Analysis of diversity amongst widely distributed and endemic *Atalantia* (family Rutaceae) species from Western Ghats of India

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ABSTRACT

The genus *Atalantia* Correa is represented in India with four species and two varieties. The infra - generic classification and the species limits in *Atalantia* are, however, not well defined, due to the occurrence of intermediate forms. Two species, *A. racemosa* Wight and *A. monophylla* (L.) DC., are widely distributed, while the third species, *A. wightii* Tanaka is endemic in the Western Ghats, a well-known biodiversity hotspot. PCR-based methods have been commonly used for the assessment of genetic diversity in plants. We report for the first time the genetic diversity within and between populations of the above three species using two Single Primer Amplification Reaction (SPAR) methods. AMOVA analysis clearly indicates the lesser diversity among the species than within them. The UPGMA tree included all of the doubtful species in one single sub-cluster within the major cluster of *A. racemosa* and *A. wightii*, suggesting that these are probably hybrids derived from these two species. At the population level, all *A. monophylla* populations grouped together in a cluster that was clearly separated from all other species and populations. [Physiol. Mol. Biol. Plants 2009; 15(3) : 211-224] E-mail : shirishranade@yahoo.com

Key words : AMOVA, *Atalantia*, diversity, PCA, Rutaceae, SPAR methods, Western Ghats

Abbreviations : AMOVA – Analysis of Molecular Variation; DAMD - Directed Amplification of Minisatellite DNA; ISSR – Inter Simple Sequence Repeat; PCA – Principal Components Analysis; RAPD - Random Amplified Polymorphic DNA; SPAR – Single Primer Amplification Reaction, UPGMA – Unweighted Pair Group Method with Arithmetic Means

INTRODUCTION

The genus *Atalantia* Correa (family Rutaceae) comprises of about 11 very closely related species occurring naturally in Indian mainland, the Andaman and Nicobar Islands, Sri Lanka, Myanmar, Thailand, Peninsular Malaya, North & South Vietnam, Laos, Cambodia, South China, Sumatra and Java (Swingle and Reece, 1967). The plants are large spinous shrubs or small to medium trees resembling the *Citrus* fruit trees in general aspects, bearing white fragrant flowers and small globose fruits that look like diminutive greenish-yellow limes or oranges. The species are rich in flavonoids, essential oils and alkaloids that have medicinal properties, are

useful in treatment for chronic rheumatism and paralysis, as an antidote to snake bite, as antibacterials and antifungals and even as an antifeedant activity against *Spodoptera litura* larvae (The Wealth of India, 2000; Kumar *et al.*, 2009).

Atalantia is considered a close wild relative of *Citrus* L., and both these genera belong to the subtribe *Citrinae* of the tribe *Citreae* of the orange subfamily, *Aurantioideae*. Because of their close affinities with *Citrus*, the species of *Atalantia* are valued as rootstocks for grafting commercially grown Citrus fruit trees. The genus is represented in India with four species, some of which also include varieties: *Atalantia monophylla* (L.) DC. var. *monophylla*, *A. monophylla* (L.) DC. var. *macrophylla* Oliver, *A. racemosa* Wight var. *racemosa*, *A. racemosa* Wight var. *bourdillonii* Narayanan *et al.* Nayar, *A. simplicifolia* (Roxb.) Engl. and *A. wightii*

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Tanaka (Nair and Nayar, 1997). Of these, *Atalantia simplicifolia* is an extremely rare species known so far only from a few sporadic collections made in N.E. India such as Cachar (Assam), Khasi hills (Meghalaya), Lushai hills (Mizoram) and Naga hills (Nagaland). The other three species have wider occurrence in the Western and Eastern Ghats of peninsular India and of these only *Atalantia wightii* is endemic to the Western Ghats. The infra generic resolution of the *Atalantia* species as well as the inter-species relationships have not been well understood. The species limits are also not well defined, presumably due to the occurrence of intermediate forms of two or more closely related species with sympatric distribution. *Atalantia monophylla* and *A. racemosa* are good examples to demonstrate the extent of morphological plasticity that these two species exhibit throughout their natural range in Southern peninsular India. It is suspected that the frequent occurrence of intermediate forms of *Atalantia monophylla* and *A. racemosa* could be due to inter-specific hybridization (Stone, 1985).

