Rheedea
Vol. 26(1)
37–51
2016
ISSN: 0971 - 2313

Molecular differentiation and phylogenetic relationship of the genus *Punica* (Punicaceae) with other taxa of the order Myrtales

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Abstract

Phylogenetic analyses were carried out in two species of *Punica* L. (*P. granatum* L. and *P. protopunica* Balf.f.), and twelve closely related taxa of the order Myrtales based on sequence of the Internal Transcribed Spacer (ITS) and the 5.8S coding region of the nuclear ribosomal DNA. All the accessions of the *Punica* grouped into a distinct clade with strong support in Bayesian, Maximum Likelihood and Maximum Parsimony analyses. Trapaceae showed the most distant relationship with other members of Lythraceae *s.l.* Phylogenetic tree exclusively generated for 42 representative taxa of the family Lythraceae *s.l.,* revealed similar clustering pattern of Trapaceae and Punicaceae in UPGMA and Bayesian trees. All analyses strongly supported the monophyly of the family Lythraceae *s.l.,* nevertheless, the sister relation with family Onagraceae is weakly supported. The analyses of the ITS sequences of *Punica* in relation to the other taxa of the family Lythraceae *s.l.,* revealed that the genus *Punica* is distinct under the family Lythraceae, however this could be further substantiated with comparative sequencing of other phylogenetically informative regions of chloroplast and nuclear DNA.

Keywords: 5.8S Coding Region, ITS Regions, nrDNA, Punicaceae, Satellite Family

Introduction

The genus *Punica* L. consists of two species, viz., *P. granatum* L. and *P. protopunica* Balf.f. However, some authors recognise the ornamental dwarf pomegranate, *P. nana* L., as distinct species (Jbir *et al.,* 2008). *Punica protopunica* is endemic to the Socotra Island, Yemen (Guarino *et al.,* 1990). As a crop, *P. granatum* is extensively cultivated in Iran, Afghanistan, India, and Mediterranean countries and to some extent in the USA, China, Japan and Russia (Mars, 2000). It is believed that Iran may be the primary centre of origin of *P. granatum* and from there it might have migrated to other regions of the world (de Candolle, 1885). In India, pomegranate is found growing wild in the warm valleys and outer hill ranges of the Western Himalaya at elevations between 900 and 1800 m in the states of Himachal Pradesh, Jammu and Kashmir, and Uttarakhand (Misra *et al.,* 1983; Pandey *et al.,* 2008; Narzary *et al.,* 2010).

Placement of the genus *Punica* under different families such as Punicaceae, Lythraceae and Myrtaceae has been a long standing taxonomic problem. Baillon (1880) included this genus under the family Myrtaceae. However, Bentham & Hooker (1862–83) did not agree with this inclusion because of the absence of dotted glands and infra-marginal veins on leaves, presence of valvate calyx lobes and absence of aromatic principles in green parts. They included *Punica* as an anomalous genus in the family Lythraceae. Metcalfe & Chalk (1950) also justified the inclusion of *Punica* in Lythraceae due to its affinity with that family in the similarity of the anatomical characters, especially the occurrence of intraxylary phloem. However, due to morphological plasticity of the genus *Punica,* several authors (Hutchinson, 1926; Warming & Potter, 1932; Rendle, 1938; Gundersen, 1950; Lawrence, 1951) justified its inclusion in a separate family, Punicaceae. The salient features on which Punicaceae was established as a

separate family distinct from the Lythraceae are the union of the ovary with the receptacle of the thalamus and unique morphological feature of the fruit, especially termed as ''balusta'' (Nath & Randhawa, 1959).

Punica is characterised by several morphological features such as fruit with leathery pericarp and pulpy seeds with edible sarcotesta (Dahlgren & Thorne, 1984). Besides, ovules of *Punica*, with their thick, multi-layered outer integument and unicellular archesporium, differ from those of Lythraceae *s.s.* (Huang & Shi, 2002). These unique characteristics prompted most of the authors to treat Punicaceae as a separate family, but wood anatomy (Bridgewater & Baas, 1978; Graham *et al.*, 1993) and pollen morphology (Patel *et al.*, 1984; Graham *et al.*, 1990) suggested the inclusion in, or at least a close relationship with, Lythraceae. The family Lythraceae *s.l.* comprises of four subfamilies, with one, Punicoideae, sometimes regarded as a satellite family of Lythraceae *s.s.* Recently, APG III (2009) has placed *Punica* under the family Lythraceae on the basis of morphological as well as molecular data. The objective of the present study was to reconstruct the phylogeny of the genus *Punica* and its relationship with various components under the order Myrtales using sequences of the Internal Transcribed Spacer (ITS) and the 5.8 S coding region of the nuclear ribosomal DNA.

Material and Methods

Plant materials

In the present study 6 wild pomegranates representing different populations and 11 cultivated pomegranates representing important cultivars, and 12 closely related taxa, including 1 Socotra pomegranate (*P. protopunica*), were included (**Table 1**). The tissue samples were dried over silica gel. Voucher specimens collected for all the samples have been deposited in the herbarium of CSIR – National Botanical Research Institute, Lucknow (LWG). In the case of *P. protopunica*, only the DNA was procured from the DNA Bank at Royal Botanic Gardens (RBG), Kew, UK as detailed below.

