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Phylogenetic relationships within the Lythraceae in Iran

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Abstract

The Lythraceae consists of a group of plants with a complex taxonomy. The difficulty of classifying them becomes particularly clear when morphological characteristics are used. In the Flora Iranica and the Flora of Iran, this family is represented by five and four genera, respectively. In the present study, three genera *Lythrum*, *Ammannia*, and *Rotala*, and six species of Lythraceae growing in Iran were analyzed, using nine cpDNA *trn*H-*psb*A and 21 nrDNA ITS sequences retrieved from GenBank. Phylogenetic relationships within the family were evaluated based on cpDNA *trn*H-*psb*A and nrDNA ITS, as well as combined datasets using maximum parsimony, maximum likelihood, and Bayesian inference methods. Most of the reconstructed trees revealed the monophyly of the above-mentioned genera in Iran. All trees confirmed the distinctiveness of species within these three genera. The results of this study indicate a phylogenetic alliance of *L. thesioides* with *L. silenoides* and the three species *L. thymifolia*, *L. junceum* and *L. hyssopifolia.* Moreover, *A. coccinea* is evolutionarily related to *A. multiflora* and *A. baccifera* to *A. auriculata.* The results indicated that, a taxonomic revision of these genera is required. In addition, the evolutionary patterns of some diagnostic morphological and micromorphological characters were evaluated in this study.

Keywords: Monophyly, morphological characteristics, phylogeny, sequence, taxonomy

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خلاصه

گل‌حنائیان، تیره‌ای متشکل از گروهی از گیاهان، دارای رده‌بندی پیچیده‌ای است. دشواری در رده‌بندی آن‌ها به ویژه وقتی مشخص می‌شود که از صفات ریخت‌شناسی استفاده شود. در فلورا ایرانیکا و فلور ایران، به ترتیب پنج و چهار جنس از این تیره گزارش شده است. در مطالعه حاضر، سه جنس *Lythrum*، *Ammannia* و *Rotala* و شش گونه از گل‌حنائیان در ایران، با استفاده از نُه توالی کلروپلاستی *trn*H-*psb*A و ۲۱ توالی هسته‌ای ITS که از بانک ژن دریافت شد، مورد بررسی قرار گرفتند. روابط فیلوژنتیکی این تیره براساس داده‌های توالی کلروپلاستی *trn*H-*psb*A، توالی هسته‌ای ITS و ترکیبی از این توالی‌ها با استفاده از روش‌های بیشینه صرفه‌جویی، بیشینه احتمال و بیزین مورد ارزیابی قرار گرفت. بیشتر درخت‌های بازسازی شده، تک‌تباری سه جنس مذکور را در ایران نشان دادند. همه درخت‌ها، متمایز بودن گونه‌ها در سه جنس مذکور را تایید کردند. یافته‌های این تحقیق بیانگر اتحاد فیلوژنتیکی *L. thesioides* با *L. silenoides* و سه گونه *L. thymifolia*، *L. junceum* و *L. hyssopifolia* بود. به علاوه، از لحاظ تکاملی، *A. coccinea* با *A. multiflora* و *A. baccifera* با *A. auriculata* رابطه خویشاوندی نشان داد. نتایج حاصل، نیاز به بازنگری آرایه‌شناختی این جنس‌ها را آشکار ساخت. همچنین، در این بررسی، الگوهای تکاملی برخی صفات تشخیصی ریخت‌شناسی و ریزریخت‌شناسی مورد ارزیابی قرار گرفت.

واژه‌های کلیدی: آرایه‌شناسی، تبارزایی، تک‌نیایی، توالی، صفات ریخت‌شناسی

Introduction

The Lythraceae belongs to the order Myrtales within the Eudicots in the clade Malvids forms a monophyletic group with the Geraniales (APG IV 2016) with around 28 genera and 700 species (POWO 2024). Most genera of this family are found growing in wetland, aquatic, or marshy habitats, especially in northern and western Iran (Graham 2007, Yousef Naanaie 2010).