The Western Ghats (along with Sri Lanka) is identified as one of the 34 global biodiversity hotspots (Mittermeier *et al.*, 2004) and includes several endemic or rare and threatened plants and animals. We have compared the genetic diversity within and between populations of species of *Atalantia* collected from different Western Ghat localities. Of the three species occurring in Western Ghats, two are widely distributed elsewhere in peninsular India also while the third species is endemic to the Western Ghats. Single Primer Amplification Reaction (SPAR) methods namely Directed Amplification of Minisatellite DNA (DAMD; Heath *et al.*, 1993), PCR for Inter-Simple Sequence Repeat regions (ISSR; Zietkiewicz *et al.*, 1994) and Random Amplified Polymorphic DNA (RAPD; Welsh and McClelland 1990; Williams *et al.*, 1990) are powerful and capable of resolving even individual-specific profiles. We have recently used such methods successfully for diversity analysis in mulberry, betel vine, papaya, mango, jatropha and pomegranate varieties (Bhattacharya and Ranade, 2001; Ranade and Farooqui, 2002; Ranade *et al.*, 2002; Verma *et al.*, 2004; Bhattacharya *et al.*, 2005; Saxena *et al.*, 2005; Srivastava *et al.*, 2007; Ranade *et al.*, 2008; Ranade *et al.*, 2009; Narzary *et al.*, 2009). The RAPD profiles are due to several genomic regions while the DAMD profiles are mostly from the minisatellite-rich regions. Thus combining the data from two or more such methods we will have a comprehensive assessment of the diversity (Powell *et al.*, 1996; Parsons *et al.*, 1997). Elsewhere, the SPAR methods (RAPD, ISSR-PCR,

DAMD) have been used for population genetic and structure analysis in case of *Changium smyrnioides* Wolff. (Fu *et al.*, 2003), for assessment of genetic diversity of *Ginkgo biloba* L. populations (Fan *et al.*, 2004), for distinction between wild and cultivated enset gene pools (Birmeta *et al.*, 2004), for determining population structures in a tropical shrub, *Erythroxylum havanense* (Dominguez *et al.*, 2005) and for determining the genetic structure of the endangered plants like *Ammopiptanthus* species (Ge *et al.*, 2005), *Neolitsea sericea* (Wang *et al.*, 2005) and *Monimopetalum chinense* (Xie *et al.*, 2005). These references testify to the usefulness of the SPAR methods, especially RAPD, for population genetic analyses. In the present paper, we have used DAMD and RAPD together for the analysis of inter- as well as intra-species diversity in *Atalantia* species. In this paper we show the distribution of diversity amongst different populations of *Atalantia* species collected from W. Ghats in India using data generated by two SPAR methods from the same set of accessions and combined together for a comprehensive analysis followed by assessment of the population level diversity and its partitioning. Our results clearly indicate the lesser diversity among the species than within them.

MATERIALS AND METHODS

Plant material

The young non-senescing leaves were collected from plants of all the *Atalantia* accessions, from different locations (Figure 1, Table 1). In all cases, clean and healthy leaf tissue was stored as tissue paper wrapped packets over silica gel at room temperature.

Isolation of DNA

Total genomic DNAs from individual plants were isolated from the silica gel-dried leaf tissue with a DNeasy Plant Mini Kit (QIAGEN, USA) according to the instructions of the manufacturer. DNA concentrations were estimated by gel electrophoresis on 0.7 % agarose, staining with ethidium bromide, and comparison with a set of concentration standards.

SPAR primers

Four minisatellite core sequence primers for DAMD reactions were custom synthesized from Bangalore Genei, India. Decamers from kits U and M (procured from Qiagen Operon Technologies Inc., USA) were used as primers for RAPD reactions. Details of the primers used are given in Table 2.

Table 1. Accessions of *Atalantia* species collected from different locations for the study using SPAR methods. Sample names used are the names assigned to the isolated DNA samples for the experimental work. In all cases where herbarium vouchers have been prepared, the voucher numbers correspond to the numbers at NBRI, Lucknow Herbarium (LWG).