DNA extraction, amplification and sequencing

Total genomic DNAs were extracted from silica dried leaves using the CTAB method (Doyle & Doyle, 1990). In case of *P. protopunica,* DNA was procured from the RBG, Kew (Acc. No. 1905). The primer sequences of P4 (5′-TCCTCCGCTTATT GATATGC-3′) and P5 (5′-GGAAGTAAAAGTCGT AACAAGG-3′) (White *et al*., 1990; Baldwin, 1992; Baldwin *et al*., 1995) were custom synthesized from Bangalore Genei, India. These primers were used to amplify ITS-1 and ITS-2 including 5.8S gene. The amplification of ITS regions including 5.8S gene was carried out following the reaction conditions of Allan & Porter (2000). Final concentrations or amounts of each reagent in 25 µl reaction volume were as follows: P4 and P5 primers (0.4 µM each), dNTPs (200 µM each in equimolar ratios), sterile water (13.0 µl), 1× *Taq* polymerase buffer (containing 10 mM Tris – HCl (pH 8.3), 50 mM KCl and 1.5 mM $MgCl_2$), 1 unit of *Taq* DNA polymerase (Bangalore Genei, India), and genomic DNA (about 50 ng). An MJ Research (Watertown, Massachusetts, USA) thermal cycler was programmed for 3 min pre-denaturation at 94°C, then 35 cycles of 1 min denaturation (94°C), 1 min annealing (50°C) and 1.5 min primer extension (72°C). A final 5 min incubation cycle (72°C) completed the primer-template extensions. Reactions were cross-checked by the inclusion of negative controls in each sample set.

The amplified products obtained were electrophoresed through 0.8% (w/v) standard agarose gels in 0.5× TBE buffer at a constant voltage of 5 V/cm. After electrophoresis gel was stained in ethidium bromide, visualized and archived using Uvitec gel documentation system (UV Technology, UK). A known DNA ladder of 100 base pair differences was also loaded on the first well of the gels. Since the size of ITS region is known (approximately 600–700 base pairs), the bands of interest were identified with reference to the DNA ladder in the gels and were excised and put into the fresh eppendorf tubes for purification of DNA. PCR products were eluted from the agarose gel and then purified using QIAquick Gel Extraction Kit (QIAGEN). The quality and quantity of the purified products were estimated loading on the 1% agarose gel in 0.5× TBE buffer at constant voltage of 5 V/cm.

Sequencing was conducted using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) and the ABI 3730 (Applied Biosystems). After the completion of capillary gel electrophoresis the fluorescence data were displayed as an electropherogram with the help of data collection software (ABI PRISM® DNA Sequencing Analysis Software version 5.0).

Only the sequence data with reliable read lengths were included in the present study. The sequences determined for the amplified ITS regions were uploaded to the EMBL/GenBank

nucleotide database for storage and archiving. These nucleotide sequences as well as those from other plants under order Myrtales that were already available in the database were used for phylogenetic analysis as detailed below.

Phylogenetic analysis

In the phylogenetic analyses, a total of 73 accessions including ITS sequences of 18 pomegranates and 55 out-groups representing almost all the families (Lythraceae *s.l.*, Myrtacaee, Rhynchocalycaceae, Psiloxylaceae, Crypteroniaceae, Alzateaceae, Oliniaceae, Penaeaceae, Onagraceae, Memecylaceae, Melastomataceae, Combretaceae and Vochysiaceae) under the order Myrtales (Graham *et al.*, 2005, APG III, 2009) were included (**Table 1**). These sequences of interest available in the NCBI global services were downloaded to use as a supplement in the phylogenetic study of *Punica*. The names of the taxa downloaded from the GenBank have been presented in Table 1, along with their accession numbers.

The ITS sequences were aligned with Clustal W (Thompson *et al.*, 1994) using MEGA5 software (Tamura *et al.*, 2011) and MUSCLE (Edgar, 2004). The alignment was further examined and edited manually as well. The boundaries of the ITS regions (ITS1-5.8S-ITS2) were determined by aligning with the existing sequence reported in the database (Huang & Shi, 2002) as a reference. The basic sequence statistics, including conserved sites, variable sites, parsimony informative sites, singleton sites, and transition/transversion ratio were also analysed with MEGA5 software. Analyses of evolutionary divergence between sequences were conducted using the maximum likelihood method in MEGA5. Phylogenetic trees were also generated with the UPGMA method (Sneath & Sokal, 1973) carrying 1000 bootstrap replicates.

The dataset was also analysed using maximum likelihood, Bayesian and parsimony approaches. Maximum Likelihood (ML) analyses were carried out using PhyML 3.0 (Guindon & Gascuel, 2003) with 1000 non-parametric bootstrap replicates (Felsenstein, 1985) in order to assess confidence of the nodes.

The MRBAYES 3.1.2 programme (Huelsenbeck & Ronquist, 2001) was employed to sample trees using a MCMC method. The analyses were performed assuming the general time-reversible model of nucleotide substitution (Rodriguez *et al.*, 1990) including estimation of invariant sites, assuming a discrete gamma distribution with six rate categories and allowing site-specific rates (GTR+I+G) for the single-gene and the combined analyses. A nucleotide substitution model was selected using the Akaike information criterion as implemented in the program jModelTest (Posada, 2008). No molecular clock was assumed. Two parallel runs of 5 million generations were made, starting with a random tree and employing 12 simultaneous chains each. Every 100th tree was saved into a file. The first 500000 generations (i.e., 5000 trees) were deleted as the "burn in" of the chains.

