academicJournals

Vol. 8(2), pp. 88-94, 10 January, 2014
DOI: 10.5897/JMPR09.334
ISSN 1996-0875 ©2014 Academic Journals
http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Comparative assessment of genetic diversity among the Asclepiadaceous species using randomly amplified polymorphic DNA (RAPD) markers and numerical taxonomy system (NTSYS) cluster analysis

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Accepted 29 December, 2009

Asclepiadaceae is an economically important family species which are the source of fiber, rubber and dyes. In this study, genetic variability has been determined in three species, that is, *Tylophora hirsuta, Wattakaka volubilis* and *Cryptolepis buchananii.* The aim of present study was to understand the extent and pattern of genetic diversity among the individuals of same and different species of Asclepiadaceae. To asses the level of polymorphism within the species and members of different species, randomly amplified polymorphic DNA (RAPD) markers were used. Sixty RAPD primers of OPA, OPC, OPF and OPG series were used; only eight primers of OPC series gave amplification. Maximum polymorphism at interspecific and intraspecific levels was shown by OPC 09 and minimum polymorphism was observed in OPC 05. The data was analyzed using software numerical taxonomy system (NTSYS) cluster analysis PC version 2.20. In total 190 monomorphic and 78 polymorphic bands were produced from all primers. Therefore, out of 322 amplified products, 59% were monomorphic and 24.22% were polymorphic. Low genetic diversification was found both at intraspecific and interspecific level. Mixed pattern of grouping in the analyses indicated the close affinities of species with each other.

Key words: Comparative assessment, genetic diversity, Asclepiadaceous species, randomly amplified polymorphic DNA (RAPD) markers on Asclepiadaceae.

INTRODUCTION

Asclepiadaceae have 175 to 180 genera and 2000 species which are distributed mainly in the tropical and subtropical regions of the world. In Pakistan, it is represented by 23 genera and 41 species. *Tylophora* have 50 species, represented in Pakistan by 2 species, namely, *Tylophora hirsuta* and *Tylophora tenerrima* (Nasir and Ali, 1983). There are two species of *Wattakaka* in Pakistan, *Wattakaka volubilis* and *Asclepias volubilis*. Genus *Cryptolepis* have 12 species. Only one species is known from Pakistan, which is *Cryptolepis buchananii* (Nasir and Ali, 1983). Members of Asclepiadaceae are distributed in paleotropical regions. It is distributed throughout India, Nepal, Cylon, Malaysia, South China, tropical Asia and Africa. In Pakistan, they are distributed in North Punjab, Azad Kashmir, Hazara, Rawalpindi, Attock and salt range (Nasir and Ali, 1983). Members of this family are mostly herbs, lianas, shrubs or trees (rarely) which are laticiferous. Normal plants

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have very peculiar vegetative form. Some plants are cactoid; with leaves modified as pitchers in *Dischidia rafflesiana.* They are well developed or much reduced, opposite (decussate, usually) or whorled (rarely and rarely spiral) and fleshy or membranous or modified into spines. Lamina is entire (often reduced) containing one-vein or pinnate or palmate. They are stipulate or exstipulate (Watson and Dallwitz, 1992). The basic chromosome number x = 11 is the most predominant number in Asclepiadoideae, Periplocoideae and Secamonoideae. Only 3.5% of the studied species and taxa deviate from this basic chromosome number. Among the deviant ones, x = 10 is the most frequent followed by x = 9 and the sporadic increasing deviates x = 12, 13, and 14 (Albers and Meve, 2001).

Molecular markers have been looked upon as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. They have also become extremely popular markers for phylogenetic analysis adding new dimensions to the evolutionary theories (Joshi et al., 1999). Restriction fragment length polymorphism (RFLP), DNA sequencing, and a number of polymerase chain reaction (PCR) based markers are being used extensively for reconstructing phylogenies of various species. The techniques are speculated to provide path-breaking information regarding the fine time scale on which closely related species have diverged. Furthermore, these studies hold a great promise for revealing more about the pattern of genetic variation within species (Avise, 1994). Coding sequences played an important role, however, non coding sequences are also an important resource for phylogenetic reconstruction. There are six different kinds of nuclear DNA marker systems: (1) internal transcribed spacer (ITS), (2) external transcribed spacer (ETS), (3) simple sequence repeats (SSRs), (4) transposable elements (TE), (5) nuclear introns, and (6) promoter regions. Among them, the two most widely used non coding DNA marker systems are the nuclear encoded ITS (Internal Transcribed Spacer of nuclear encoded RNA) and the plastidic *trnLF* region (*trnL intron, trnLF* intergenic spacer) (Calonje et al., 2008).