Phylogenetic, morphological, and embryological data indicate a close relationship between Lythraceae and Onagraceae (Dahlgren & Thorne 1984, Johnson & Briggs 1984). Subsequent molecular analyses support this relationship (Conti *et al*. 1997, Sytsma *et al*. 2004, APGIV 2016). Several members of the family have undergone rapid evolutionary changes, as suggested by molecular studies and fossil evidence (Graham *et al*. 2005). The family is geographically widespread and exhibits great diversity, including aquatic plants, shrubs, and trees. While most genera have only one or two species, some are placed in separate subfamilies or even monogeneric families (Koehne 1903, Cronquist 1982, Dahlgren & Thorne 1984, Johnson & Briggs 1984, Takhtajan 1987). The delimitation of generic boundaries for the whole family is complicated because of diverse floral traits and natural habitats. There are only a few studies on the morphological and micromorphological characteristics of this family in Iran.

Polatschek & Rechinger (1968) introduced five genera for the family, namely, *Lawsonia* L., *Lythrum* L., *Ammannia* L., *Rotala* L., and *Peplis* L. Among these, *Lawsonia*, *Rotala*, and *Peplis* were each represented by a single species, while *Lythrum* and *Ammannia* comprised nine and four species, respectively. The diversity center of the *Ammannia*, *Lythrum*, and *Rotala* is in the Old World (Graham 2007). According to Yousef Naanaie (2010), four genera are recognized in Iran, namely, *Lawsonia, Lythrum*, *Ammannia*, and *Rotala.* These genera are distinguished on the basis of morphological characteristics such as leaf arrangement, inflorescence type, and fruit type (Yousef Naanaie 2010). *Lawsonia inermis* L. is cultivated in western and southern Iran. *Lythrum* and *Ammannia* comprise seven and four species, respectively, which are distributed in different regions of Iran (Yousef Naanaie 2010). A new species of *Ammannia*, *A. coccinea* Rottb., was reported by Naqinezhad & Naseri Larijani (2017) from Guilan Province (north of Iran).

Rotala is represented by only one species, *R. indica* (Willd.) Koehne, which occurs in northern Iran. For two other species, *R. densiflora* (Roth.) Koehne and *Peplis hyrcanica* Sosn., which are listed in the Flora Iranica (Polatschek & Rechinger 1968), there is no corresponding herbarium evidence. Their absence suggests that, they have not been observed or collected in Iran. It is assumed that, their inclusion in the Flora Iranica is based more on reports from the flora of the Soviet Union than on direct evidence from Iranian habitats. Moreover, Yousef Naanaie (2010) found no evidence for the occurrence of these species in the natural habitats of Iran.

Based on recent studies, microscopic pollen and leaf examinations provide important taxonomic data for Lythraceae research. According to Mahmoodi *et al.* (2022a), qualitative pollen characteristics are essential for distinguishing generic boundaries; while Mahmoodi *et al.* (2022b) showed how leaf micromorphology is used in species identification.

Molecular research has expanded our knowledge of the systematics of the Lythraceae. Morris (2007) analyzed molecular data from two chloroplast regions, specifically *atp*B-*rbc*L and *trn*K-*mat*K, together with previously sequenced data from *trn*L-*trn*F, *rbc*L, *psb*A-*ycf*3 and nrDNA ITS for 29 genera belonging to the Lythraceae. It was found that, several convergent developments had taken place in both morphological features and life history traits during the evolutionary history of the family (Morris 2007). Following Gu *et al*. (2019), a comparison of the chloroplast genomes of 22 species of the Lythraceae was carried out. The phylogenetic analyses included 42 Myrtales species. It was found that, within the order Myrtales, the Lythraceae and Onagraceae diverged later compared to the Melastomataceae and Myrtaceae.

The taxonomic complexity of the Lythraceae leads to uncertainties regarding genera and species relationships. As mentioned earlier, there is considerable uncertainty regarding the status of *Lythrum*, *Ammannia*, and *Rotala* in Iran. Therefore, the aims of this study were: 1) to carry out a comprehensive analysis of the systematic organization of above-mentioned three genera in Iran; 2) to construct a phylogenetic tree to clarify their evolutionary relationships; and 3) to evaluate trait evolution based on the current phylogenetic study.

Materials and Methods

- Plant sampling

Six species of Lythraceae growing in Iran were examined in this study. Specimens were obtained from the herbaria of the Research Institute of Forests and Rangelands (TARI), the University of Tehran (TUH), and the University of Guilan (GUH). Furthermore, some specimens were collected from their natural habitats (Table 1). The collected specimens were carefully evaluated for taxonomic identification using Flora Iranica (Polatschek & Rechinger 1968), the Flora of Iran (Yousef Naanaie 2010), and the Flora of Turkey (Davis 1988). Additionally, sequence data from previous studies were retrieved from GenBank, including nine taxa for the *trn*H-*psb*A region and 21 (including outgroups) for the nrDNA ITS (Appendix 1). Based on Morris (2007), *Ludwigia grandiflora* (Michx.) Greuter & Burdet and *Lu. peruviana* (L.) H. Hara (Onagraceae) were used as outgroups.