We plotted the log-likelihood scores of sample points against generation time using the programme TRACER 1.0 (http://evolve.zoo.ox.ac.uk/software.; Rambaut & Drummond, 2003) to ensure that stationarity was achieved after the first 500000 generations by checking whether the log-likelihood values of the sample points reached a stable equilibrium (Huelsenbeck & Ronquist, 2001). Additionally, we used the program AWTY (Nylander *et al.*, 2007) to compare split frequencies in the different runs and to plot cumulative split frequencies to ensure that stationarity was reached. Of the remaining (after burn-in), 90000 trees (45000 from each parallel run), a majority-rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes.

The parsimony analysis was carried out in PAUP 4.0b10 (Swofford, 2003) performed at www.bioportal.uio.no, using heuristic search and 4000 bootstrap replicates. Phylogenetic trees were drawn with the program TreeView (Page, 1996). Since the topologies of the ML, Bayesian and MP analyses did not show any supported conflicts, only the 50% majority-rule consensus tree of Bayesian tree sampling is shown in **Fig. 3.** Bayesian posterior probabilities (PP) equal or above 0.95 are indicated above the branches and MP/ML bootstrap values equal or above 70% are mentioned below the branches. Bold branches have PP, MLBS or MPBS values above these thresholds and are considered as well supported.

Results

ITS regions of about 700bp were amplified successfully with primer P4 and P5 from all the 29 accessions. The sequences were aligned using Clustal W program in MEGA software and the ITS-1, 5.8S and ITS-2 regions were determined with reference to the earlier sequence reported on *P. granatum* (Huang & Shi, 2002). These nucleotide sequences were submitted to EMBL nucleotide data bank. The sequences were used in the analysis

Table 1. Taxa belonging to the Order Myrtales considered for phylogenetic studies using nuclear ribosomal ITS sequences

Sl. No.	Plant Name	Family	Voucher No./ Source	EMBL Acc. No.
1	*Punica granatum* L.	Punicaceae	–	AY035760
2	*Punica granatum* L.	Punicaceae	LWG 227911	FM886992
3	*Punica granatum* L.	Punicaceae	LWG 248005	FM886994
4	*Punica granatum* L.	Punicaceae	LWG 227938	FM886995
5	*Punica granatum* L.	Punicaceae	LWG 227978	FM886996
6	*Punica granatum* L.	Punicaceae	LWG 248114	FM886997
7	*Punica granatum* L.	Punicaceae	LWG 228720	FM886998
8	*Punica granatum* L.	Punicaceae	LWG 247828	FM886999
9	*Punica granatum* L.	Punicaceae	LWG 247852	FM887000
10	*Punica granatum* L.	Punicaceae	LWG 228767	FM887001
11	*Punica granatum* L.	Punicaceae	LWG 247803	FM887002
12	*Punica granatum* L.	Punicaceae	LWG 228761	FM887003
13	*Punica granatum* L.	Punicaceae	LWG 247814	FM887004
14	*Punica granatum* L.	Punicaceae	LWG 247807	FM887005
15	*Punica granatum* L.	Punicaceae	LWG 247859	FM887006
16	*Punica granatum* L.	Punicaceae	LWG 247842	FM887007
17	*Punica granatum* L.	Punicaceae	LWG 247851	FM887008
18	*Punica protopunica* Balf.f.	Punicaceae	RBG	FM887009
19	*Ammannia baccifera* L.	Lythraceae	LWG 247864	FM887010
20	*Ammannia baccifera* L.	Lythraceae	NCBI	AF420216
21	*Cuphea hookeriana* Walp.	Lythraceae	NCBI	AF420221
22	*Cuphea lanceolata* W.T. Aiton	Lythraceae	NCBI	AY035763
23	*Heimia myrtifolia* Cham. & Schltdl.	Lythraceae	NCBI	AF201693
24	*Heimia myrtifolia* Cham. & Schltdl.	Lythraceae	NCBI	AF420215
25	*Lagerstroemia speciosa* (L.) Pers.	Lythraceae	NCBI	AF163696
26	*Lagerstroemia tomentosa* C. Presl.	Lythraceae	NCBI	AF201688
27	*Lawsonia inermis* L.	Lythraceae	LWG 247869	FM887015
28	*Lawsonia inermis* L.	Lythraceae	NCBI	AY078424
29	*Lythrum salicaria* L.	Lythraceae	NCBI	AY035749
30	*Lythrum salicaria* L.	Lythraceae	NCBI	AY035750
31	*Nesaea luederitzii* Koehne	Lythraceae	NCBI	AY035753
32	*Pemphis acidula* J.R. Forst. & G. Forst.	Lythraceae	NCBI	AY035762
33	*Woodfordia fruticosa* (L.) Kurz.	Lythraceae	LWG 247874	FM887020
34	*Woodfordia fruticosa* (L.) Kurz.	Lythraceae	NCBI	AF201692
35	*Woodfordia fruticosa* (L.) Kurz.	Lythraceae	NCBI	AF420222
36	*Duabanga grandiflora* (DC.) Walp.	Sonneratiaceae	NCBI	AF163695
37	*Duabanga grandiflora* (DC.) Walp.	Sonneratiaceae	NCBI	AF208695
38	*Sonneratia alba* Sm.	Sonneratiaceae	NCBI	AF163701
39	*Sonneratia apetala* Buch.-Ham.	Sonneratiaceae	NCBI	AF163697
40	*Trapa natans* var. *bispinosa* (Roxb.) Makino	Trapaceae	LWG 247873	FM887019