Accession Number ^a	Voucher No.	Location where collected	Altitude from MSL (m)	Latitude	Longitude	Taxon identity
ARLN1	225701	Lonavala	808 m	N 18° 42. 49	E 73° 23.43	<i>Atalantia racemosa</i> var. <i>racemosa</i>
ARLN2	225702	-Do-	811 m	N 18° 42.43	E 73° 23.44	-Do-
ARLN3	225703	-Do-	811 m	N 18° 42.43	E 73° 23.44	-Do-
ARLN4	225704	-Do-	808 m	N 18° 42.46	E 73° 23.42	-Do-
ARLN5	225705	-Do-	808 m	N 18° 42.46	E 73° 23.42	-Do-
ARLN6	225706	-Do-	773 m	N 18° 42.40	E 73° 23.04	-Do-
ARLN7	225707	-Do-	773 m	N 18° 42.40	E 73° 23.04	-Do-
ARLN8	225708	-Do-	773 m	N 18° 42.40	E 73° 23.04	-Do-
ARLN9	225709	-Do-	773 m	N 18° 42.40	E 73° 23.04	-Do-
ARLN10	225710	-Do-	726 m	N 18° 37.18	E 73° 22.50	-Do-
ARPL11	225727	Pallivasal	525 m	N 08° 54.73	E 77° 10.87	<i>Atalantia racemosa</i> var. <i>bourdillonii</i>
ARPL12	225728	-Do-	525 m	N 08° 54.75	E 77° 10.83	-Do-
ARPL13	225729	-Do-	525 m	N 08° 54.75	E 77° 10.84	-Do-
ARPL14	225730	-Do-	525 m	N 08° 54.76	E 77° 10.82	-Do-
ARPL15	225733	-Do-	525 m	N 08° 54.77	E 77° 10.86	-Do-
AMMA16	225711	Maruthamalai	702 m	N 11° 02.75	E 76° 51.07	<i>Atalantia monophylla</i> var. <i>monophylla</i>
AMMA17	225712	-Do-	745 m	N 11° 02.70	E 76° 51.04	-Do-
AQMA18	225713	-Do-	745 m	N 11° 02.70	E 76° 51.04	<i>Atalantia racemosa</i> ? or <i>A. wightii</i> ?
AQMA19	225714	-Do-	810 m	N 11° 02.71	E 76° 51.05	-Do-
AQMA20	225715	-Do-	810 m	N 11° 02.71	E 76° 51.05	-Do-
AQMA 21	225716	-Do-	810 m	N 11° 02.71	E 76° 51.05	-Do-
AMMA22	225717	-Do-	756 m	N 11° 02.71	E 76° 50.05	<i>Atalantia monophylla</i> var. <i>monophylla</i>
AMMA23	225718	-Do-	756 m	N 11° 02.71	E 76° 50.05	-Do-
AMMA24	225719	-Do-	701 m	N 11° 03.40	E 76° 50.94	-Do-
AWIG25	-	Indira Gandhi Wild Life Sanctuary	716 m	N 10° 28.22	E 76° 50.46	<i>Atalantia wightii</i>
AWIG26	-	-Do-	716 m	N 10° 28.22	E 76° 50.46	-Do-
AWIG27	-	-Do-	721 m	N 10° 28.23	E 76° 50.45	-Do-
AWIG28	-	-Do-	718 m	N 10° 28.23	E 76° 50.45	-Do-

Table 1. Continued

Accession Number ^a	Voucher No.	Location where collected	Altitude from MSL (m)	Latitude	Longitude	Taxon identity
AWIG29	-	-Do-	725 m	N 10° 28.25	E 76° 50.48	-Do-
AWIG30	-	-Do-	730 m	N 10° 28.25	E 76° 50.48	-Do-
AWRM31	225723	Rosemala	525 m	N 08° 54.75	E 77° 10.83	-Do-
ARRM32	225724	-Do-	525 m	N 08° 54.75	E 77° 10.83	<i>Atalantia racemosa</i> var. <i>bourdillonii</i>
ARRM33	225725	-Do-	525 m	N 08° 54.75	E 77° 10.83	<i>Atalantia racemosa</i> var. <i>bourdillonii</i>
ARRM34	225726	-Do-	525 m	N 08° 54.75	E 77° 10.83	<i>Atalantia racemosa</i> var. <i>bourdillonii</i>
AWAY35	225734	Aryankavu	350 m	ND	ND	<i>Atalantia wightii</i>
AWAY36	225735	-Do-	355 m	ND	ND	-Do-
AWAY37	225736	-Do-	355 m	ND	ND	-Do-
AWME38	225739	Merchinston Estate	826 m	N 08° 44.23	E 77° 08.24	-Do-
AWME39	225740	-Do-	826 m	N 08° 44.23	E 77° 08.24	-Do-
AWME40	225741	-Do-	826 m	N 08° 44.23	E 77° 08.24	-Do-
AWME41	225742	-Do-	826 m	N 08° 44.23	E 77° 08.24	-Do-
AWME42	225743	-Do-	824 m	N 08° 44.23	E 77° 08.24	-Do-
AWME43	225745	-Do-	824 m	N 08° 44.23	E 77° 08.24	-Do-
AWME44	225746	-Do-	825 m	N 08° 44.23	E 77° 08.24	-Do-
AWME45	225747	-Do-	830 m	N 08° 44.23	E 77° 08.24	-Do-
AWME46	225748	-Do-	826 m	N 08° 44.23	E 77° 08.24	-Do-
AWME47	225749	-Do-	826 m	N 08° 44.23	E 77° 08.24	-Do-
AWME48	225750	-Do-	825 m	N 08° 44.23	E 77° 08.24	-Do-
AQAK49	225601	Anakatti	ND	ND	ND	<i>Atalantia racemosa</i> ? or <i>A. wightii</i> ?
AQAK50	225602	-Do-	ND	ND	ND	-Do-
AMKU51	225609	Kunjapanai	517 m	N 11° 21.07	E 76° 56.40	<i>Atalantia monophylla</i>
AMKU52	225611	-Do-	672 m	N 11° 21.43	E 76° 56.71	-Do-
AMKU53	225631	-Do-	520 m	N 11° 21.07	E 76° 56.40	-Do-
AMKY54	225645	Karayar	217 m	N 08° 39.80	E 77° 20.53	-Do-
AMKY55	225646	-Do-	217 m	N 08° 39.80	E 77° 20.53	-Do-
AQKY56	225647	-Do-	217 m	N 08° 39.80	E 77° 20.53	<i>Atalantia racemosa</i> ? or <i>A. wightii</i> ?
AQKU57	225616	Kunjapanai	940 m	N 11° 21.58	E 76° 56.32	-Do-
AQKU58	225621	-Do-	1009 m	N 11° 21.46	E 76° 55.79	-Do-

