
velu/1-468 168 TAAAATTTGGTTTCTATATGATAAACGTACAAATGAGCATCTTATCTTTGAGCAAGGAAT
juli/1-472 171 TAAAATTTGGTTTCTATATGATAAACGTACAAATGAGCATCTTATCTTTGAGCAAGGAAT
arti/1-471 171 TAAAATTTGGTTTCTATATGATAAACGTACAAATGAGCATCTTATCTTTGAGCAAGGAAT
glan/1-470 170 TAAAATTTGGTTTCTATATGATAAACGTACAAATGAGCATCTTATCTTTGAGCAAGGAAT
alba/1-468 169 TAAAATTTGGTTTCTATATGATAAACGTACAAATGAGCATCTTATCTTTGAGCAAGGAAT
laev/1-465 167 TAAAATTTGGTTTCTATATGATAAACGTACAAATGAGCATCTTATCTTTGAGCAAGGAAT
tama/1-464 161 TAAAATTTGGTTTCTATATGATAAACGTACAAATGAGCATCTTATCTTTGAGCAAGGAAT
Pipt/1-475 179 TAAAATTTGGTTTCTATATGATAAA GTACAAATGACATCTTATCTTTGAGCAAGGAAT
hybr/1-162 1 -----
pall/1-164 1 -----
consensus 181 taaaatttggtttctatatgataaacgtacaaatgagcatcttatctttgagcaaggaat

chil/1-470 230 CCTCATTTGACTGATTAACAATACATATCATTACTCCGACTGAAACATACAAAGTCTTAT
velu/1-468 228 CCTCATTTGACTGATTAACAATACATATCATTACTCCGACTGAAACATACAAAGTCTTAT
juli/1-472 231 CCTCATTTGACTGATTAACAATACATATCATTACTCCGACTGAAACATACAAAGTCTTAT
arti/1-471 231 CCTCATTTGACTGATTAACAATACATATCATTACTCCGACTGAAACATACAAAGTCTTAT
glan/1-470 230 CCTCATTTGACTGATTAACAATACATATCATTACTCCGACTGAAACATACAAAGTCTTAT
alba/1-468 229 CCTCATTTGACTGATTAACAATACATATCATTACTCCGACTGAAACATACAAAGTCTTAT
laev/1-465 227 CCTCATTTGACTGATTAACAATACATATCATTACTCCGACTGAAACATACAAAGTCTTAT
tama/1-464 221 CCTCATTTGACTGATTAACAATACATATCATTACTCCGACTGAAACATACAAAGTCTTAT
Pipt/1-475 239 CCTCATTTGACTGATTAACAATACATATCATTACTCCGACTGAAACATACAAAGTCTTAT
hybr/1-162 1 -----
pall/1-164 1 -----
consensus 241 cctcatttgactgattaacaatacatatcattactccgactgaaacatacaaagtcttat

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chil/1-470 290 TTTGGAAGATCCAAGAAATTACAGGGCTTGGATCAAACATAT----TTTTTTTTTCGTCTT
velu/1-468 288 TTTGGAAGATCCAAGAAATTACAGGGCTTGGATCAAACATAT----TTTTTTTTTCGTCTT
juli/1-472 291 TTTGGAAGATCCAAGAAATTACAGGGCTTGGATCAAACATAT----TTTTTTTTTCGTCTT
arti/1-471 291 TTTGGAAGATCCAAGAAATTACAGGGCTTGGATCAAACATAT----TTTTTTTTTCGTCTT
glan/1-470 290 TTTGGAAGATCCAAGAAATTACAGGGCTTGGATCAAACATAT----TTTTTTTTNCGTCTT
alba/1-468 289 TTTGGAAGATCCAAGAAATTACAGGGCTTGGATCAAACATAT----TTTTTTTTTCGTCTT
laev/1-465 287 TTTGGAAGATCCAAGAAATTACAGGGCTTGGATCAAACATAT----TTTTTTTTTCGTCTT
tama/1-464 281 TTTGGAAGATCCAAGAAATTACAGGGCTTGGATCAAACATATGTAATTTTTTTTTTCGTCTT
Pipt/1-475 299 TTTGGAAGATCCAAGAAATTACAGGGCTTGGATCAAACATATGTAATTTTTTTTTTCGTCTT
hybr/1-162 1 -----CAGCCGGTTACTCTCAATATTTATCCATAT---C-TCTT
pall/1-164 1 -----CAGCCGGTTACTCTCAATATTTATCCATATTTT-TCTT
consensus 301 tttggaagatccaagaattaCaGGGcTtggATCaAAcTaT ttTtTtTtcgTCTT

chil/1-470 346 TTTTTTTAGTTGACATATACTCAAGTAATCTCTTAAAAATGAGGATGATGCGTCACGAAN
velu/1-468 344 TTTTTTTAGTTGACATATACTCAAGTAATCTCTTAAAAATGAGGATGATGCGTCACGAAN
juli/1-472 347 TTTTTTTAGTTGACATATACTCAAGTAATCTCTTAAAAATGAGGATGATGCGTCACGAAN
arti/1-471 347 TTTTTTTAGTTGACATATACTCAAGTAATCTCTTAAAAATGAGGATGATGCGTCACGAAN
glan/1-470 346 TTTTTTTAGTTGACATATACTCAAGTAATCTCTTAAAAATGAGGATGATGCGTCACGAAN
alba/1-468 345 TTTTTTTAGTTGACATATACTCAAGTAATCTCTTAAAAATGAGGATGATGCGTCACGAAN
laev/1-465 343 TTTTTTTAGTTGACATATACTCAAGTAATCTCTTAAAAATGAGGATGATGCGTCACGAAN
tama/1-464 341 TTTTTTTAGTTGACATATACTCAAGTAATCTCTTAAAAATGAGGATGATGCGTCACGAAN
Pipt/1-475 358 TTTTTTTAGTTGACATATACTCAAGTAATCTCTTAAAAATGAGGATGATGCGTCACGAAN
hybr/1-162 40 TTTTTTTAGTTGACATATACTCAAGTAATCTCTTAAAAATGAGGATGATGCGTCACGAAT
pall/1-164 42 TTTTTTTAGTTGACATATACTCAAGTAATCTCTTAAAAATGAGGATGATGCGTCACGAAT
consensus 361 TTTTTTTAGTTGACATATACTCAAGTAATCTCTTAAAAATGAGGATGATGCGTCACGAAN

chil/1-470 406 GGTCCGGATAGCTCAGCTGGTAGAGCAAAGGACTGAAAAATCCTCGTGCCCCANNTTCAA
velu/1-468 404 GGTCCGGATAGCTCAGCTGGTAGAGCAAAGGACTGAAAAATCCTCGTGCCCCNNTTCAA
juli/1-472 407 GGTCCGGATAGCTCAGCTGGTAGAGCAAAGGACTGAAAAATCCTCGTGCCCCNNGTTCAA
arti/1-471 407 GGTCCGGATAGCTCAGCTGGTAGAGCAAAGGACTGAAAAATCCTCGTGCCCCNNTTCAA
glan/1-470 406 GGTCCGGATAGCTCAGCTGGTAGAGCAAAGGACTGAAAAATCCTCGTGCCCCNNGTTCAA
alba/1-468 405 GGTCCGGATAGCTCAGCTGGTAGAGCAAAGGACTGAAAAATCCTCGTGCCCCNNTTCAA
laev/1-465 403 GGTCCGGATAGCTCAGCTGGTAGAGCAAAGGACTGAAAAATCCTCGTGCCCC-ANNTTCAA
tama/1-464 401 GGTCCGGATAGCTCAGCTGGTAGAGCAAAGGACTGAAAAATCCTCGTGCCCCNNGTTNAAA