As very little work has been done on the molecular aspect of *T. hirsuta, C. buchananii* and *W. volubilis,* the need was felt to explore and study the genetic variations of these plants and their taxonomic status at molecular level. The present thesis aims to study the pattern of genetic diversity in *T. hirsuta, C. buchananii* and *W. volubilis,* using RAPD markers in order to establish polygenetic relationship among these genera.

MATERIALS AND METHODS

Plants of three different genera of Asclepiadaceae *(Tylophora, Cryptolepis, Wattakaka*) were collected from Quaid-i-Azam University campus Islamabad. Young leaves were collected and stored at 4°C in sealed plastic bags. Total genomic DNA was extracted from fresh leaves by CTAB method (Richards et al., 1997) with few modifications. Two to three leaves of the plant material were ground with the help of pestle and mortar, by adding 1 ml of preheated (65°C) 2X Cetyl trimethyl ammonium bromide (CTAB) buffer. All the purified DNA samples were stored at -20°C for further use. The presence of DNA and its quality was checked by running it on 1% agarose gel prepared in 0.5X Tris acetate ethylene diamine tetra acetic acid (TAE) buffer.

The concentration of DNA was measured with the help of spectrophotometer at 260 nm wavelength and ratios of OD_{260}/OD_{280} were recorded. The ratio 1.8 to 2.0 was taken as an indication of good quality DNA (Pich and Schubert, 1993).

The amount of DNA was quantified using the formula:

$$\text{DNA concentration } (\mu g/ml) = \frac{O.D_{260} \times 100 \text{ (dilution factor)} \times 50}{1000}$$

Sixty primers of different RAPD series (Gene link) were used to analyze the genetic variation among the sample plants. These primers were from OPA, OPC, OPF and OPG series. Only the random primers of OPC series gave amplification profiles. Ten random primers of OPC series were tested in RAPD analysis, namely, OPC 01, OPC 02, OPC 03, OPC 04, OPC 05, OPC 06, OPC 07, OPC 08, OPC 09, and OPC 10. Scoreable amplification profiles were given by all the aforementioned primers except OPC 03 and OPC 10. The sequence of ten primers (OPC series) is given as shown in Table 1.

Table 1. The sequence of ten primers (OPC series).

Primer	Base sequence (5´-3´)
OPC1	TTCGAGCCAG
OPC2	GTGAGGCGTC
OPC3	GGGGGTCTTT
OPC4	CCGCATCTAC
OPC5	GATGACCGCC
OPC6	GAACGGACTC
OPC7	GTCCCGACGA
OPC8	TGGACCGGTG
OPC9	CTCACCCTCC
OPC10	TGTCTGGGTG

Different PCR conditions were used for the optimization of amplification, however, the best suitable conditions were as follows: initial denaturation at 94°C for 1 min followed by 44 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 1 min and extension at 72°C for 2 min. Final cycle was same except extension for 7 min at 72°C. After PCR, contents were held at 4°C till use. The genomic DNA was amplified by selected primers in 25 µl reaction mixture containing 25 to 50 ng/µl DNA, 25 pmol primer, 12.5 µl 2x PCR master mix (Fermentas) and 10.5 µl of PCR water (Fermentas) using thermal cycler (Labnet, multigene gradient).

Amplified products were run on 1.5% agarose gel run in 0.5X TAE buffer. Gel documentation was carried out by Dolphin Doc Plus gel documentation system (Wealtech). The size of each band was estimated by using 100bp DNA ladder plus (Fermentas). Bands were recorded as present (1) or absent (0). Cluster analysis was carried out based on similarities within class and dissimilarities between different classes, that is, samples showing good correlation have been placed in same group. All monomorphic and polymorphic bands were scored and included in the analysis.