Table 1. Continued

Accession Number ^a	Voucher No.	Location where collected	Altitude from MSL (m)	Latitude	Longitude	Taxon identity
AQAK59	225603	Anakatti	ND	ND	ND	-Do-
AQAK60	225604	-Do-	ND	ND	ND	-Do-
AQAK61	225607	-Do-	ND	ND	ND	-Do-
AQAK62	225608	-Do-	ND	ND	ND	-Do-
AQKU63	225610	Kunjapanai	517 m	N 11° 21.07	E 76° 56.40	-Do-
AQKU64	225619	-Do-	1009 m	N 11° 21.46	E 76° 55.79	-Do-
OGMP65	225738	Quilon	525 m	N 08° 54.75	E 77° 10.83	<i>Murraya paniculata</i>

^aThe four character alphabetical prefix in each accession number indicates appropriate population based on Taxon identity and location for collection. ARLN, ARPL and ARRM accessions are *A. racemosa* accessions collected from Lonavala, Pallivasal and Rosemala respectively; AMMA, AMKU and AMKY are *A. monophylla* accessions collected from Maruthamalai, Kunjapanai and Karayar respectively; AWIG, AWRM, AWAY and AWME are *A. wightii* accessions collected from Indira Gandhi Wild Life Sanctuary, Rosemala, Aryankavu and Merchinston Estate respectively; AQMA, AQAK, AQKY and AQKU are accessions with doubtful species identities collected from Maruthamalai, Anakatti, Karayar and Kunjapanai respectively.

ND : Not determined

Table 2. Different primers used for the two SPAR methods, DAMD and RAPD. The numbers of bands that were scored only amongst the *Atalantia* accessions are summarized.

Sr.No.	Primer Name	Primer Sequence (5'-3')	Annealing Temperature (°C)	Number of bands	
				Total	Polymorphic
DAMD					
1.	M-13	GAGGGTGGCGGTTCT	55 ^a	15	15
2.	HVR	CCTCCTCCCTCCT	55 ^a	9	9
RAPD					
1.	OPU-02	CTGAGGTCTC	35	7	7
2.	OPU-06	ACCTTTGCGG	35	8	8
3.	OPU-07	CCTGCTCATC	35	6	6
4.	OPU-09	CCACATCGGT	35	5	5
5.	OPU-10	ACCTCGGCAC	35	5	5
6.	OPU-11	AGACCCAGAG	35	7	7
7.	OPU-13	GGCTGGTTCC	35	7	7
8.	OPU-14	TGGGTCCCTC	35	13	12 ^b
9.	OPU-17	ACCTGGGGAG	35	6	6
10.	OPU-19	GTCAGTGCGG	35	10	10
11.	OPM-07	CCGTGACTCA	35	4	4

^aThe optimum annealing temperature was determined after testing different temperatures 3-10 °C less than the denaturing temperature. For the DAMD primers, the denaturing temperature was calculated according to Berger & Kimmel 1987 by adding 2 °C for every A or T and 4 °C for every G or C in the primer sequence.

^bPrimer OP-U14 profile includes a band corresponding to DNA fragment of ~900 bp that is present in all the *Atalantia* species accessions and is indicated in Figure 1 by small arrows on one of the lanes in each gel panel.