Sl. No.	Plant Name	Family	Voucher No./ Source	EMBL Acc. No.
41	*Trapa maximowiczii* Korsh.	Trapaceae	NCBI	AY035756
42	*Trapa maximowiczii* Korsh.	Trapaceae	NCBI	AY035757
43	*Callistemon salignus* (Sm.) Colv. ex Sweet	Myrtaceae	LWG 247865	FM887011
44	*Callistemon comboynensis* Cheel	Myrtaceae	NCBI	AM234140
45	*Callistemon viminalis* (Sol. ex Gaertn.) G. Don	Myrtaceae	NCBI	EF041510
46	*Eucalyptus globulus* Labill.	Myrtaceae	NCBI	AF058463
47	*Psidium cinereum* Mart. ex DC.	Myrtaceae	NCBI	AM234079
48	*Psidium cattleianum* Afzel. ex Sabine	Myrtaceae	NCBI	AM234080
49	*Syzygium arboreum* (Baker f.) J.W. Dawson	Myrtaceae	NCBI	EF026621
50	*Syzygium jambos* (L.) Alston	Myrtaceae	NCBI	EF026629
51	*Rhynchocalyx lawsonioides* Oliv.	Rhynchocalycaceae	NCBI	AM235850
52	*Psiloxylon mauritianum* (Bouton ex Hook.f.) Baill.	Psiloxylaceae	NCBI	EF026606
53	*Crypteronia paniculata* Blume	Crypteroniaceae	NCBI	AM235848
54	*Alzatea verticillata* Ruiz & Pav.	Alzateaceae	NCBI	AM235849
55	*Olinia emarginata* Burtt Davy	Oliniaceae	NCBI	AM235852
56	*Olinia radiata* J. Hofmeyr & Phill.	Oliniaceae	NCBI	AM235853
57	*Penaea cneorum* Meerb.	Penaeaceae	NCBI	AM235865
58	*Penaea dahlgrenii* Rourke	Penaeaceae	NCBI	AM235870
59	*Ludwigia hyssopifolia* (G. Don) Exell	Onagraceae	NCBI	AY035747
60	*Memecylon lateriflorum* (G. Don) Bremek.	Memecylaceae	NCBI	AY903365
61	*Memecylon roseum* H. Perrier	Memecylaceae	NCBI	AY903366
62	*Memecylon thouarsianum* Naudin	Memecylaceae	NCBI	AY903367
63	*Pternandra caerulescens* Jack	Memecylaceae	NCBI	EF683154
64	*Pternandra echinata* Wall.	Memecylaceae	NCBI	EF683155
65	*Votomita guianensis* Aubl.	Memecylaceae	NCBI	AY903371
66	*Rhexia cubensis* Griseb.	Melastomataceae	NCBI	DQ985627
67	*Rhexia lutea* Walter	Melastomataceae	NCBI	DQ985628
68	*Rhexia mariana* L.	Melastomataceae	NCBI	DQ985630
69	*Combretum wallichii* DC.	Combretaceae	NCBI	AF208731
70	*Terminalia arjuna* (Roxb. ex DC.) Wight & Arn.	Combretaceae	LWG 247871	FM887017
71	*Terminalia arjuna* (Roxb. ex DC.) Wight & Arn.	Combretaceae	NCBI	AF338255
72	*Terminalia bellirica* (Gaertn.) Roxb.	Combretaceae	NCBI	AF334768
73	*Vochysia tucanarum* Mart.	Vochysiaceae	NCBI	DQ787415

EMBL European Molecular Biology Laboratory Database
LWG Herbarium, National Botanical Research Institute, Lucknow
NCBI National Center for Biotechnology Information Database
RBG Royal Botanic Gardens, Kew

along with other ITS sequences pertaining to order Myrtales that were downloaded from the NCBI data bank.

The ITS sequences of all the accessions were aligned using Clustal W program in MEGA5 software. The average frequencies of Thymine (T) and Adenine (A) were 18.3% and 20.7%, respectively; showing 39% mean A+T contents in 635.2 bases average length of ITS sequence throughout the taxa under study. Similarly, the average frequencies of Guanine (G) and Cytosine (C) were 30.3% and 30.8% respectively; showing 61% mean G+C contents throughout the entire ITS sequences. The mean A+T and G+C contents observed exclusively in Punicaceae were 33.75% and 66.25%, respectively. The basic statistics such as conserved sites, variable sites, parsimony informative sites and singleton sites were calculated after the complete deletion of the missing/gap sites from all the sequences. This was done separately for higher to lower group, i.e., Myrtales, Lythraceae *s.l.,* and Punicaceae. The aligned ITS sequences (including 5.8S) formed in a matrix of 565 nucleotide sites after the deletion of missing/gap sites, of which 398 sites were variables and 351 sites were parsimony informative in Myrtales. In Lythraceae *s.l.*, 272 variable sites and 237 parsimony informative sites were recorded from 565 nucleotide sites. Again, in Punicaceae that included 17 accessions of *P. granatum* and 1 accession of *P. protopunica*, there were 34 variable sites and only 4 parsimony informative sites out of 565 nucleotide sites studied. The nucleotide pair frequencies like identical pairs, transitional pairs (si), transversional pairs (sv), and transition/transversion ratio (R) of ITS sequences were also calculated among the groups of Myrtales, Lythraceae *s.l.* and Punicaceae. The average base substitutions recorded in Myrtales was 113 (*i.e.*, si = 53, sv = 60) with transition/transversion ratio (R) of 0.9 throughout the taxa under study. In the family Lythraceae *s.l.*, the si/sv ratio was R = 0.9 with an average substitution of 72 bases where si and sv values were 34 and 38, respectively. Interestingly, in the monogeneric family Punicaceae, the mean base substitutions for all the taxa was low (only 4) with an equal proportion of transition and transversion events (R = 1.0).