Table 2. Total number of bands produced from six primers of OPC series.

S/N	Lane No.	OPC 04	OPC 05	OPC 06	OPC 07	OPC 08	OPC 09	Total bands
1	1	5	5	3	3	2	3	21
2	2	5	7	5	3	3	3	26
3	3	5	6	5	3	2	2	23
4	4	5	5	5	3	3	3	24
5	5	5	4	4	3	3	3	22
6	6	5	2	2	3	1	4	17
7	7	5	4	4	3	4	3	23
8	8	5	1	4	3	4	7	24
9	9	5	1	3	3	3	6	21
10	10	5	2	2	3	3	3	18
11	11	5	5	3	3	4	2	22
12	12	5	5	2	3	3	3	21
13	13	5	6	2	3	4	1	21
14	14	5	5	4	3	1	2	20
15	15	5	5	1	3	2	3	19
Total		75	63	49	45	42	48	322

Presence or absence of unique and rare bands was used to generate genetic similarity coefficients and then similarity coefficients were used to construct dendrograms by computer programme (NTSYS) pc version 2.20 (Rohlf, 2002).

RESULTS AND DISCUSSION

Genomic DNA was isolated from leaves of *T. hirsuta, C. buchananii* and *W. volubilis*. Isolated genomic DNA was run on 1% agarose gel after treatment with RNase A for determining the quality and quantity of DNA. In PCR, high quality of DNA is required for amplification purpose; therefore, good quality DNA was confirmed by agarose gel electrophoresis. By using this genomic DNA as a template, amplification was carried out by using different RAPD primers of OPC series. Amplified products were confirmed by running it on 1.5% agarose in 0.5X TAE at constant voltage.

The size range of all the amplified fragments produced by the aforementioned primers was 200 to 1200 bp. In total, 322 bands were produced by all the primers used in this study. Among these 322 bands, 190 monomorphic and 78 polymorphic bands were observed (Figure 1). Therefore, out of 322 amplified products, 59% were monomorphic and 24.22% were polymorphic with an average of 11% polymorphism per primer. Maximum number of bands was produced by OPC 04 and minimum number of bands was produced by OPC 08. In one lane, maximum number of seven and minimum number of one band was found. Maximum polymorphism was observed in OPC 09 and minimum polymorphism was observed in OPC 05. However, no polymorphic band was observed in OPC 04 and OPC 07, therefore have shown 100% similarity (Table 2).

Cluster analysis of bands produced by all primers

Similarity indices were developed on the basis of amplified products of six RAPD primers with samples of three different species (*T. hirsuta*, *C. buchananii* and *W. volubilis*). These similarity coefficients are shown in Table 2. The range of genetic similarity values were from 0.42 to 0.85 with the mean of 0.63. The lowest similarity value was present between the sample number 2 and 8, while the highest similarity value was found between sample number 2 and 5. UPGMA cluster analysis has also revealed the same results. In cluster analysis of all primers sample number, 2 and 5 were 100% similar while sample number 2 and 8 had shown very low similarity level. Both of them (2 and 8) were so distant from each other that they were present in different clusters.

UPGMA cluster analysis of all primers revealed two major clusters in this cladogram (Figure 2). Data from six primers of OPC series were analyzed in this cluster analysis and samples had showed 63% similarity level and 37% divergence among them. Cluster 1 was characterized by ten samples (1, 2, 3, 4, 5, 11, 12, 13, 14 and 15). All samples of *T. hirsuta* (1, 2, 3, 4 and 5) were clustered together in cluster 1. All samples of *W. volubilis* (11, 12, 13, 14 and 15) were also clustered together in cluster 1. 70% similarity level was present in all the samples of cluster 1. Cluster 1 was further divided into subcluster 1 and subcluster 2. Subcluster 1 contained all samples of *W. volubilis* and they had shown 74% similarity. Two groups were present in subcluster 1, that is, G 1 and G 2. Sample number 14, 15 and 11, 13 were clustered in groups 1 and 2, respectively. Both of them had shown 84% similarity. These were the least diverse samples of *W. volubilis*. Sample 12 was closely related to