Amplification reactions

The SPAR with minisatellite core sequence primers was carried out essentially according to Zhou *et al.* (1997) except for primer M13 where the reactions were essentially according to Lorenz *et al.* (1995), after first determining the optimum annealing temperatures in the range 40 °C to 55 °C. The SPAR for RAPD primers was carried out essentially following Williams *et al.* (1990) after first optimizing most of the PCR parameters (data not shown). All amplifications were carried out in Thermal Cycler (Model PTC-200™, M.J. Research Inc., USA) and the products were separated by electrophoresis through 1.2 % (in case of DAMD profiles) or 1.0 % (in case of RAPD profiles) agarose gels in 0.5X TBE buffer, visualized and imaged using Nighthawk gel documentation system (pdi Inc., USA) after staining with ethidium bromide. Only distinct and well-separated bands were included in the analysis.

Data Analysis

Data (fragment sizes of all amplification products, estimated from the gel by comparison with low range DNA ruler (molecular weight marker, Bangalore Genei, India) were scored as discrete variables, using “1” to indicate presence and “0” to indicate absence of a band. A pair wise matrix of similarities between accessions was determined for the cumulative band data from the two methods using Jaccard coefficient (Jaccard, 1901) in the FREETREE program (ver. 0.9.1.5; Pavlicek *et al.* 1999). These pair wise similarity data were used to generate a UPGMA (Unweighted Pair Group Method with Arithmetic Means) tree after allowing a 1000 replicate bootstrap test using the same program. The tree was viewed, annotated and printed using TREEVIEW (ver. 1.6.5; Page, 2001). The robustness of the SPAR methods was tested in each case with a suitable non-*Atalantia* out group DNA, *Murraya paniculata* (L.) Jack. included in the analysis, and in all cases out group was resolved as distinct from the *Atalantia* DNAs.

Diversity assessment in groups of varieties

For this only the *Atalantia* accessions were considered in 14 populations based on the taxon identity as well as the locations where they were collected from and for the three species, the populations were grouped for the species concerned, and all other populations of unidentified accessions were considered together as a separate fourth group. Thus both, single population as well as grouped population, analyses were carried out based on the cumulative band data by both SPAR

methods using the program POPGENE (ver. 1.32, Yeh *et al.*, 1997) that allowed estimation of Nei's (1973) and Shannon-Weaver (1949) index of diversity. PCA analysis was carried out using the program GenAlEx (ver. 6.1, Peakall and Smouse 2006). Finally AMOVA (Analysis of Molecular Variations) was performed with the program ARLEQUIN ver. 2.000 (Schneider *et al.*, 2000) in order to determine the partitioning of SPAR profile variation within and between population groups.

RESULTS

The SPAR analysis (DAMD and RAPD) were carried out on *Atalantia* accessions after an initial optimization of the reaction conditions as well as thermocycler program based on the original references so as to ensure consistent and reproducible profiling (data not shown). From all accessions between three and five DNA preparations were made and each of these were tested in PCR. However, amplification product data were scored only for those accessions where at least two out of three DNA preparations resulted in identical profiles with the same primer used in the reaction. Once these optimizations were completed, the different minisatellite core sequence primers for DAMD and the arbitrary sequence decamers from Qiagen-Operon kits U and M were tested with 4-5 template DNAs and here also only those primers resulting in profiles from 80 % or more of the accessions used were selected for the entire set of *Atalantia* accessions. By these selections we were finally able to generate scorable profiles from 64 accessions of three species of *Atalantia* and another Rutaceae plant, *Murraya paniculata* used as the out group plant with two DAMD primers and 11 of the RAPD primers and these data along with the polymorphism scored are given in Table 2. A typical gel profile for one DAMD and two RAPD primers of the entire set of 64 accessions and out group is shown in Figure 2 a-c respectively. One RAPD primer, OP-U14 has revealed a ~900 bp amplification product that is present in all the 64 *Atalantia* accessions (Fig. 2b) and may represent a potentially *Atalantia*-specific fragment that could be developed into a SCAR marker. No two accessions were identical in all SPAR profiles and this shows that all plants that were collected were unique and that they were not duplicates of other accessions. The agarose gel profile band data, scored cumulatively for both DAMD and RAPD methods together resulted in a matrix of 102 bands amongst the *Atalantia* accessions. The pair wise comparison of the band data resulted in a matrix of similarity coefficients (SI) in the range from 0.14 to 0.93 for the entire set of *Atalantia*

Fig. 1. Schematic map outlining the Western Ghats region and showing the different populations that were collected. Map not to scale. The Western Ghats region has been indicated separately and its range is indicated as a dark shaded region in the outline map of peninsular India. The scale for this map is indicated along with North Direction and five of the states of India, namely, Maharashtra, Karnataka, Kerala, Tamil Nadu and Andhra Pradesh.