Nucleotide substitution and deletion appeared to be the main sources of variability. The 5.8S region in nuclear DNA is highly conserved and any base changes in this region determine the structural conformation of rRNA. A base substitution (C↔T) at the 269 site of 5.8S region exclusive to the members of Punicaceae was observed. Three important indel sites, viz., 86 to 110 positions in ITS1 region, 365 to 367 and 395 to 403 positions in ITS2 region, were also observed exclusive to the ITS regions of the family Punicaceae in comparison to other families under the order Myrtales (data not shown). However, the indel site 365 to 367 in *P. protopunica* was not in accordance with the *P. granatum* rather it was in accordance with the other families of the order Myrtales. Unlike *P. granatum*, *P. protopunica* exhibited similar base substitutions to some members of Lythraceae at the positions such as 15, 298, 323 and 516.

Pair-wise sequence divergence estimation among the 73 sequences was also conducted using the maximum likelihood method (Tamura *et al.,* 2011) in MEGA5 programme. Within the family Punicaceae, the *P. protopunica* exhibited 0.03 divergences to all the accessions of *P. granatum*. The pomegranate accession (AY035760) from China, showed 0.01 divergences to all the accessions of Indian pomegranates, while no sequence divergence was observed among the Indian pomegranates (**Table 2**) indicating that there is still no significant ITS sequence divergence among the Indian pomegranates. In the present study, the genus *Punica* showed highest ITS sequence divergence to *Trapa* L. (0.18–0.20) and the lowest divergence to *Lawsonia* L. (0.07–0.08) and *Woodfordia* Salisb. (0.08–0.09) under the family Lythraceae *s.l.* (**Table 3**).

The mean sequence divergences between the families were estimated based on the pair-wise sequence analysis of 73 taxa representing different families under the order Myrtales. The maximum divergence (0.49) was observed between the families Vochysiaceae and Melastomataceae, whereas the lowest (0.09) was between Oliniaceae and Penaeaceae. The families Oliniaceae and Penaeaceae also showed relatively low distances in the range of 0.10 to 0.14 with the families Rhynchocalycaceae, Alzateaceae and Crypteroniaceae. Similarly, the family Lythraceae *s.s.* showed reasonably low distances with its satellite families Punicaceae (0.10) and Sonneratiaceae (0.12), except Trapaceae (0.19). The distance between Punicaceae and Sonneratiaceae was 0.10 and that in between Sonneratiaceae and Trapaceae was 0.16. Such low distance values are critical in the delimitation of closely related families or the morphologically intractable groups (**Table 3**).

ITS sequences of 42 representatives of the family Lythraceae *s.l.* (including Punicaceae, Sonneratiaceae and Trapaceae) were analyzed exclusively to generate Phylogenetic tree. Here, the clustering

Table 2. Estimates of evolutionary divergence between the accessions of the family Punicaceae based on ITS sequence data. Analyses were conducted using the Maximum Likelihood method in MEGA5. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 297 positions in the final dataset. Values in parentheses are the serial number of the accessions as given in Table 1

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
Punica granatum (1)	-																	
Punica granatum (2)	0.01	-																
Punica granatum (3)	0.01	0	-															
Punica granatum (4)	0.01	0	0	-														
Punica granatum (5)	0.01	0	0	0	-													
Punica granatum (6)	0.01	0	0	0	0	-												
Punica granatum (7)	0.01	0	0	0	0	0	-											
Punica granatum (8)	0.01	0	0	0	0	0	0	-										
Punica granatum (9)	0.01	0	0	0	0	0	0	0	-									
Punica granatum (10)	0.01	0	0	0	0	0	0	0	0	-								
Punica granatum (11)	0.01	0	0	0	0	0	0	0	0	0	-							
Punica granatum (12)	0.01	0	0	0	0	0	0	0	0	0	0	-						
Punica granatum (13)	0.01	0	0	0	0	0	0	0	0	0	0	0	-					
Punica granatum (14)	0.01	0	0	0	0	0	0	0	0	0	0	0	0	-				
Punica granatum (15)	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	-			
Punica granatum (16)	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-		
Punica granatum (17)	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	
Punica protopunica (18)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	-

Table 3. Estimates of evolutionary divergence between the families of the order Myrtales. All results are based on the pair-wise analysis of 73 ITS sequences given in table 1. Analyses were conducted using the Maximum Likelihood method in MEGA5 considering uniform rates of transitions and transversions. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 297 positions in the final dataset. Values in parentheses are the number of accessions in a family