Pipt/1-475 417 GGTCCGGATAGCTCAGCTGGTAGAGCAAAGGACTGAAAAATCCTCGTGCCCCACCAGTTCAA-
hybr/1-162 100 GGTCCGGATAGCTCAGCTGGTAGAGCAAAGGACTGAAAAATCCTCGTGCCCCACCAGTTCAA
pall/1-164 102 GGTCCGGATAGCTCAGCTGGTAGAGCAAAGGACTGAAAAATCCTCGTGCCCCACCAGTTCAA
consensus 421 GGTCCGGATAGCTCAGCTGGTAGAGCAAAGGACTGAAAAATCCTCGTGcCccc TtcAa

chil/1-470 466 ANAAA-
velu/1-468 464 ATAAA-
juli/1-472 467 AANAAA
arti/1-471 467 ANAAA-
glan/1-470 466 AAAAA-
alba/1-468 465 AAAAA-
laev/1-465 462 ATAA--
tama/1-464 461 NAAA--
Pipt/1-475 -----
hybr/1-162 160 AAA---
pall/1-164 162 AAA---
consensus 481 a aa

```

Figure 4.2: Boxshade representation of the alignment of the intergenic spacer region between *trnL* 3'exon and *trnF* sequences. The dark shade indicates homologous sequences and the gaps are indicated as dashes. Substitutions and insertions are also indicated as light/grey shades in the alignment. Unknown bases are represented by the letter 'N'.

Table 4.1 - Position of indel events in the multiple sequence alignment. Species names hybr- hybrid, palli- *Prosopis pallida*, glan- *P. glandulosa*, alba- *P. alba*, tama- *P. tamarugo*, velu- *P. velutina*, laev- *P. laevigata*, juli- *P. juliflora*, arti- *P. articulata*, Pipt- *Piptadenia floribunda* and chil- *P. chilensis*

Species	Insertion/deletion	Sequence	Position
hybr, palli	deletion	AAGG....GAAA	4 to 318
Pipt	insertion	CAA	1 to 3
glan, alba, tama	deletion	C/G	10
velu, laev	deletion	TT	13,14
Pipt	insertion	A	15
juli, arti, glan, Pipt	insertion	A	24
laev	deletion	T	39
Pipt	deletion	T	53
tama	insertion	T	54
Pipt	insertion	CACAA	140 to 144
tama	deletion	ATGGA	160 to 164
tama	deletion	TAGA	175 to 178
tama, Pipt, palli	insertion	G/TTA	342 to 344
tama, Pipt	insertion	A/T	345
hybr	deletion	TT	353, 354
hybr, palli	deletion	G	356
Pipt	deletion	T	368
laev	deletion	C	472
Pipt	deletion	AAT/AAAA	480 to 486
Pipt, hybr, palli	deletion	A	484
chil, velu, juli, arti, glan	insertion	A	485

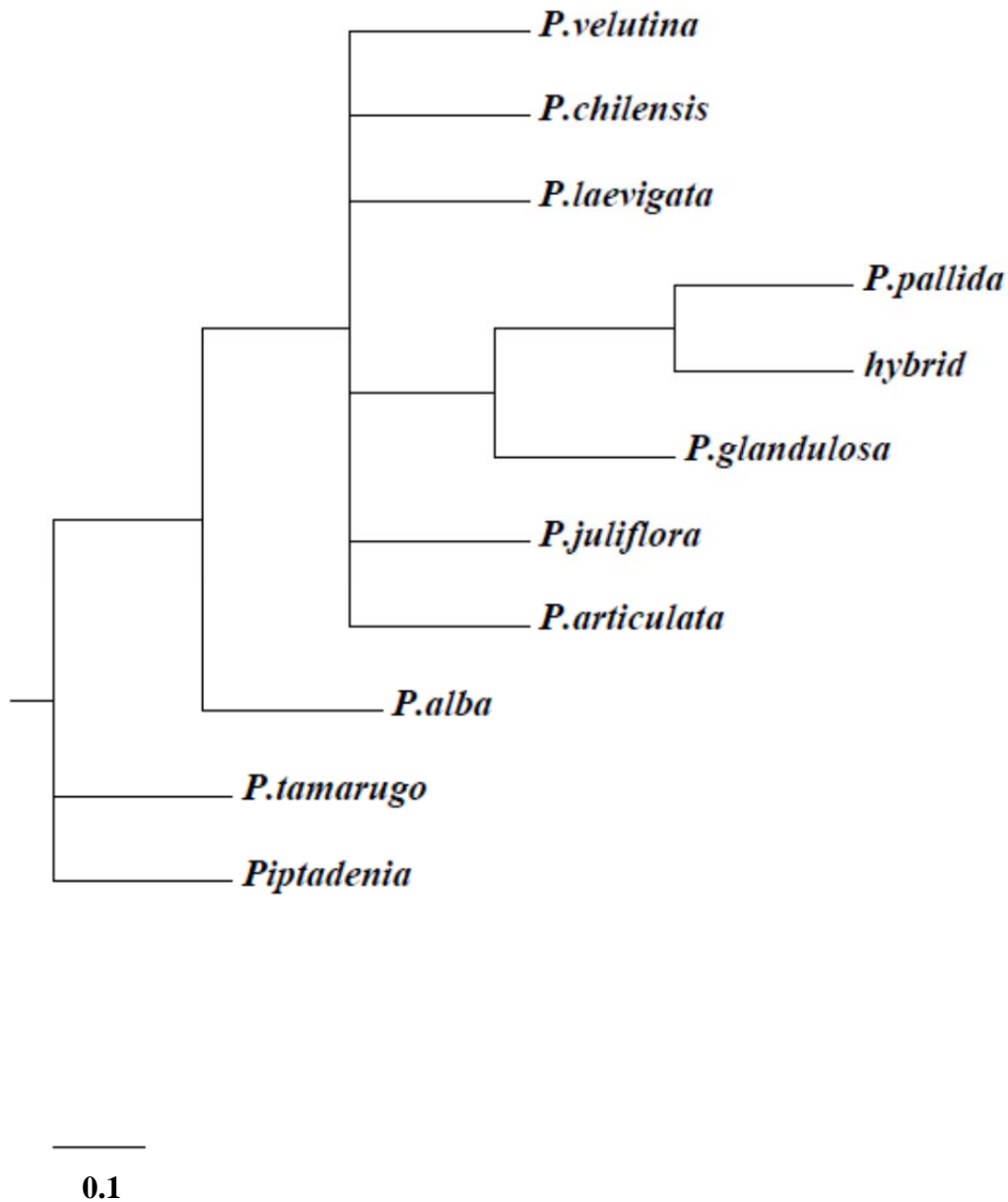


Figure 4.3: Phylogenetic tree showing the relationships among *Prosopis* species. The tree was created using PhyloDendron, a tree printing program (scale bar, 0.1 amino acid substitutions per site). *Piptadenia floribunda* is used to root the tree.

4.4 Discussion and conclusions

The utility of non-coding chloroplast sequences in inferring the relationship status of *P. juliflora* and *P. pallida* have been confirmed in this study. The genetic affinities among the species in section Algarobia of the genus has been a subject of debate and suggestions have been made for the regrouping of species within the section (Ramírez *et al.* 1999, Landeras *et al.* 2006, Burghardt and Espert 2007). This study also conducted a preliminary attempt to define their phylogenetic relationship.

The sequencing of the trnL-trnF intergenic spacer using universal primers designed by Taberlet *et al.* (1991) was successful in all the species analysed. The total length of this region varied from 162 to 472 bp in the *Prosopis* species studied. The pairwise similarity analysis of *P. pallida* and hybrid sequences using the program Dotlet revealed a high percentage of similarity between the two sequences. RAPD marker analysis on the same hybrid accession by Landeras *et al.* (2006) also revealed specific identifiable markers for the identification of *P. pallida* and the putative hybrid.

Multiple sequence alignments are useful for comparison of many nucleotide sequences simultaneously (Claverie and Notredame 2007). Clustal W multiple sequence alignment on the *Prosopis* non-coding sequences reveals many insertions, deletions and substitutions. Except for *P. pallida* and the hybrid, large variations in the lengths were not observed among other species. The 318 bp deletion in *P. pallida* and the hybrid is of interest as *P. pallida* is one of its putative parents (Landeras *et al.* 2006). The suggested other parent of the hybrid, *P. chilensis* showed no variation in length. *P. pallida* has its native range overlapping with *P. chilensis* in southern Peru