Table 1. Plant species sequenced in the present study, along with their corresponding herbarium details and GenBank accession numbers in Iran

Taxon	GenBank accession No. (*trn*H-*psb*A)	Locality along with related information
Ammannia auriculata Willd.	LC822553	Lorestan prov.: Khorramabad, Cham-Divan, 1000 m, Veise Karami 24108 (TUH)
A. multiflora Roxb.	LC822554	Gilan prov.: Someh Sara, Hendekhaleh District, Nokhaleh Akbari Village, 21 m, 2017, Ashouri 8553 (GUH)
Lythrum silenoides Boiss. & Noë	LC822552	Lorestan Prov.: Khorramabad, Cham-e Divan, 1000 m, 1998, Veise Karami 24113 (TUH)
L. thesioides M. Bieb.	LC822551	Fars Prov.: 6 km south of Jahrom, between Hood and Kooreh Villages, 800 m, Assadi & Akhani 61867 (TARI)
L. virgatum L.	LC822550	W. Azerbaijan Prov.: Between Salmas and Qushchi, Khan Takhti, 1400 m, 1993, Ghahreman & Mozaffarian 17444 (TUH)
Rotala indica (Willd.) Koehne	LC822549	Gilan Prov.: Siahkal, Khararud District, Salash Village, 55 m, 2017, Ashouri 8555 (GUH)

- DNA extraction and Sanger sequencing

The total DNA was extracted from fresh and dried leaf tissues using the 2 x CTAB procedure developed by Doyle & Doyle (1987). Additionally, the DNeasy Plant Mini Kit (Qiagen Valencia, CA, USA) was used following the manufacturer's instructions. For molecular investigations, the plastid *psb*A-*trn*H intergenic spacer region and the nuclear ribosomal ITS markers were selected.

Amplification of the *psb*A-*trn*H region was carried out with the primers *trn*Hf-05 (CGCGCATGGTGGATTCACAATCC) and *psb*A3-f (GTTATGCATGAACGTAATGCTC), developed by Tate & Simpson (2003) and Sang *et al.* (1997), respectively. PCR reactions were conducted in a volume of 25 µL, which included 9.5 µL deionized water, 12.5 µL of 2x Taq DNA polymerase master mix RED (Amplicon, Cat No. 180301), 1 µL of

each primer (10 pmol/µL), and 1 µL of template DNA (20 ng/µL). The amplification of the *psb*A-*trn*H region included an initial denaturation step at 93 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 48–56 °C for 45 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 5 min. Sequencing was performed using an ABI 3730xl capillary sequencer (Thermo Fisher Scientific, Waltham, USA; Applied Biosystems, USA) at the NiaGen company (Tehran, Iran).

- Phylogenetic analyses

Sequences were aligned using MAFFT (available online at https://ngphylogeny.fr), with subsequent minor manual adjustments. Phylogenetic relationships were analyzed using maximum parsimony (MP), maximum likelihood (ML) methods, and Bayesian inference (BI). The maximum parsimony analysis was performed using PAUP* 4.b10 software (Swofford 2002), Bayesian inference was conducted using MrBayes on the CIPRES gateway (Miller *et al.* 2010), and maximum likelihood analysis was carried out on the IQ-TREE web server (http://iqtree.cibiv.univie.ac.at). For the maximum parsimony method, a heuristic search option was conducted with 1,000 replications of random addition sequences with ten trees retained at each step. Tree bisection-reconnection (TBR) branch swapping was used, with Mul-Trees enabled and steepest descent disabled. Branch support was evaluated using 1,000 bootstrap replicates, providing bootstrap percentages (bootstrap support, BS), following the same settings as those used for the heuristic searches (Felsenstein 1985). The rescaled consistency index (RC), consistency index (CI), and retention index (RI) were calculated using PAUP* 4.b10 software (Swofford 2002) to assess tree quality. To determine the appropriate evolutionary model for the data, the IQ-TREE web server was utilized. Based on the Akaike information criterion (AIC), the TVM+F+I model was selected for the cpDNA *trn*H-*psb*A data, the GTR+F+G4 model for the nrDNA ITS data, and the combined data in both ML analysis and Bayesian inference.