	Punicaceae	**Lythraceae**	**Sonneratiaceae**	**Trapaceae**	**Myrtaceae**	**Rhynchocalycaceae**	**Psiloxylaceae**	**Crypteroniaceae**
Punicaceae (18)	0							
Lythraceae (17)	**0.10**	0						
Sonneratiaceae (4)	**0.10**	0.12	0					
Trapaceae (3)	0.19	0.19	0.16	0				
Myrtaceae (8)	0.22	0.21	0.19	0.24	0			
Rhynchocalycaceae (1)	0.23	0.24	0.22	0.25	0.21	0		
Psiloxylaceae (1)	0.28	0.27	0.24	0.30	0.16	0.29	0	
Crypteroniaceae (1)	0.25	0.26	0.24	0.28	0.24	0.17	0.34	0
Alzateaceae (1)	0.25	0.24	0.21	0.27	0.24	0.14	0.29	0.16
Oliniaceae (2)	0.24	0.25	0.22	0.27	0.25	0.12	0.35	0.14
Penaeaceae (2)	0.21	0.21	0.21	0.26	0.25	**0.10**	0.33	0.12
Onagraceae (1)	0.20	0.22	0.22	0.25	0.26	0.25	0.31	0.28
Memecylaceae (6)	0.30	0.30	0.30	0.32	0.30	0.27	0.38	0.25
Melastomataceae (3)	0.26	0.26	0.26	0.27	0.30	0.22	0.36	0.27
Combretaceae (4)	0.24	0.25	0.25	0.29	0.27	0.28	0.34	0.29
Vochysiaceae (1)	0.38	0.39	0.41	0.45	0.35	0.37	0.42	0.40

(*continued*)

	Alzateaceae	**Oliniaceae**	**Penaeaceae**	**Onagraceae**	**Memecylaceae**	**Melastomataceae**	**Combretaceae**	**Vochysiaceae**
Punicaceae (18)								
Lythraceae (17)								
Sonneratiaceae (4)								
Trapaceae (3)								
Myrtaceae (8)								
Rhynchocalycaceae (1)								
Psiloxylaceae (1)								
Crypteroniaceae (1)								
Alzateaceae (1)	0							
Oliniaceae (2)	0.12	0						
Penaeaceae (2)	**0.11**	**0.09**	0					
Onagraceae (1)	0.26	0.25	0.23	0				
Memecylaceae (6)	0.24	0.25	0.24	0.36	0			
Melastomataceae (3)	0.25	0.24	0.21	0.31	0.21	0		
Combretaceae (4)	0.30	0.28	0.30	0.26	0.35	0.36	0	
Vochysiaceae (1)	0.42	0.42	0.39	0.41	0.44	**0.49**	0.29	0

pattern of Trapaceae and Punicaceae was similar to that of the phylogenetic tree obtained in order Myrtales. All the accessions of *Punica* clustered together in a distinct clade with a strong bootstrap support of 99%. The genus *Woodfordia* showed closer affinities to *Punica* followed by *Pemphis* J.R. Forst. & G. Forst., in the phylogenetic tree of Lythraceae *s.l.*, in contrast to the close affinities of *Punica* to *Lawsonia* shown in the phylogenetic tree of order Myrtales. The genera *Sonneratia* L.f., and *Duabanga* Buch.-Ham., clustered with *Lagerstroemia* L., in both the trees of Lythraceae *s.l.,* and Myrtales raising doubts about the existence of the satellite family Sonneratiaceae or the genera included in this family. The genus *Cuphea* P. Browne was observed distinct from the other representatives of the family Lythraceae *s.s.*, and showed sister relationships to the family Trapaceae in the phylogenetic trees. The analysis of the ITS sequences of *Punica* in relation to the other members of the family Lythraceae *s.l.,* revealed that the genus *Punica* is distinct under the family Lythraceae (**Fig. 1**).

In Bayesian phylogenetic tree, Lythraceae *s.l.,* formed a strongly supported monophyletic group and Onagraceae was a sister group but without significant support. Within this group four major strongly supported clades were found. Clade I included monophyletic lineages of *Punica*, *Woodfordia* and *Cuphea*, the latter two are grouped together forming a sister clade to *Punica*. Species of *Lagerstroemia* and the members of Trapaceae, Sonneratiaceae were clustered together in clade II and *Ammannia* L., *Nesaea* Kunth, and *Lawsonia* were grouped in clade III. *Lythrum* L., and *Heimia* Link were grouped in a separate clade IV. *Pemphis* was included in the Lythraceae *s.l.,* with unresolved relationship (**Fig. 2**).

The family Psiloxylaceae was strongly supported as a sister group to the family Myrtaceae. The monogeneric families such as Alzateaceae, Oliniaceae and Rhynchocalycaceae showed monophyletic status with Crypteroniaceae and Penaeaceae where they altogether formed a strongly supported distinct clade and the sister group relation to the family Myrtaceae lacked support. The family Memecylaceae which had been treated under the family Melastomataceae (APG III, 2009) formed a strongly supported monophyletic lineage.

Discussion

The delimitation of the family Lythraceae has been problematic historically, whereas a general consensus exists that the genera of the Lythraceae *s.s.,* are closely related to each other. There has been considerable disagreement whether the satellite genera such as *Duabanga*, *Punica* and *Sonneratia* should be included in the Lythraceae, all of which have been placed in separate families at various times (Koehne, 1903; Melchior, 1964; Hutchinson, 1973; Dahlgren, 1980; Takhtajan, 1980, 1986; Cronquist, 1981, Thorne, 1983; Tobe & Raven, 1983; Dahlgren & Thorne, 1984; Johnson & Briggs, 1984). There is a common agreement that *Punica* is closely related to, and most probably derived from, Lythraceae, from which it is distinguished by several specialised features, such as fruits with leathery pericarp and pulpy seeds with edible sarcotesta (Dahlgren & Thorne, 1984; Graham *et al.,* 1990, 2005). These unique features prompted some authors to treat Punicaceae as a separate family. In contrast, the ovules of *Punica*, with their thick, multilayer outer integument and unicellular archesporium, differ from those of Lythraceae *s.s.* Wood anatomy (Bridgewater & Baas, 1978; Graham *et al.,* 1993), chromosome data (Tobe *et al.,* 1986), and pollen morphology (Patel *et al.,* 1984; Graham *et al.,* 1990), however, suggested a close relationship with Lythraceae *s.l*. The combination of all these features leads Tobe & Raven (1983) to suggest *Punica* as being a distinct archaic offshoot within Lythraceae *s.l.* This placement of *Punica*, was also indicated by the rbcL gene tree but with weak support (Conti *et al.,* 1997).