but it is not known whether sympatry exists among them or whether interspecific hybrids form in that region (Pasicznik *et al.* 2001). A similar deletion event was observed in some mimosoid species in a study by Catalano *et al.* (2008). Common mutation events like deletion or addition of sequences of few hundred base pairs in length serve as characteristic molecular markers in phylogenetic analysis potentially differentiating species (Palmer 1985). The noticeable variation in the length of the two species suggests an inheritance of maternal chloroplast DNA from *P. pallida* to the hybrid.

In most angiosperms, chloroplast and mitochondria are strictly maternally inherited (Corriveau and Coleman 1988) and this allows for the elucidation of parent species in the case of hybrids (Erickson, Straus and Beversdorf 1983). Biparental inheritance of chloroplasts is a rare occurrence but studies have proved that relaxed control mechanism of organelle inheritance to the zygote could result in this (Harris and Ingram 1991, Reboud and Zeyl 1994). In the present study, although DNA was extracted and phylogenetically analysed from only a limited number of seeds of putative *P. pallida* × *P. chilensis* hybrid, all the samples appeared to have inherited the chloroplast from *P. pallida*. However, the transfer of cpDNA gene from *P. chilensis* could not be proved. In fact, none of the other species except *P. pallida* show close relationship to the hybrid. This result also proves a study by Sherry *et al.* (2011) in which RAPD analysis shows similar banding pattern in both *P. pallida* and the hybrid. The RAPD data for the above study is detailed in Chapter 3. A more detailed study on chloroplast DNA transfer using different molecular markers would provide additional evidence regarding biparental inheritance which may be overlooked in this report.

The phenogram generated by the tree drawing software shows the relationship among the *Prosopis* species analysed in this study. *Piptadenia floribunda* of the tribe Mimoseae is used to root the tree and is considered more highly evolved than any other species in the study. A recent phylogenetic study on the genus *Piptadenia* supports the hypothesis that the genus is polyphyletic with three independent lineages (Jobson and Luckow, 2007). *P. tamarugo* belonging to the section *Strombocarpa* is relatively more divergent than the rest of the species belonging to section *Algarobia*. Genetic variation is less in *P. tamarugo* and this can be attributed to its partial self-compatibility (Hunziker *et al.* 1975). The position of *P. alba* in the phylogram suggests that it is distinct from the other *Algarobia* species. *P. alba* belongs to the series *Chilenses* and would be expected to group with any other *Prosopis* species in the same series. The seeds of *P. alba* sample analysed in this study were not collected from its native range. This could be attributed to the relative divergence shown by the species. Interestingly *P. alba* and *P. chilensis*, both from series *Chilenses* appeared to cluster together in a study by Ramírez *et al.* (1999). *P. chilensis* from South America forms a clade with the North American *Prosopis* species, *P. velutina*, *P. articulata*, *P. laevigata*, *P. glandulosa*, along with the tropical *P. juliflora* and *P. pallida*. This result is incongruent with its natural distribution. Except for *P. pallida* and *P. articulata*, the other species are included in the series *Chilenses* by Burkart (1976). Despite their morphological and ecological divergence, they appear to be genetically similar. Incongruencies between the morphological and chloroplast data are not uncommon and have been reported in many studies (Wendel, Stewart, and Rettig 1991, Shaw and Allen 2000, Draper, Hedenas, and Grimm 2007). This could be due to an ancestral polymorphism in the

cpDNA or past introgressive events (Rieseberg and Brunsfeld 1992, Soltis and Kuzoff 1995).