Four Markov chain Monte Carlo (MCMC) heuristic searches were conducted for Bayesian analyses, with each search comprising 10 million generations and sampling every 1,000 generations. Tracer version 1.6 (Rambaut *et al.* 2014) was used to check the convergence of parameter estimates, with the effective sample sizes exceeding 200 for all parameters. The first 25% of trees were discarded as burn-in. Support for nodes was calculated using the posterior probability (PP). The remaining trees were utilized to create a consensus tree, which was visualized using TreeView v.1.6.1 (Page 2001).

- Characters evolution

The evolutionary patterns of three palynological traits, two morphological and micromorphological traits of leaves and the type of inflorescence (Table 2), were mapped onto the concatenated ML tree. These morphological traits are taxonomically informative and are used for genus classification (Polatschek & Rechinger 1968, Yousef Naanaie 2010, Mahmoodi *et al.* 2022a, b).

Results

All phylogenetic analyses typically generated consistent tree topologies. Analysis of the combined data led in higher resolution and node support than the results obtained from individual nrDNA ITS and *trn*H-*psb*A trees. Given the absence of discrepancies among the analyses, authors of the paper have exclusively focused on describing and discussing the maximum likelihood tree derived from the nrDNA ITS, *trn*H-*psb*A, and combined datasets. Bootstrap values and posterior probabilities are indicated along the branches in figures 1–3.

Table 2. Morphological and micromorphological traits used to evaluate trait evolution

Leaf wax ornamentation	Pollen			Inflorescence type	Leaf arrangement
	Number of pseudocolpi	Pseudocolpi membrane ornamentation	Exine sculpturing		
Crust/striate epicuticular folding (Type I)	3	Micro-verrucate to micro-baculate	Striate	Solitary, or with axillary cyme	Opposite
Smooth layer with long and short striate epicuticular folding and granule (Type II)	6	Psilate	Striate-regulate	Spike-like, with multi-flowered or solitary cyme	Alternate
Irregular platelets (Type III)			Rugulate		
Tuberculate and granule (Type IV)					
Crust/platelets/rodlets (Type V)					
Crus/granule (Type VI)					
Alveolate with striate epicuticular folding, granule (Type VII)					
Reticulate-alveolate tuberculate (Type VIII)					
Reticulate (Type IX)					

- Cladistic analysis of *trn*H-*psb*A intergenic spacer data

The length of the *trn*H-*psb*A sequences of the taxa varied from 150 bp (in *A. coccinea*) to 387 bp (in *Lu. grandiflora*). In the maximum likelihood tree, the main node A is strongly supported (MP BS = 100, ML BS = 100, PP = 1). *Lythrum* species exist separately on lower clades, forming an unsupported non-monophyletic group. The two species, *L. virgatum* and *L. salicaria*, diverged first from the other species in the group (MP BS = 80, ML BS = 78, PP = 0.87). Subsequently, the remaining species are found in sub-clades A1 (above) and A2 (below). Sub-clade A2, which includes *L. thesioides* and *L. silenoides* (MP BS = 78, ML BS = 87, PP = 0.93), while A1, which has low support and includes species of *Rotala* and *Ammannia*, as well as *Sonneratia alba*, *Trapa natans*, *Punica granatum*, and *Lawsonia inermis*. Additionally, *Ammannia* forms a monophyletic group (MP BS = 81, ML BS = 85, PP = 0.97) (Fig. 1).