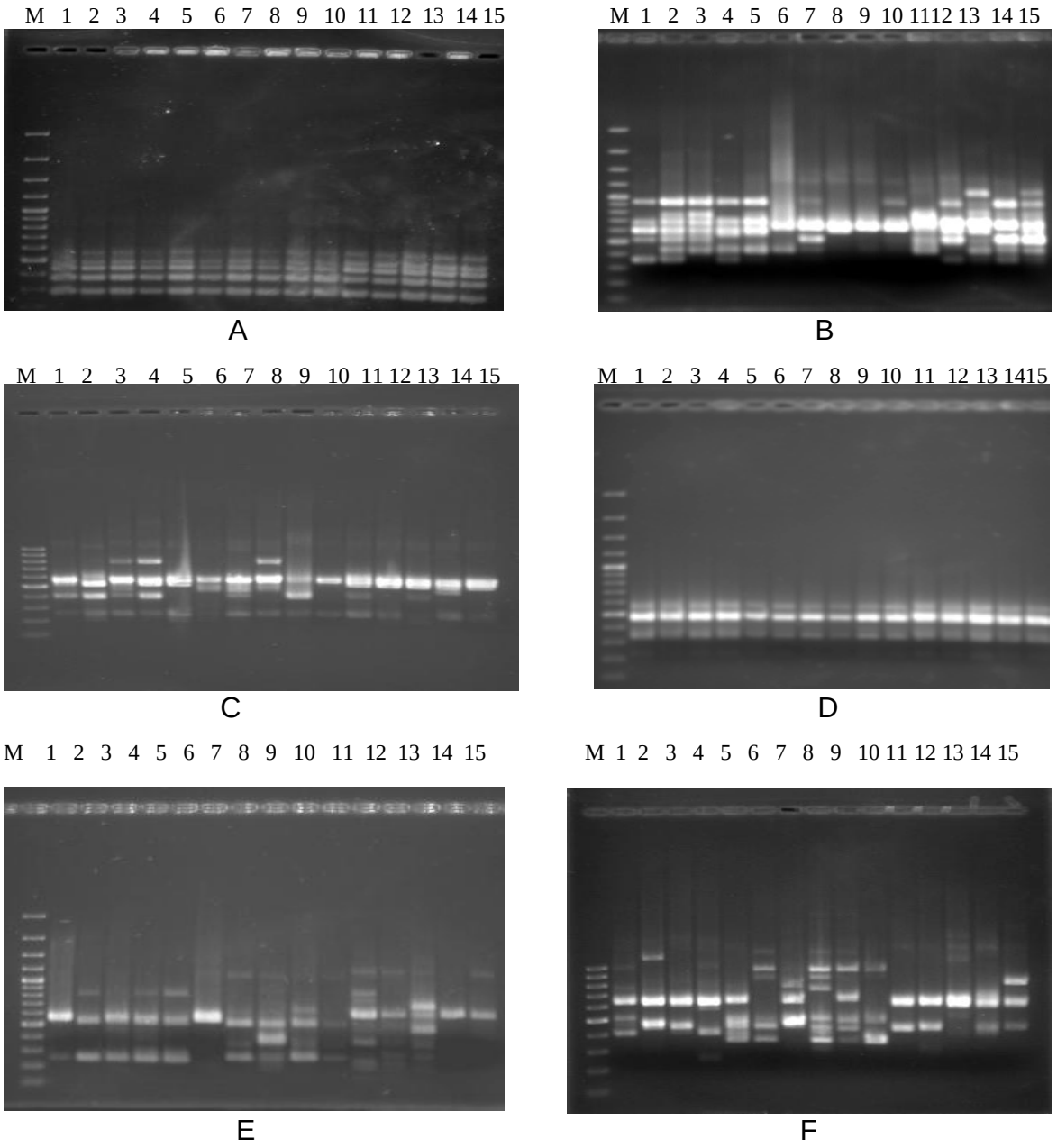

Figure 1. RAPD amplification profiles of fifteen samples of different species with different primers. A: OPC 4, B: OPC 5, C: OPC 6, D: OPC 7, E: OPC 8, and F: OPC 9. M: marker (DNA ladder), *Tylophora hirsuta* (sample 1-5), *Cryptolepis buchananii* (sample 6-10) and *Wattakaka volubilis* (sample 11-15).

group 1, but it was unresolved. It means that its origin was not clear. Only one sample of *T. hirsuta* (1) was present in subcluster 1, but it was also unresolved.