The discordance between a gene tree constructed using copies of gene sampled from various species and the actual phylogeny of the species derived from the gene tree is common (Maddison 1997). This disagreement between the species tree and gene tree might be due to hybridisation, lineage sorting, gene duplication and gene extinction (Maddison 1997). The allelic polymorphism within the species is a factor which causes this discordance (Takahata and Nei 1985). With higher level phylogenies, a gene tree might be a near accurate representation of the species tree, but at species level, it is necessary to combine data obtained from multiple genes that have evolved independently to accurately estimate the species tree (Pamilo and Nei 1988). In the case of nuclear genes, a large number of nucleotides are necessary to accurately construct a species tree since the nucleotide substitution rate is 10times lower than in organelle DNA such as mitochondrial or chloroplast DNA. (Pamilo and Nei 1988). The available data on the cpDNA trnL-trnF intergenic spacer region in *Prosopis* species do not allow for a straightforward comparison. However, this study provides an insight into the taxonomy of the confused genus *Prosopis*. A robust phylogeny can be achieved by analyzing additional nuclear and chloroplast DNA regions. This will allow broader sampling of the *Prosopis* species.

P. juliflora has its native range in Central America, Carribean islands and northern South America (Burkart 1976). The species is known to have close affinity to the other North American species, *P. velutina*, *P. glandulosa* and *P. laevigata* (Pasicznik *et al.* 2001). In the phylogram, *P. juliflora* is grouped into a single clade

with other species from the section *Algarobia*. Thus the natural distribution of species does not seem to have any impact on their phylogenetic relationship. Except for *P. alba*, all the other *Algarobia* species analysed are included in a clade. The division of *Algarobia* into series was also not clarified in this study.

The non-coding regions of cpDNA tend to evolve more rapidly than coding sequences, thus making it extremely useful in phylogenetic studies at lower taxonomic levels (Wolfe *et al.* 1987, Gielly and Taberlet 1994). But, it must be noted that in closely related species, the amount of variation found even in non coding cpDNA region might be too less for analysis of large number of accessions or for species of recent origin (Friesen *et al.* 1999). Hence RAPD was carried out to reveal even small genetic differences since a large part of the nuclear DNA will be analysed (Rieseberg *et al.* 1993, Powell *et al.* 1997). However, the limitations of RAPD such as low reproducibility of some bands and the uncertain homology of comigrating bands during gel electrophoresis (Rieseberg 1996). Previous studies using isozyme and RAPD on the North American species *P. glandulosa* and *P. velutina* showed high genetic variability among their populations (Bessega, Saidman, and Vilardi 2000). The flavenoid contents of *P. laevigata* and *P. glandulosa* var. *glandulosa* and *P. glandulosa* var. *torreyana* were very similar (Solbrig *et al.* 1977). Similarly isozyme data on the three species *P. laevigata*, *P. velutina* and *P. glandulosa* var. *glandulosa* revealed them to be closely related (Solbrig and Bawa 1975). RAPD studies have revealed *P. laevigata* to be similar to *P. glandulosa* with higher similarity coefficient than the former compared with *P. juliflora* (Juárez-Muñoz, Carrillo-Castaneda, and Rubluo 2006).

The relationship between *P. juliflora* and *P. pallida* is of interest since the trnL/F sequence of the species show length difference. *P. pallida* is not closely related to the *P. juliflora* sample from Galapagos Islands. This in fact agrees with the classification of Burkart (1976), where he placed *P. pallida* in a separate series from that of *P. juliflora*. RAPD and microsatellite studies on the relationship between *P. juliflora* and *P. pallida* by Sherry *et al.* (2011) concluded that the two species are genetically dissimilar. The results for this RAPD study can be found in Chapter 3. Although sympatry may exist between *P. juliflora* and *P. pallida* in their native ranges in northern Peru and Ecuador, no conclusive evidence was obtained from previous studies to support this hypothesis. Burkart (1976) describes a putative hybrid between *P. juliflora* and *P. pallida*. But he suggested that this could actually be a form of *P. juliflora* var. *horrida*. Recent molecular work suggests regrouping the series to include both the species together (Landeras *et al.* 2006), but our data do not support this. This result also does not agree with the proposition by (Pasiiecznik *et al.* 2001) to include both the species into a complex namely, the '*P. juliflora*-*P. pallida*' complex due to their similarities although more accessions of each species from their native ranges would give a conclusive result.

The *Prosopis* species in this study were selected from their native and introduced ranges. The eight species from Algarobia show discordance between their grouping and taxonomic status proposed by Burkart (1976). This is not the first time that this section has been a subject of debate. Electrophoretic seed protein analysis on the section Algarobia revealed discordance between the biochemical and morphological data (Saidman 1986, Hunziker *et al.* 1986). Section Algarobia is not considered as a natural group by many authors (Bessega, Saidman, and Vilardi 2005, Burghardt and

Espert 2007, Catalano *et al.* 2008). Most of the species in the section Algarobia are self-incompatible (Solbrig and Cantino 1975) and hence highly variable genetically (Simpson 1977, Hunziker *et al.* 1986). Section Strombocarpa and section Algarobia are thought to be highly differentiated in terms of speciation process and adaptive strategies (Saidman *et al.* 1996). This is evident from the phenogram since *P. tamarugo* is clearly separated from the rest of the Algarobia species in the present work. A more detailed study including more species from Strombocarpa would provide more evidence on this. Interestingly, morphological and biochemical data involving seven species from Strombocarpa suggest that it constitutes a natural taxon (Burghardt and Espert 2007) unlike section Algarobia. Moreover isozymic and morphological data shows that the species of section Strombocarpa are clearly differentiable unlike species from Algarobia where frequent hybridisation has resulted in discordance between morphological and biochemical data (Saidman *et al.* 1996). Hence suggestions have been made to treat the two sections as subgenera (Hunziker *et al.* 1986, Saidman *et al.* 1996).