The close relation of the family Onagraceae with the strongly supported monophyletic lineage Lythraceae *s.l.,* found in this investigation was in agreement to the previous studies (Hung & Shi, 2002; Graham *et al.,* 2005). The six "crown" clades recovered by all analyses of Graham *et al*. (2005), were congruent to the clades found in the present study, except unresolved relation of *Pemphis* and the strongly supported clade IV included *Heimia* and *Lythrum* (**Fig. 2**). In phylogenetic tree (**Fig. 1**) *Pemphis* appeared sister to the *Punica* and *Woodfordia* clade, however the relation lacked support. The outside position of *Pemphis* from the "crown" clade III labelled by Graham *et al.* (2005), makes sense, because no morphological evidences of affinities between *Pemphis* and *Punica* were found in previous cladistic analyses (Johnson & Briggs, 1984; Graham *et al.,* 1993). In the Lythraceae lineage, the satellite monogeneric family Trapaceae was clustered with Sonneratiaceae, whereas another satellite family Punicaceae was grouped together with two genera (*Woodfordia, Cuphea*) of the family Lythraceae in a distinct clade. The paraphyly of the family Lythraceae, lack of morphological synapomorphies in the clades, and weak support

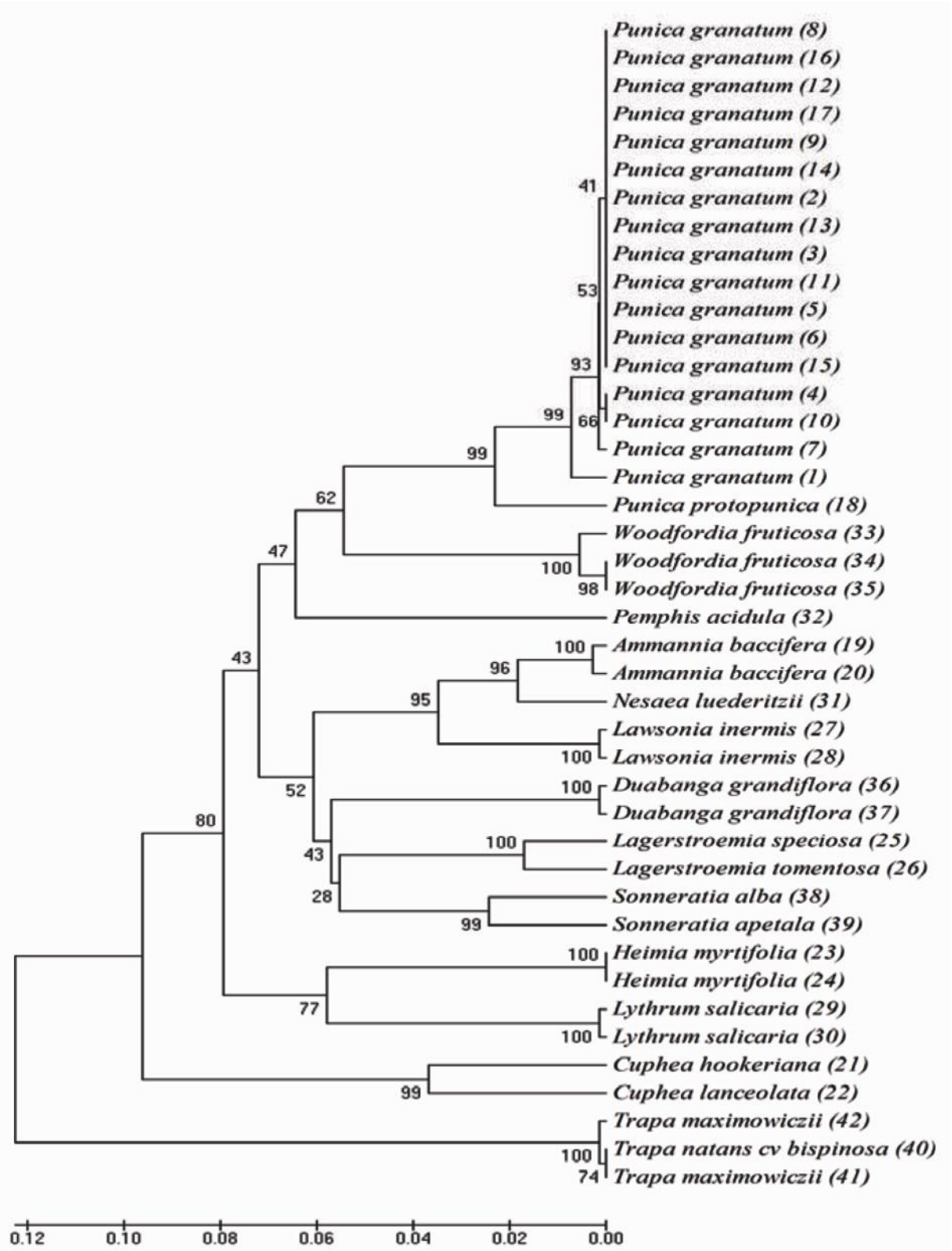

Fig. 1. Phylogenetic tree of the family Lythraceae *s.l.* based on ITS sequences of nuclear rDNA generated using UPGMA.