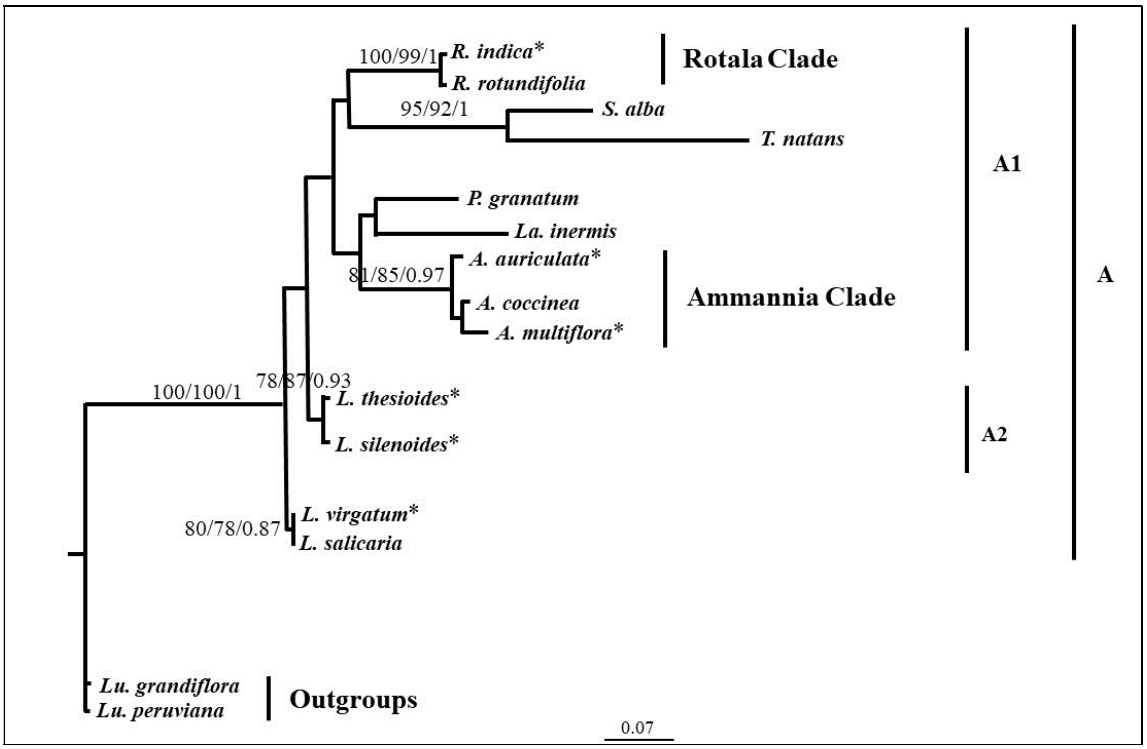

Fig. 1. A maximum likelihood phylogenetic tree inferred from *trn*H-*psb*A data of Lythraceae species. Bootstrap support values from maximum parsimony and maximum likelihood analyses greater than 50%, as well as posterior probabilities from Bayesian inference greater than 0.5, are indicated above the branches. Asterisks denote newly sequenced samples in this study.

- Cladistic analysis of nrDNA ITS data

The length of the ITS sequences of the taxa ranged from 198 bp (in *L. thesioides*) to 534 bp (in *S. alba* and *La. inermis*). The maximum likelihood tree based on nrDNA ITS data revealed that, the Lythraceae is monophyletic (node A) with high support (MP BS = 100, ML BS = 100, PP = 1). The relationships among genera remained unresolved, as they formed a polytomy with three sub-clades: A1, A2, and A3. Sub-clade A1 includes all the examined *Lythrum* species in the "Lythrum" clade, with three species of *L. hyssopifolia*, *L. junceum*, and *L. thymifolia*, along with *L. virgatum* and *L. salicaria* forming a monophyletic group (MP BS = 97, ML BS = 96, PP = 1). Sub-clade A2 contains the *Ammannia* species in the "Ammannia" clade with high support (MP BS = -, ML BS = 100, PP = 1), along with *La. inermis*, *D. grandiflora*, *S. alba*, and *T. natans*. Sub-clade A3 comprises the "Rotala" clade (*R. indica* and *R. rotundifolia*) (MP BS = 100, ML BS = 100, PP = 1), and *P. granatum* (Fig. 2).