Fig. 2. Typical agarose gel electrophoresis profiles of amplification products with one DAMD primer (a) M13 and two RAPD primers, (b) OP-U14 and (c) OP-U19. Lane marked as Marker contains the Low Range DNA ruler (Bangalore Genei, Bangalore, India) as the size marker. The individual *Atalantia* DNAs are named as per accession list given in Table 1. The small light arrows on one lane in each gel panel in (b) indicate an amplification product of ~900 bp size in RAPD with primer OP-U14.

Fig. 3. UPGMA dendrogram after a 1000 replicate bootstrap analysis showing the clear separation of the out group plant and the rest of the *Atalantia* accessions. The small numbers at the nodes (only 50 % and higher values have been shown) are the bootstrap support percentages while the OTU names are abbreviated as in Table 1 and are given to the right of each branch.

accessions (data not shown), indicating the presence of tremendous diversity amongst the individual accessions. Based on the SI values, the UPGMA tree given in Figure 3 was computed. The UPGMA tree was tested for its robustness with a 1000 replicate bootstrap and the tree consistently separated the out group plant *Murraya paniculata* from the *Atalantia* accessions. The cumulative band data was also used for the POPGENE, GenAlEx and ARLEQUIN analyses. The Table 3 includes the Nei and Shannon-Weaver indices of diversity and clearly shows that the *Atalantia wightii* accessions were the most diverse while the *Atalantia racemosa* accessions had the least diversity amongst the three identified species groups. However, the group of accessions with doubtful species status was also with low diversity indices. Individually within populations of the different species, the Pallivasal set of *A. racemosa*,

the Maruthamalai set of *A. monophylla*, the Merchinston Estate set of *A. wightii* and the Kunjapanai set of *Atalantia* accessions with doubtful species identity were the most diverse. In all of these sets, the proportion of polymorphic bands was also higher than that in the other sets (data not shown). As an indicator of the diversity, inter- population identities were measured as Nei's Original as well as unbiased statistics (Table 4) while the inter- as well as intra-species identities were averaged and are given in Table 5. This table clearly shows that the inter-species identities were less than the intra-species identities. But the range of identities (as an indicator of diversity) was greater at intra-species levels (0.66-0.97) than that at inter-species levels (0.55-0.82). The inter-population statistics were used to generate a tree depicting the relationship between the different populations (Figure 4). Here the three

Fig. 4. The inter-population analysis tree based on Nei's unbiased distances between the populations. The branches are identified by population names based on the locations and species identity of accessions as given in Table 1.

populations of *A. racemosa* and four of *A. wightii* grouped together in one major cluster that also included a sub-cluster of all four populations of the unidentified *Atalantia* accessions. The other major cluster included only the three populations of *A. monophylla*. PCA analysis was carried out using the program GenAlEx and the PCA figure for all accessions considered as 14 populations is given in Figure 5. The PCA analysis clearly shows that the accessions from AMKU, AMMA,

Fig. 5. PCA analysis shows the distribution of all the accessions of *Atalantia* species, considered according to their populations, along the two principal coordinates. The population names are abbreviated as in Table 1 and are indicated in the key panel to the right of the PCA. Coordinates 1 and 2 were based on the input cumulative band data for the SPAR methods. PCA analysis was done using GenAlEx program.

Table 3. Grouped population diversity index and other statistics for the *Atalantia* species accessions computed for cumulative 102 loci from the band data of the two SPAR methods.

Group (populations)	Sample Size	Mean H^a (St. Dev.)	Mean I^b (St. Dev.)	Gst	Nm ^c	No. of polymorphic loci (%)
<i>A. racemosa</i> (ARLN, ARPL, ARRM)	18	0.2224 (0.1922)	0.3393 (0.2673)	0.6089	0.3212	75 (73.53)
<i>A. monophylla</i> (AMMA, AMKU, AMKY)	10	0.1941 (0.2159)	0.2843 (0.3042)	0.7010	0.2133	52 (50.98)
<i>A. wightii</i> (AWIG, AWRM, AWME, AWAY)	21	0.2497 (0.1870)	0.3781 (0.2609)	0.5696	0.3777	78 (76.47)
Doubtful species (AQAK, AQKU, AQMA, AQKY)	15	0.2008 (0.1848)	0.3097 (0.2629)	0.6241	0.3011	69 (67.65)
All groups (14 POPULATIONS)	64	0.3226 (0.1452)	0.4883 (0.1828)	0.3507	0.9256	101 (99.02)

^aH is Gene diversity (Nei 1973)

^bI is Shannon's Information index, $I = H_{pop}/H_{sp}$ where H_{pop} and H_{sp} are population and species diversity indices respectively (Shannon and Weaver 1949)]

^cNm is the estimate of gene flow, $Nm = 0.5(1 - Gst)/Gst$ where Gst is the gene differentiation index (McDermott and McDonald 1993).