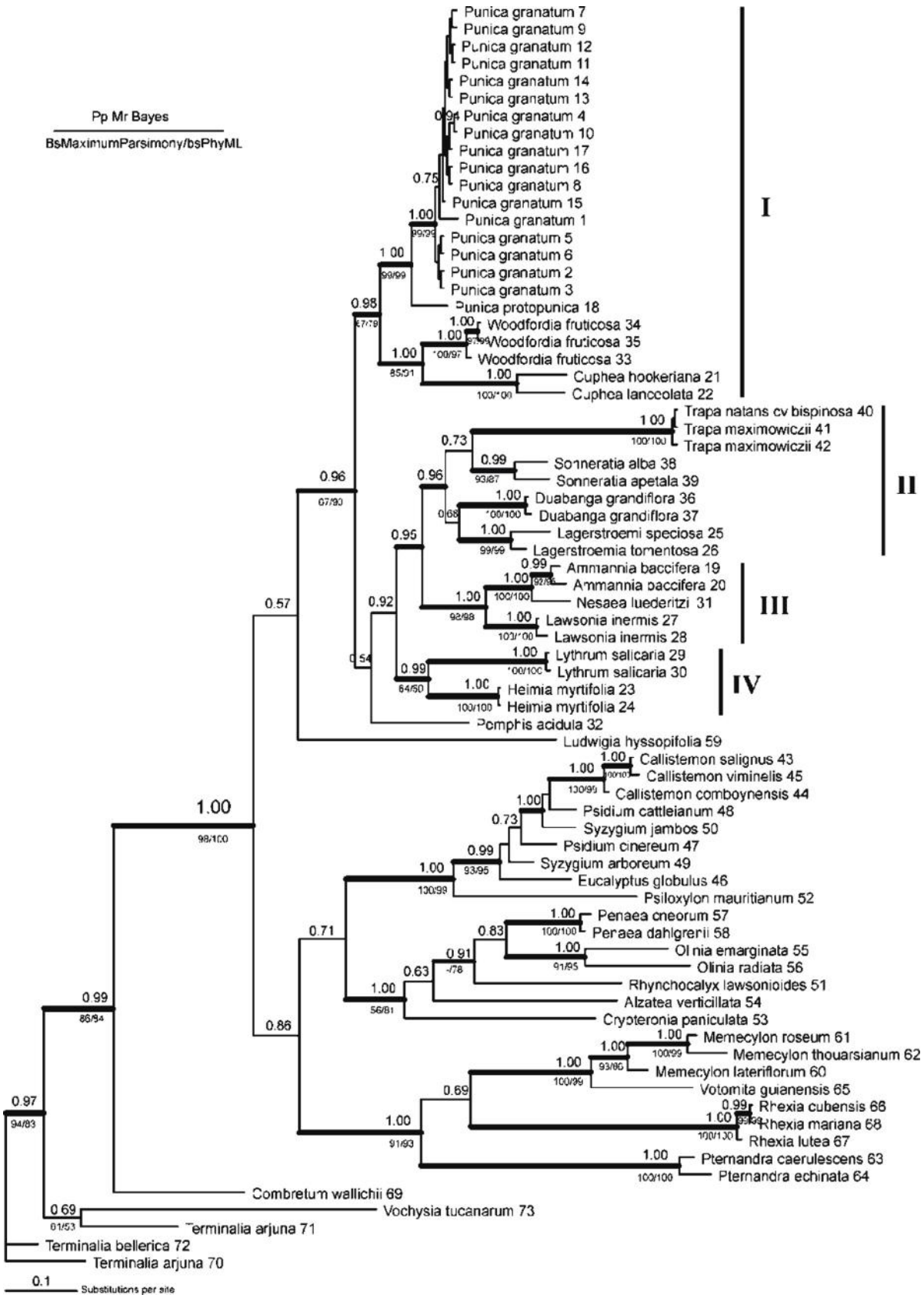

Fig. 2. Consensus (50%-majority-rule) based on 90,000 trees from B/MCMC tree sampling procedure from nuclear ITS rDNA dataset.

on the node included Trapaceae, Sonneratiaceae and Lythraceae *p.p.* (**Fig. 2**), support an early pattern of rapid radiation in the family (Huang & Shi, 2002).

Phylogenetic tree exclusively generated for 42 representative taxa of the family Lythraceae *s.l.* (**Fig. 1**), showed similar clustering pattern of Trapaceae and Punicaceae to that of the phylogenetic tree obtained in order Myrtales (**Fig. 2**). However, the reorganization of the genera under the families Lythraceae *s.s.,* and Sonneratiaceae were observed. All the accessions of *Punica* clustered together in a distinct clade with a strong bootstrap support (99%). The present findings however could further be substantiated with comparative sequencing of other phylogenetically informative regions of chloroplast and nuclear DNA.

Acknowledgements

Partial financial support from the Department of Science and Technology, New Delhi (to TSR) is gratefully acknowledged. We thank the anonymous reviewer for constructive suggestions and modifications in the manuscript.

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Received: 21.3.2016
Revised and Accepted: 9.6.2016