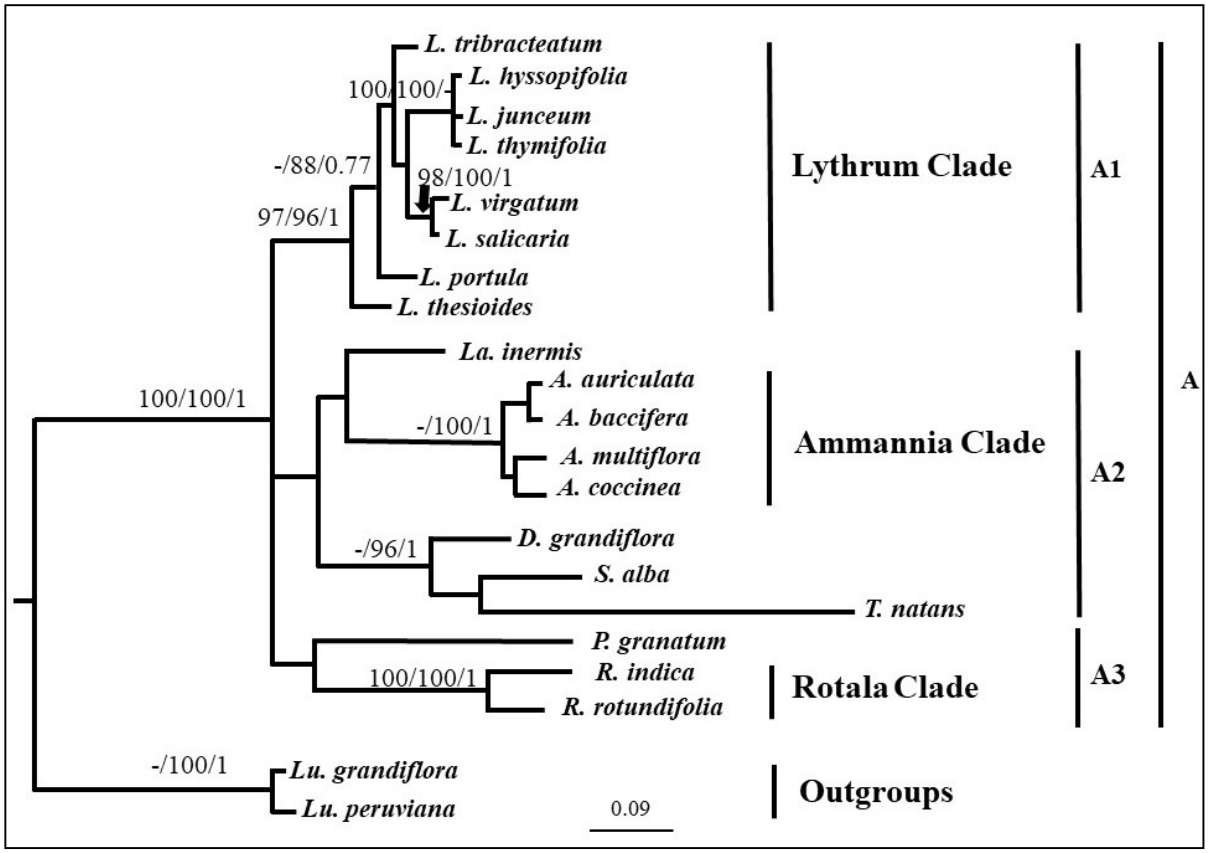

Fig. 2. A maximum likelihood phylogenetic tree inferred from ITS data of Lythraceae species. Bootstrap support values from maximum parsimony and maximum likelihood analyses greater than 50%, as well as posterior probabilities from Bayesian inference greater than 0.5, are indicated above the branches.

- Cladistic analysis of combined data

The phylogenetic tree reconstructed from the combined data exhibits high-support for node A (MP BS = 100, ML BS = 100, PP = 1) that contains clades A1 and A2. Clade A1 further divides into two sub-clades, A1a and A1b. A1a, also known as the "Rotala" clade, includes two species, *R. indica* and *R. rotundifolia*, which form a monophyletic group (MP BS = 100, ML BS = 100, PP = 1), along with *P. granatum* on a separate branch. Additionally, three species, *T. natans*, *S. alba* (in a small sub-group), and *D. grandiflora* (on a separate branch), are positioned within this clade (MP BS = -, ML BS = 99, PP = 1).

Sub-clade A1b includes species of *Ammannia* in the "Ammannia" clade (MP BS = -, ML BS = 100, PP = 1), along with *La. inermis* (MP BS = 73, ML BS = 100, PP = 0.93). The species of *Lythrum* are placed in sub-group A2 or the "Lythrum" clade (MP BS = 98, ML BS = 95, PP = 1). Among them, two species, *L. silenoides* and *L. thesioides* are grouped in sub-group A2b (MP BS = 97, ML BS = 99, PP = 0.93), while three species, *L. hyssopifolia*, *L. junceum*, and *L. thymifolia*, are placed in sub-group A2a (MP BS = 92, ML BS = 100, PP = 1). Finally, two species, *L. virgatum* and *L. salicaria*, are clustered together (MP BS = 99, ML BS = 100, PP =1) (Fig. 3).