In summary, all the species from section Algarobia except *P. alba* grouped into a single clade. This study is the first phylogenetic analysis on the relationship between *P. juliflora* and *P. pallida* based on non-coding trnL/F sequence. This study also attempted to identify the status of a putative *P. pallida* × *P. chilensis* hybrid on the basis of trnL/F sequence analysis. *P. pallida* is identified as one of the parents of the hybrid species, though the putative relation of the latter to *P. chilensis* could not be determined. The *P. pallida* and *P. juliflora* samples in this study shows significant variation in sequence length as determined by their cpDNA trnL-trnF sequence analysis. It must be noted that the data presented in this study are preliminary and

additional work should be done to assess the phylogeny of the genus fully. Not all the sections of the genus are represented and the sampling of taxa is also limited in this case since the focus was on differentiating *P. juliflora* and *P. pallida*. However this study gives some information on the effectiveness of using the chloroplast DNA trnL-trnF intergenic spacer region in differentiating the species.

In general, the natural distribution of the species and their taxonomic status seems incongruent. This study supports the fact that within section Algarobia the grouping of series seems artificial, agreeing with many other studies. The fact that few of the *Prosopis* species are not from their native range should also be considered while interpreting the results. Phylogenetic analysis of wide range of species from their native range and the different sections of the genus could help in resolving the taxonomic confusion surrounding this genus and in the proper exploitation of economically important species like *P. juliflora* and *P. pallida*.

5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 Discussion

P. juliflora and *P. pallida* originate in the tropics and they share similar morphology in terms of flower, pod, leaf and tree form (Burkart 1976, Pasiecznik *et al.* 2001). Species exhibiting intermediate morphological characters originating from native ones as a result of interspecific hybridisation have caused difficulty in their delimitation (Hunziker *et al.* 1975). Ploidy studies confirmed that *P. juliflora* is entirely tetraploid and *P. pallida* is a diploid (Harris *et al.* 2003, Trenchard *et al.* 2008). In zones of sympatry, hybridisation between the two species would result in hybrid species with intermediate characters. These hybrids are sterile triploids and their identification based on morphology alone would be difficult in such zones. These hybrid populations may constitute new species, varieties and forms (Pasiecznik, Harris, and Smith 2004). The presence of such triploid *Prosopis* was reported by Harris *et al.* (2003) from material collected in Cape Verde. *P. juliflora* and *P. pallida* are sympatric in southern Ecuador and northern Peru where all the varieties of *P. juliflora* and all forms of *P. pallida* are found (Pasiecznik *et al.* 2001). Burkart (1976) also suggested a putative hybrid between *P. juliflora* and *P. pallida* in northern Ecuador. Introgression between *P. juliflora* and *P. pallida* has also been reported in southern Botswana (Muzila *et al.* 2011), where the *P. juliflora* × *P. pallida* hybrids were separated using multivariate analysis of morphological characters. Their study also describes highly unlikely *P. chilensis* × *P. juliflora* × *P. glandulosa* and an intergeneric hybrid between *Acacia karoo* Hayne and *P. juliflora*. However, their study which focused only on the morphological characters of hybrids has to be treated with caution and further research has to be undertaken for the clarification occurrence

of *Prosopis* interspecific hybrids in south-western Botswana. Phenotypic plasticity is also common as characters are known to vary within species due to environmental changes (Pasiecznik *et al.* 2001). Though the species resemble each other in morphology, they are identified as different species and differentiated using morphology, ploidy and molecular characters (Johnston 1962, Burkart 1976, Harris *et al.* 2003, Landeras *et al.* 2006, Trenchard *et al.* 2008, Palacios *et al.* 2011, Sherry *et al.* 2011).

Taxonomic studies by several authors have suggested either raising or reducing the ranks of morphologically distinct populations (Hunziker *et al.* 1986, Saidman *et al.* 1996, Burghardt and Palacios 1997). Many earlier studies insist on reducing the ranks of morphologically and genetically similar species to sub-species, geographic races or clines (Saidman *et al.* 1996, Earl 1999 cited in Pasiecznik *et al.* 2001). However, Burkart's (1976) classification of the genus into sections is supported by several studies using floral and leaf morphology, protein electrophoresis, flavanoids and isozymes (Solbrig and Bawa 1975, Solbrig *et al.* 1977, Martínez 1984, Burghardt and Palacios 1997, Ramírez *et al.* 1999).

The aim of the current research was to identify different techniques that could differentiate the two species, and several approaches were studied. The first was the cytological approach where the leaf cell characters were compared in the accessions of *P. juliflora* and *P. pallida*. Leaf characters can be altered by environmental variables and hence stomatal characters were utilised in this study as they are thought to be much less affected by these variables (Stace 1989). Foliar characters and ploidy have been successfully used to separate the two species (Harris *et al.* 2003, Trenchard

et al. 2008). Even with possible interspecific hybridisation and variation in morphological characters (Solbrig *et al.* 1977), the tetraploid *P. juliflora* and diploid *P. pallida* grouped separately in the study by Harris *et al.* (2003).

The accessions of *P. juliflora* and *P. pallida* analysed in this study had their ploidy confirmed using flow cytometry. All the accessions selected for the leaf stomatal study were identified by Trenchard *et al.* (2008) using mature leaf characters and ploidy analysis. For the molecular studies, the *P. juliflora* seedling used was from seeds obtained from Galapagos Islands. The identity of the seedling was established through leaf morphology and ploidy analysis (Trenchard *et al.* 2008). *P. juliflora* is naturalised in Galapagos Islands after many introductions from native ranges (Wiggins and Porter 1971). Mc Mullen (1999) also describes *P. juliflora* as one of the native flowering plants found in Galapagos Islands. There might be some variation in the *P. juliflora* populations found there due to the adaptive nature of *Prosopis* species and this should be considered while interpreting results from molecular data. The origin of the species selected in this study is described in detail in Tables 2.1 and 3.1.