In subcluster 2, four samples of *T. hirsuta* were clustered. They had shown 80% similarity with each other. One group was present in subcluster 2 (G 3). Sample numbers 2 and 5 were clustered together in group 3, because they had shown 100% with each other. Samples 3 and 4 were unresolved but closely related to group 3. Sample 1 was the most diverse one among all the samples of *T. hirsuta*.

Cluster 2 was characterized by five samples of *C. buchananii* (6, 7, 8, 9 and 10). They had shown 72% similarity. A group was formed in cluster 2, that is, G 4, which was characterized by two samples, that is, 9 and 10. They were the least diverse samples of *C. buchananii*, because they were at 84% similarity level. The other three samples 6, 7 and 8 were unresolved. Among the samples of *C. buchananii*, sample 6 was the most diverse sample. UPGMA cluster analysis had shown a clear picture about the position of three genera. Presence of *T. hirsuta* and *W. volubilis* in a same cluster

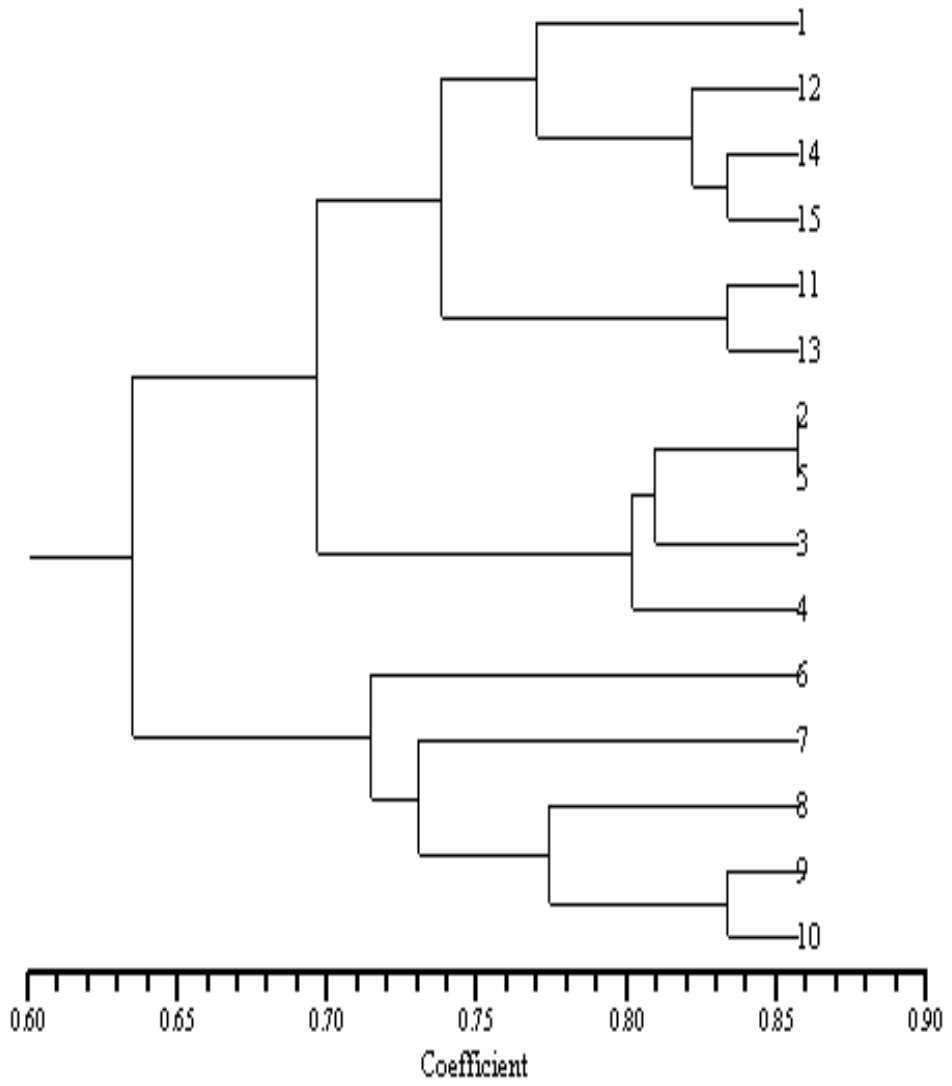

Figure 2. Dendrogram produced by amplified products of all primers. The number corresponds to the species as follows: *Tylophora hirsute* (1, 2, 3, 4, 5), *Cryptolepis buchananii* (6, 7, 8, 9, 10), *Wattakaka volubilis* (11, 12, 13, 14, 15). C: cluster, Sc: subcluster, G.

had revealed their close relationship with each other. Position of sample 1 of *T. hirsuta* in dendrogram with the samples of *W. volubilis* showed very close relationship of this sample with *W. volubilis.*

Cluster analysis of all primers has shown that genetic diversity is present within the species and between different species. Species are the units of ecological diversity and alleles are the units of genetic diversity. Samples of different species simply harbor more distinct alleles than samples of the same species. Weighting of ecological measures of species diversity on the basis of their genetic distance has been considered by several authors (May, 1990; Humphries et al., 1995). Individuals within a given species share some percentage of alleles; otherwise, they would not be considered members of the same species. This shared (or common) portion of the gene pool includes two basic classes of genes. Genes that are monomorphic within species are common to all individuals. The remainder of loci (on average, 15% in animals and 50% in plants) is polymorphic, varying among individuals in a population, and among populations within a species. The genetic profile of whole populations typically varies from place to place across a species range. These differences may arise as the result of chance occurrences, such as the genetic composition of dispersing individuals that create a new population (founder effect), or changes in allele frequencies that result from chance mating in very small populations (genetic drift) (Primack and Kang, 1989; Templeton, 1991; Meffe and Carroll, 1994; Eckert et al., 1996; Husband and Schemske, 1996).