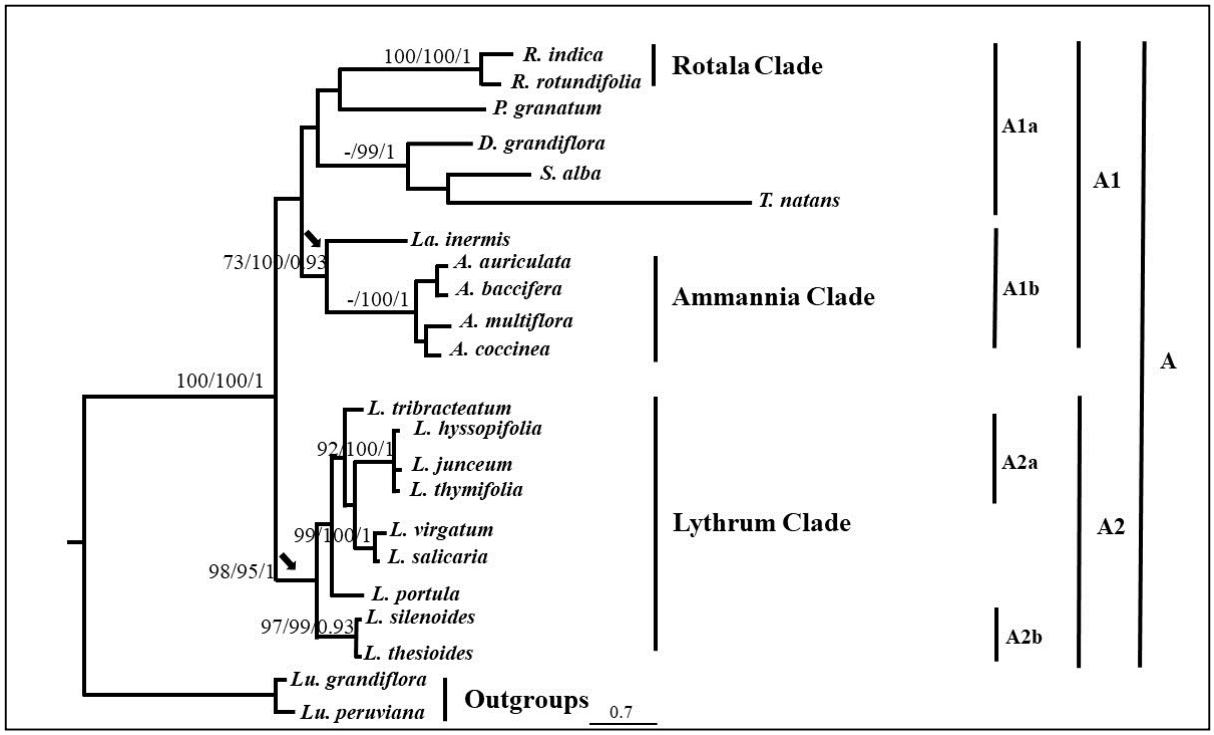

Fig. 3. A maximum likelihood phylogenetic tree inferred from combined data of Lythraceae species. Bootstrap support values from the maximum parsimony and maximum likelihood analyses greater than 50%, and posterior probabilities from Bayesian inference greater than 0.5, are indicated above the branches.