Statistical analysis of the leaf cell characters in this study identified a number of variables which could separate *P. juliflora* and *P. pallida*, being the length of the stomata, epidermal cell density and chloroplast number differed between the two species. The results are discussed in detail in chapter 2. Out of the above stated characters, stomatal length and epidermal cell density were used to develop key for better differentiation of the species. Previous morphological studies have developed identification keys for *Prosopis* species, particularly the tropical species (Harris *et al.* 2003, Pasiecznik, Harris, and Smith 2004, Trenchard *et al.* 2008). These keys are

extremely useful to foresters and collectors for initial identification. DECORANA and TWINSpan successfully separated *P. juliflora* and *P. pallida* into two groups using leaf cell characters for the first time. The results of the multivariate analysis described in this study are crucial in the understanding of the two species. The morphological character keys developed by Pasiecznik, Harris and Smith (2004) for *P. juliflora* and *P. pallida* are extremely useful for the initial identification. However, the two characters, stomatal length and epidermal cell density would allow in the further identification process to accurately differentiate the two species as shown in this study.

One of the difficulties facing taxonomists describing the two species is the morphological similarity exhibited and their separation into two 'good' species. There are problems delimiting the species of section *Algarobia* and *P. juliflora* and *P. pallida* are no exception. Species with morphological discontinuities due to frequent interspecific hybridisation have caused difficulty in delimitation of species (Hunziker *et al.* 1975). To resolve this issue Pasiecznik *et al.* (2001) grouped *P. juliflora* and *P. pallida* into a 'complex' namely the '*P. juliflora*-*P. pallida*' complex. This problem with delimitation of *P. juliflora* and *P. pallida* was addressed by Trenchard (2006), where accessions of both species were separated successfully using multivariate analysis. These results agree with the current findings and also with Burkart's (1976) classification of the two species into separate series. A recent study by Palacios *et al.* (2012) involving *P. juliflora* and *P. pallida* clarified the genetic identity of the two species in the Peruvian-Ecuadorian coast and in the Caribbean basin of Colombia and Venezuela. Their study differentiated *P. juliflora* and *P. pallida* into separate clusters using morphological and molecular characters.

The multivariate techniques, DECORANA and TWINSpan have identified diagnostic stomatal characters differentiating the two species. The keys which were developed based on the identifiable characters in the present study could be used in conjunction keys based on foliar characters to improve the morphological description of the two species. This would be aid in the proper management of introduced species which are otherwise mismanaged often due to misidentification and misnaming. The results show that *P. juliflora* and *P. pallida* are two ‘good’ species which can be differentiated using the keys based on stomatal characters. Out of the two, the potentially beneficial *P. pallida* could be utilised for its much sweeter pods, erect form and fewer thorns, thus preventing being permanently harmed by a biocontrol agent intended for the invasive and morphologically similar *P. juliflora* (Pasiiecznik *et al.* 2006). All the results are recorded and discussed in the Chapter 2 of this thesis. Further cytological and karyological work to improve the differentiation of the two species are suggested for future work.

The second approach to achieve the aim in differentiating the species was using molecular techniques. Given the morphological similarities shown by many species, it was necessary to resolve the relationship between the species at a DNA level so that the differences observed among them are not due to any phenotypic plasticity. In this study, RAPD, microsatellites and chloroplast non-coding sequences were utilised. Burkart (1976) assigned the two species into separate series within section Algarobia, but this division of Algarobia into series were not accepted by some authors (Burghardt and Palacios 1997, Ramírez *et al.* 1999). Thus, other species representing sections Algarobia and Strombocarpa were also included for molecular analyses in

addition to *P. juliflora* and *P. pallida* to assess their genetic relationship. The molecular studies described in chapter 3 and 4 analysed the relatedness of the two species.

RAPD primers revealed the genetic diversity existing among tree legumes (Gomez, Ramasubramanian, and Mohankumar 2011). In the genus *Prosopis*, these markers have been successfully used in assessing the genetic diversity between the species of different sections and in clarifying the putative parents of hybrids (Ramírez *et al.* 1999, Vázquez-Garcidueñas *et al.* 2003, Landeras *et al.* 2006). Burkart's (1976) classification of the genus into five sections based on observable vegetative characters was accepted by many authors who based their conclusions on morphological, biochemical and molecular markers (Caccavari de Filice 1972, Martinez 1984, Burghardt and Palacios 1997, Bessega, Saidman, and Vilardi 2005). However, incongruence between morphological and molecular data is also reported (Bessega, Vilardi, and Saidman 2006).

In this study, RAPD markers were capable of differentiating species from section Algarobia and Strombocarpa. Only *P. tamarugo* was included from section Strombocarpa, but the presence of unique bands distinguishing this species was noted. Section specific RAPD markers were identified by Ramírez *et al.* (1999) and Landeras *et al.* (2006). In fact, Ramírez *et al.* (1999) concluded that all the five sections could be separated using molecular markers. Landeras *et al.* (2006) suggested that *P. juliflora* and *P. pallida* are closely related owing to the high similarity index values between them, but the present study differed from their findings. The RAPD

profiles clearly differentiated them, with no indication of high similarity between the species.