Differences among species can also arise systematically,

especially if the environment in various places exposes individuals to different optima for survival and reproduction. For these and other reasons, species often diverge from one another in their genetic composition (Hartl and Clark, 1997). Each species distributes its genetic diversity (one measure of which is the total of all alleles at all loci) in a pattern reflecting both its biology and its history (Wright, 1965; Nei, 1975). Samples of two different species, that is, *T. hirsuta* and *W. volubilis* have shared some percentage of common alleles, that is why they are present in same cluster (Figure 2). These results also indicate that these two species have interspecific variations. Intraspecific variations have also been observed (Figure 2), like the individual of *T. hirsuta* (1) which is more diverse than other individuals of same species. However, this sample is more closely related to the samples of other species, that is, *W. volubilis.* Evidences from other studies had shown that within species variation might be present like in *Hoya parasitica* (Asclepiadaceae) which is one of the most common members of the section Euhoya (Hooker, 1883). *H. parasitica* is the most common, relatively widespread and extremely variable species (Rintz, 1978). Taxonomic study of the species (Kiew, 1995), included variations among the individuals of *H. parasitica.* There were variation in texture, shape, size and venation of leaves, the size of the flower and shape of corolla lobes. From these studies it was concluded that *H. parasitica* complex is composed of several undescribed taxa (Kidyue et al., 2005).

Variations might be present within the individuals of same species collected from same geographic area. Studies have indicated that all samples of Asclepiadaceae which were collected from Western North America had shown genetic variations among them. Some evolutionary lines vary less than others and more importantly, all the members of a particular line from one geographic area will often vary approximately in the same way (Stebbins, 1974). While the Asclepiadaceae were sampled more southerly in distribution, with only a few exceptions, all were taken from areas north of the Tropic of Cancer and must have been subjected to many of the same types of climatic shifts during the Tertiary and Quaternary Periods. In addition to the effect of similarity in geographic range, a considerable amount of variations within the species or between different species is observed (Gilmartin, 1980).