Table 4. Matrix of Interpopulation identities by Nei Original (above the diagonal) and Nei Unbiased (below diagonal) methods computed for cumulative 102 loci from the two SPAR methods for all the 14 populations.

Population	ARLN	ARPL	ARRM	AMMA	AMKU	AMKY	AWIG	AWRM	AWAY	AWME	AQMA	AQAK	AQKY	AQKU
ARLN		0.7336	0.7068	0.6242	0.6169	0.5987	0.7758	0.7226	0.6881	0.7824	0.7012	0.7066	0.5627	0.6942
ARPL	0.742		0.8659	0.7593	0.6362	0.6524	0.7769	0.7271	0.716	0.8008	0.8101	0.8033	0.6571	0.7934
ARRM	0.7128	0.88		0.6636	0.5761	0.5739	0.7411	0.7233	0.773	0.7621	0.6968	0.7597	0.6024	0.7727
AMMA	0.6289	0.771	0.672		0.7552	0.7096	0.6819	0.6186	0.6803	0.7774	0.7466	0.6907	0.608	0.6987
AMKU	0.6197	0.644	0.5815	0.7617		0.9336	0.5877	0.55	0.6117	0.7053	0.5598	0.6461	0.6614	0.6747
AMKY	0.604	0.6633	0.5819	0.7188	0.9427		0.591	0.5588	0.6112	0.714	0.5634	0.667	0.7048	0.6893
AWIG	0.7804	0.7876	0.7491	0.6886	0.5917	0.5976		0.8907	0.7169	0.8289	0.7288	0.754	0.6299	0.714
AWRM	0.724	0.7341	0.7281	0.6222	0.5515	0.5628	0.8944		0.734	0.7842	0.6464	0.6823	0.549	0.6373
AWAY	0.7001	0.7341	0.7902	0.6949	0.6229	0.6251	0.731	0.7455		0.832	0.6766	0.6926	0.5861	0.682
AWME	0.7886	0.8134	0.7718	0.7866	0.7115	0.7234	0.8373	0.7889	0.8501		0.7852	0.8088	0.7186	0.7797
AQMA	0.7055	0.8214	0.7045	0.7542	0.5638	0.5698	0.7349	0.6492	0.6901	0.7934		0.8137	0.6538	0.8088
AQAK	0.713	0.8168	0.7703	0.6997	0.6525	0.6766	0.7625	0.6872	0.7084	0.8195	0.8231		0.8207	0.9527
AQKY	0.5638	0.6634	0.6064	0.6115	0.6632	0.7098	0.6325	0.549	0.5953	0.723	0.6567	0.8266		0.8099
AQKU	0.703	0.8097	0.7863	0.7104	0.6838	0.7017	0.7247	0.6443	0.7001	0.7929	0.8211	0.97	0.8187	

Table 5. Inter and intra-species group Nei's unbiased Identity statistics.

Group	No. of population pairs	Range of identities	Average identity
Intra-species	18	0.66-0.97	0.81
Inter-species	73	0.55-0.82	0.69

and AMKY are close to each other. Similarly, the accessions from AWIG and ARLN also group together closely. The other accessions are more distributed in the PCA. The grouping of all the populations of *A. monophylla* in PCA also is consistent with the separate and distinct identity of this taxon when the UPGMA tree of the populations was plotted (Figure 4). The partitioning of genetic diversity in these accessions of *Atalantia* was mostly between individuals and individual populations for each species than within the species group itself as between the four groups (*A. racemosa*, *A. monophylla*, *A. wightii* and the unidentified *Atalantia* accessions), only about 22 % of the variation was measured (Table 6). This suggests that the existing demarcation of species is probably not comprehensive enough to lead to a clear distinction between them. This is actually consistent with the confusion in taxonomy of *Atalantia* species based on morphological analyses.