A putative *P. chilensis* × *P. pallida* hybrid was also included in this study. Hybrids with intermediate phenotypes are common in wide zones of sympatry (Burkart 1976, Hunziker *et al.* 1986). RAPD analysis of the hybrid provided information on the utility of molecular markers in the identification and characterisation of intermediate phenotypes and their origin. RAPD primers producing common markers in both the hybrid and *P. pallida* were detected, suggesting that the hybrid is very closely related to *P. pallida* which could mean the latter as one of the parents. Interbreeding of species from section Algarobia is known (Simpson 1977, Hunziker *et al.* 1986), creating hybrids exhibiting intermediate characters. Morphological, isoenzymatic and RAPD studies have been used in the past to identify hybrid species and their putative parents (Burghardt and Palacios 1997, Ramírez *et al.* 1999, Vázquez-Garcidueñas *et al.* 2003). However, the morphological data does not always represent results obtained from a molecular marker analysis, and incongruence between the two analyses is common (Skepnér and Krane 1998, Hansen, Elven, and Brochmann 2000). For example, the hybrid origin of *P. chilensis* var. *riojana* is not supported by RAPD analysis though morphological and geographical data suggest it could be a hybrid between *P. chilensis* var. *chilensis* and *P. flexuosa* var. *flexuosa* (Vázquez-Garcidueñas *et al.* 2003). This lack of congruence between morphological characteristics and molecular clustering patterns in hybrids and their putative parents might be explained as a result of reticulate evolution (Vázquez-Garcidueñas *et al.* 2003). In this study, RAPD data gives conclusive evidence on the relationship between the putative *P. chilensis* × *P. pallida* hybrid and *P. pallida*. It's relatedness

with *P. chilensis* were not conclusive in this study although Landeras *et al.* (2006) have suggested they are related. The data to support their claim was not shown. The seeds of *P. chilensis* selected in this study from HDRA Accession no. 314.4 are the same accession used by Landeras *et al.* (2006) for their RAPD study on the hybrid. However, no convincing RAPD data was obtained in the present study to state *P. chilensis* as one of the putative parents of the hybrid. *P. chilensis* populations exhibit high genetic variability which helps the species to adapt to changing environments without any major change in gene frequencies (Verzino *et al.* 2003). Some adaptive genetic variation among *P. chilensis* populations were shown in their study. This could mean that the *P. chilensis* DNA analysed against the hybrid could possess some degree of genetic variation. If that is the case, the need for the use of other molecular markers to clarify the origin of the hybrid taxa analysed in this study is necessary.

Cluster analysis, using the similarity index values obtained from the RAPD data, generated a dendrogram which showed the relatedness of the selected species. Section *Strombocarpa* was separated from *Algarobia*. *P. articulata* from series *Pallidae* showed strong similarity with *P. velutina* from series *Chilenses*. Although this does not agree with Burkart's (1976) division of the section *Algarobia* into series, further molecular analysis involving more species from all the sections would provide a conclusive result on the artificial grouping of *Algarobia*. While the seed proteins electrophoretic pattern analysis supported Burkart's classification of the sections into series and assignment of species to each series (Burghardt and Palacios 1997), the division of section *Algarobia* into series is not supported by studies based on isozymes and molecular markers (Bessega, Saidman, and Vilardi 2005, Bessega *et al.* 2000a, Ramírez *et al.* 1999). However, these studies were useful in differentiating the

species belonging to each section though the taxon sampling in the above studies were not adequate to critically analyse Burkart's (1976) classification.

The North American species clustered together and this grouping is in accordance with their geographical proximity. *P. glandulosa* and *P. velutina* are naturalised in Australia, and hybrids between the two are observed (van Klinken 2012). *P. articulata* is known to hybridise with *P. glandulosa* and *P. velutina* (Johnston 1962). *P. glandulosa* var. *glandulosa* was suggested to be treated as a variety of *P. laevigata* due to morphological similarities (Johnston 1962). The North American *P. glandulosa* and *P. velutina* were differentiated using isozyme and RAPD profiles (Bessega, Saidman, and Vilardi 2000), and a further study ruled out a common origin between the two species (Bessega, Saidman, and Vilardi 2005). Interestingly, it has been observed that genetic similarity between *Prosopis* species is irrespective of their geographic closeness (Bessega, Saidman, and Vilardi 2000, Bessega *et al.* 2000b, Hamza 2010).

From the dendrogram it was clear that *P. juliflora* and *P. pallida* were not identical in terms of RAPD profiles. The similarity index value between them is only 0.27, while the highest similarity obtained in the study was 0.67 between the North American species. The low similarity value obtained between *P. juliflora* and *P. pallida* do not agree with the results shown by Landeras *et al.* (2006). However, RAPD analysis was useful in differentiating the two species. In an earlier study by Bukhari, Koivu and Kanerva (1998), seed storage protein size showed variation between *P. juliflora* and *P. pallida*.

The six microsatellite pairs developed in *P. chilensis* and *P. flexuosa* from series Chilenses of section Algarobia failed to produce amplification in *P. tamarugo* from section Strombocarpa. The transferability rate during cross-species amplification was however, high in all the Algarobia species tested. This agrees with the results obtained by Mottura *et al.* (2005) where they detected low transferability rate in Strombocarpa species compared to Algarobia species.

Sequencing of the trnL-trnF intergenic spacer region in the chloroplast DNA yielded important information about *P. juliflora* and *P. pallida*, and their relationship with other species. Large length mutation ie, deletion of 318bp observed in *P. pallida* but not in *P. juliflora* is considered as phylogenetically informative while differentiating the two species. Multiple sequence alignment gave valuable data on the sequence difference among the species. For example, the length of sequence deletion in both the hybrid and *P. pallida* suggest that they are very closely related. Since chloroplast DNA is maternally inherited (Corriveau and Coleman 1988), the result could mean that *P. pallida* is the maternal plant. However, the collector of the putative hybrid, Nick Pasiiecznik notes the mother tree as *P. chilensis*, surrounded by flowering *P. pallida*. Landeras *et al.* (2006) also suggested the relatedness between the putative *P. chilensis* × *P. pallida* hybrid and *P. pallida* using RAPD data.

The phenogram generated using the sequence alignment data did not confirm the separation of species into series in section Algarobia as proposed by Burkart (1976). *P. alba* from series Chilenses did not group with any other species. This is in discordance with the RAPD data described in Chapter 3 and results showed by Ramírez *et al.* (1999). However section Strombocarpa and Algarobia were grouped

separately. *P. juliflora* and *P. pallida* showed genetic distance though they were part of a large cluster. A recent study involving morphological and AFLP data were able to differentiate between *P. juliflora* and *P. pallida* in the Peruvian-Ecuadorian coast and in the Caribbean basin of Colombia and Venezuela (Palacios *et al.* 2011). All the North American species grouped into a large cluster. Previous studies based on isoenzymes and RAPD also successfully differentiated the North American *Prosopis* (Solbrig and Bawa 1975, Juárez-Muños, Carrillo-Castañeda, and Rubluo 2006). Though the cpDNA region analysed in this study is relatively small, additional work utilising larger DNA regions would clarify the taxonomy of the species.

This study does not conclusively suggest any taxonomic revisions in the genus, but did succeed in assessing different approaches aimed at differentiating *P. juliflora* and *P. pallida*, and the results presented and discussed in Chapters 2, 3 and 4 add to information regarding the taxonomy of the genus.