All three species which are studied in this research project share same habitat and occur sympatrically. *T. hirsuta* and *W. volubilis* both are from tribe Marsdeieae. It was evident that members of Asclepiadaceae are insect pollinated. This could have affected the evolutionary processes to produce the resulting like-patterns of the overall variability within the constituent species and taxa. Studies had revealed that tribes Marsdenieae and tribe Asclepiadeae are the most prominently *Diptera* serviced groups (Ollerton and Liede, 1997). Variations might be present at interspecific levels due to the same insects, during the process of pollination. In cluster 2 (Figure 2), sample of *C. buchananii* have shown a narrow range of diversity among them. Same is the case with the samples of *W. volubilis* in cluster 1. This low range of diversity is due to same soil and climatic conditions. *C. buchananii* is the member of sub family Periplocoideae and *T. hirsuta* and *W. volubilis* are the members of Asclepiadoideae. All the members of three species have 63% similarity among them. These results indicate that all the members are related to each other and have shown low genetic diversification. During the course of evolution, they have adapted to the most suitable environmental conditions, this might be the cause of their genetic divergence.

Cladistic analyses using morphological (Judd et al., 1994; Struwee et al., 1994) and molecular (Civeyrel, 1996; Civeyrel et al., 1998; Sennblad, 1997; Sennblad and Bremer, 1996, 2002; Sennblad et al., 1998) data have supported the contention that Asclepiadoideae and Periplocoideae form monophyletic tribes. In a study involving 15 taxa from the *Apocynacs* and 22 taxa from the *Asclepiads,* the three main subdivisions within the *Asclepiads*: Periplocoideae, Secamonoideae and Asclepiadoideae were supported (Civeyrel et al., 1998). In addition, the *Asclepiads* formed a monophyletie group (Potgieter and Albert, 1998b; Potgieter, 1999), and there is even some indication that the *Asclepiads* may be biphyletic. Civeyrel et al. (1998) reported the monophyly of the family Asclepiadaceae and of the three subfamilies, Periplocoideae, Secamonoideae, and Asclepiadoideae. Cluster analysis of *T. hirsuta, W. volubilis* and *C. buchananii* have also shown the mixed pattern of grouping among the individuals. These results indicate that these species have a very close affinity with each other. This is because they belong to a same family. These three genera belong to the two different subfamiles within Asclepiadaceae. *T. hirsuta* and *W. volubilis* are present in subfamily Asclepiadoideae and *C. buchananii* is present in subfamily Periplocoideae. Our results have also proved that *T. hirsuta* and *W. volubilis* which are present in same cluster and showed a close relationship, belonged to the same subfamily, even of the same tribe, that is, Marsdenieae. Species of this cluster have shown close affinities with each other. *C. buchananii* belonged to different subfamily, that is, Periplocoideae, and in our analysis, it is also present in different cluster.

Conclusion

Genetic variations among three species of different genera of Asclepiadaceae (*T. hirsuta, C. buchananii* and *W. volubilis*) were analyzed. For this purpose, different series of RAPD marker had been used to study the polymorphism among the species of three genera and also among the samples of same species. Results have

shown that low genetic diversification is present at interspecifc and intraspecific levels. Species have shown mixed pattern of grouping with each other while using different RAPD primer. Samples of *T. hirsuta* and *W. volubilis* are grouped together in one cluster, while samples of *C. buchananii* are grouped together in the second cluster. These interspecific relationships indicated that three genera belong to two subfamilies of Asclepiadaceae, that is, Asclepiadoideae and Periplocoideae. High level of similarity at intraspecific level has also shown that all the samples of each species are monophyletic. In future, if this work has been done on broader spectrum, then a complete gene pool of Asclepiadaceae would be developed. New species and varieties could be discovered. It would also help us to do an authentic and synthetic characterization of Asclepiadacea

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