DISCUSSION

The taxonomy of the orange subfamily (*Aurantioideae*) in family *Rutaceae* has always been complicated and a challenging task for several reasons. Notably the sub tribes within the two tribes viz. *Clauseneae* and *Citreae* also have confusing taxonomic status primarily due to

morphological plasticity and facile inter-generic and inter-species hybridizations amongst the taxa included in them. Recent studies have addressed the phylogeny and taxonomy of the tribe *Citreae* using molecular methods. Earlier phylogenetic analysis of the *Aurantioideae*, based on cp DNA sequence data, however, provided two contrasting views as to the phyletic origin and taxonomic alignments of the constituent genera included under this subfamily. For example, Chase *et al.* (1999) considered the *Aurantioideae* as a well-defined group with monophyletic origin, whereas in a more recent study Morton *et al.* (2003) maintained that the tribes *Citreae* and *Clauseneae* and the five out of the six subtribes within these tribes were not monophyletic. Morton *et al.* (2003), therefore, convincingly argued that a more comprehensive study on all the genera of the *Aurantioideae* using both the organellar as well as nuclear gene sequence data is needed to circumscribe and realign the exact taxonomic position and phyletic relationships among and between the genera, tribes and subtribes of the orange subfamily. Within the tribe *Citreae*, the genus *Atalantia* has been found to have a puzzling position in the phylogeny, since it grouped with another genus *Severinia* in all analysis of cp-DNA regions, even though this genus (*Atalantia*) also shared other molecular and botanical features with the genus *Citrus* (Araujo *et al.*, 2003). These and earlier studies based on isozymes (Herrero *et al.*, 1996) have actually led to an inference of polyphyletic origin and evolution of *Atalantia* along with *Citrus* (Araujo *et al.*, 2003). We have also observed tremendous variations in morphological characters of collected *Atalantia* accessions so that nearly 25% of them could not be clearly identified at the species level. This was particularly true in localities where *A. monophylla*, *A. racemosa* and *A. wightii* were growing in close proximities. We were, therefore, constrained to place

Table 6. AMOVA analysis of partitioning of diversity within and between population groups and species groups. For this analysis from the band data of the two SPAR methods, it was assumed that all populations have the same mating pattern and AMOVA was then computed using ARLEQUIN program as described by Huff *et al.* 1993.

Source of Variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among species groups	3	361.390	4.31422	21.79
Among populations within species groups	10	390.040	7.78694	39.33
Within populations	50	384.898	7.69797	38.88
Total	63	1136.328	19.79913	

these incompletely identified specimens under a separate group of unidentified *Atalantia* accessions. Interestingly in the SPAR profile data UPGMA tree (Fig. 3), all these unidentified accessions clustered together as a distinct group and further, the different populations included in this group were clustered along with populations of *A. racemosa* and *A. wightii* while the populations of *A. monophylla* were entirely separated in a different cluster (Fig. 4). This suggests that the accessions with doubtful species identities are probably complex hybrids involving mostly *A. wightii* or *A. racemosa*. However, these unidentified putative hybrid accessions were not primary hybrids since these never clustered along with any of the putative parental species, namely, *A. racemosa* and *A. wightii*. The results also suggest that the unidentified accessions have seemingly diverged considerably subsequent to original primary hybridization event. This seems possible because we have frequently observed several plants with intermediate morphological characters especially in places, where the putative parental species are to be found in close proximity to each other in sufficiently large numbers, such as in Maruthamalai, Anakatti, Kunjapanai and Karayar in the Western Ghats. This is further supported by the results obtained from PCA analysis (Figure 5). Our results suggest the relatedness of the suspected hybrids as above, because even in the UPGMA dendrogram for individual accessions (Fig 3), the *A. monophylla* accessions were separated from those of the rest of the species. In order to determine whether accessions collected from any specific locality are more differentiated than the others, the grouping of populations was carried out on basis of only the locality of collection (Fig. 1) and completely ignoring their taxonomic identification. Here all the accessions were included in 10 groups, namely, Lonavala, Pallivasal, Rosemala, Maruthmalai, Kunjapanai, Karayar, Indira Gandhi WLS, Aryankavu, Merchinston Estate and Anakatti. Analysis of these 10 groups showed that the accessions from Lonavala, Pallivasal, Indira Gandhi WLS, Aryankavu, Merchinston Estate and Anakatti exhibited low genetic differentiation while the highest differentiation was amongst the accessions from Karayar followed by Rosemala (data not shown). The intermediate nature and range of characters in these accessions that precluded clear identification of the species concerned and the results of the SPAR analysis show that considerably much more work with a comprehensive multi-character (molecular + morphological) survey of these accessions along with putative parents *in situ* in the natural habitats is required in order to confirm the hybridity of these accessions. Since this study has shown that the individual populations of the *Atalantia*

species have greater variation, therefore, a proper circumscription of the different species of *Atalantia* will need to closely examine several populations. In general we have found greater diversity amongst the southern collections of the *Atalantia* accessions than from the northern regions of the Western Ghats. The different species are able to hybridize to each other in nature and they do not seem to be isolated by any mating barriers. However, more studies are required into the reproductive biology of these to ascertain specific post- and or pre-mating barriers if any. Such natural inter-species hybridization not only makes their precise identification difficult but also explains why inter-species level variation is much lesser than the intra-species variation.

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