5.2 Conclusions

The following conclusions can be drawn from the present study.

- Leaf stomatal characters and chloroplast number can be successfully utilised to differentiate *P. juliflora* and *P. pallida*.
- Stomatal length and epidermal cell density were diagnostic leaf cytological characters enabling the development of an identification key.
- The multivariate analysis using TWINSpan and DECORANA divided all tetraploid *P. juliflora* and all diploid *P. pallida* to separate groups.
- RAPD markers produced good amplification profiles in *P. juliflora* and *P. pallida* and were useful in differentiating them as two ‘good’ species.

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- RAPD primers distinguished between nine tested species of section Algarobia and Strombocarpa.
 - The putative *P. chilensis* x *P. pallida* hybrid and *P. pallida* produced common diagnostic amplification bands.
 - Cluster analysis based on a similarity index grouped eight Algarobia species and a Strombocarpa species into two separate clades.
 - The value for the similarity index between *P. juliflora* and *P. pallida* was less, and were grouped into two different clades.
 - The putative hybrid and *P. pallida* are very closely related as they formed a discrete clade.
 - The microsatellite primer pairs were highly transferable in Algarobia species, though were much less transferable in *P. tamarugo* from Strombocarpa.
 - The chloroplast DNA trnL/F intergenic spacer sequence analysis reveals phylogenetically informative sequence variation that can be used to differentiate *P. pallida* and *P. juliflora*.
 - *P. pallida* is one of the parents of the putative hybrid as they show sequence similarity of more than 70%.
 - The division of section Algarobia into series is not confirmed in this study.

To conclude, the approaches undertaken in this study aimed to differentiate the two tropical *Prosopis* species, and add to the wealth of knowledge on resolving the taxonomical status of *P. juliflora* and *P. pallida*. Leaf stomatal characters and molecular markers have proven to be useful tools in differentiating *P. juliflora* and *P. pallida* populations, and their utilisation in resolving the identity of introduced

populations of the two species is highly beneficial. This work also describes keys based on stomatal length and epidermal density to successfully differentiate the two species. This is the first study ever reported on the utilisation of leaf stomatal characters in differentiating *P. juliflora* and *P. pallida* populations. The techniques described and the keys developed based on those characters can be used in conjunction with foliar character key (Harris *et al.* 2003, Pasiecznik, Harris, and Smith 2004, Trenchard 2006) and ploidy (Harris *et al.* 2003, Trenchard *et al.* 2008) to differentiate tropical *Prosopis* species. RAPD markers proved to be helpful in differentiating *P. juliflora* and *P. pallida* and found that they are not closely related species. However, a wider taxon sampling which includes species from the native ranges and overlapping geographical ranges would help to solve the taxonomical relationship between the two species and with other *Prosopis* species. This approach is important while trying to classify hybrids with intermediate characters and identifying the parentage of tetraploid *P. juliflora*. The length mutation observed in the trnL-trnF intergenic spacer region in the cpDNA between *P. juliflora* and *P. pallida* is of phylogenetic significance since the two species can be differentiated based on this sequence. Phylogenetic analysis of *Prosopis* spp using additional nuclear and cytoplasmic markers would help in resolving their taxonomic status. However, the different approaches whether cytological or molecular, have proved that the two species can be clearly demarcated and that there are specific characters that clearly distinguish between them.

5.3 Suggestions for future work

Many questions on the taxonomy of the genus are still unanswered or remain ambiguous. To resolve the uncertainty, especially with the two tropical species, *P.*

juliflora and *P. pallida*, some research directions that could be pursued are noted below.

A thorough phylogenetic investigation utilising a number of nuclear and chloroplast gene sequences would resolve much of confusion regarding the taxonomic relationship between species in the genus. For this, adequate taxon sampling is needed. Phylogenetic analysis involving both coding and non-coding fragments of both nuclear and chloroplast regions might explain incongruencies caused by hybridisation and introgression. This would also shed some light on the origin of polyploidy in *P. juliflora*.

Interspecific hybridisation is frequent between *Prosopis* species of section Algarobia and its role in speciation and evolution cannot be disputed. The origin of tetraploid *P. juliflora*, whether via hybridisation between species (allopolyploidy) or between genetically differentiated populations of single species followed by gene duplication (autopolyploidy), is of interest. The use of molecular markers to detect the origin of tetraploid species is known and this is an area which has yet to be studied in *P. juliflora*.

Cytotaxonomic studies are useful in collecting information on relationships at the species and genus levels. Since studies on the chromosome structure of *P. juliflora* and *P. pallida* are less, molecular cytogenetics can be utilised to analyse the genome structure of the two species. Advanced techniques like GISH (Genomic in situ hybridisation) and FISH (Fluorescent in situ hybridisation) could clarify the structure, function and origin of the tetraploid genome.

AFLP (amplified restriction fragment length polymorphism) DNA analysis is a reliable technique that can not only distinguish between related species, but also differentiate accessions of a single species. AFLP analysis could be useful in identifying the different accessions of *P. juliflora* and *P. pallida* collected from across the world and in tracing the historical and current introduction of their germplasm.

To conclude, the methods and analysis used in this study could be used further to resolve the confusion in distribution of *P. juliflora* and *P. pallida*. Cytotaxonomic studies are helpful in resolving the polyploid origin of *P. juliflora* and studies on ploidy could be pursued. The need to promote the productive and potentially beneficial *P. pallida* is imminent and the methods described here could be advantageous in differentiating the species since they have similar morphology and are easily confused. Polyploidisation of both the species followed by foliar and stomatal character analysis could be carried out to clarify the difference between normal and polyploidised samples. Foliar and stomatal character analysis on putative triploids between *P. juliflora* and *P. pallida* have to be carried out to analyse if the characters are intermediate for the triploids. This would be hugely beneficial for the identification of hybrids in native and introduced regions. All these approaches would allow for future selection and genetic improvement programs for potentially beneficial *Prosopis* species.

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The research article RAPD and microsatellite transferability studies in selected species of *Prosopis* (section *Algarobia*) with emphasis on *Prosopis juliflora* and *P. pallida* has been removed as it is published. The unabridged version of the thesis can be viewed at the lanchester Library, Coventry University