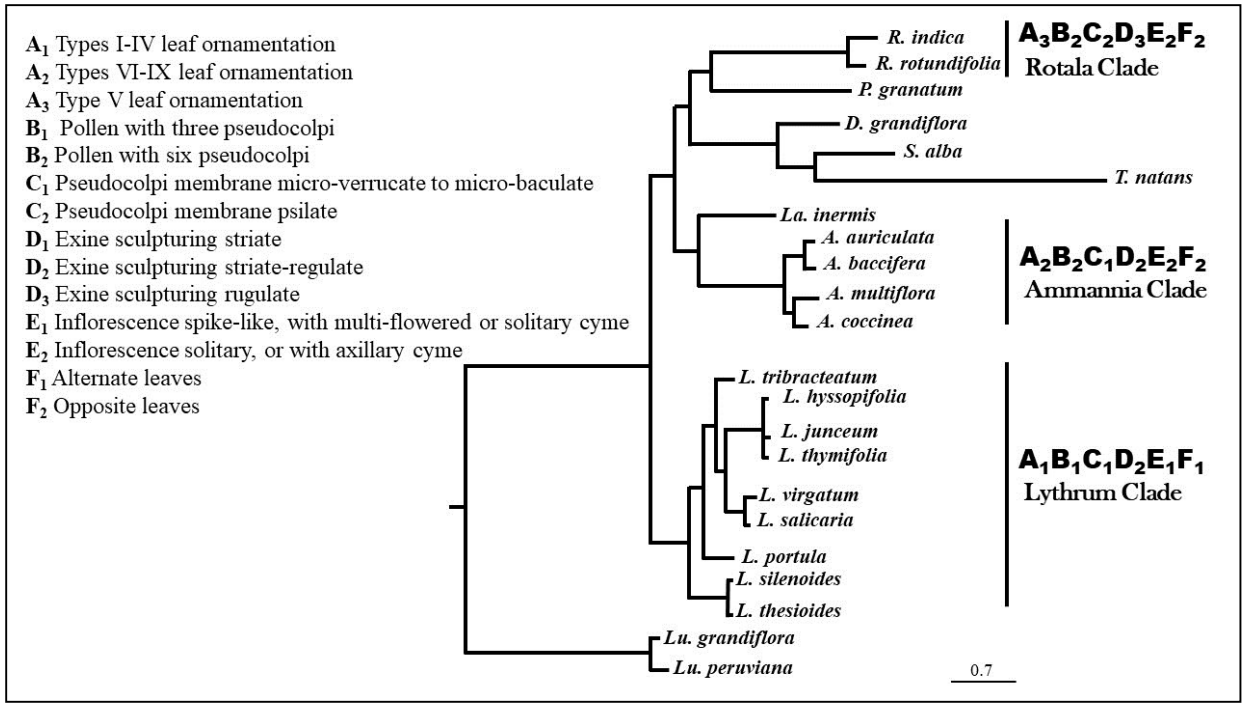

Fig. 4. Character reconstruction (including leaf epicuticular wax ornamentation, number of pseudocolpi, pseudocolpi membrane and exine sculptures, inflorescence type, and leaf arrangement) based on the ML tree topology.

Discussion

The phylogenetic trees generated using cpDNA, nrDNA, and combined sequences demonstrated monophyly of *Lythrum*, *Ammannia*, and *Rotala* in Iran, except the cpDNA ML tree. In all the resulting trees, species of these three genera were entirely separated into distinct groups. These findings agree with previous studies (Graham *et al.* 2005, Morris 2007). The genus *Lythrum* is characterized by its alternate upper leaves, spike-like inflorescence, and multi-flowered or solitary cymes (Yousef Naanaie 2010). In all phylogenetic analyses, *L. virgatum* and *L. salicaria* were consistently grouped in one clade, which was also observed in Morris (2007). Koehne (1903) classified these two species under the subgenus *Salicaria* due to their inflorescence type and heterostyly. Cytogenetic studies have shown that, these species possess a higher ploidy level than other species in this genus (n = 5, 10). The basic chromosome number is reported as n = 15 in *L. virgatum* and n = 15, 25, and 30 in *L. salicaria* (Graham & Cavalcanti 2001). Both *L. virgatum* and *L. salicaria* are perennial herbs sharing similar morphological features such as up to eight axillary flowers, winged-quadrangular stem, sessile leaves, 12 stamens, heterostyle, and capitate stigma (Yousef Naanaie 2010). The close relationship between these two species is strongly supported by shared leaf micromorphological features (e.g., stomata width, outer pristomata, and inner stomata rim types), as well as palynological traits, including similar colpi width, pollen grain outlines from both equatorial and polar views, colpi and pseudocolpi membrane types, and exine sculpturing (Mahmoodi *et al.* 2022a, b).

Lythrum thesioides and *L. silenoides* were also grouped in the chloroplast tree. Both species are annual with 2–8 stamens (Yousef Naanaie 2010) and share several affinities in leaf micromorphology, including glabrous leaves, a raised outer periclinal layer, and a sinuolate-erose inner stomatal rim. Additionally, they exhibit similar palynological features, such as tricolporate pollen grains with six pseudocolpi, colpi, and pseudocolpi membranes micro-verrucate to micro-baculate, and colpi longer than pseudocolpi (Mahmoodi *et al.* 2022a, b). These common traits are in agreement with the phylogenetic findings.

Lythrum hyssopifolia, *L. thymifolia*, and *L. junceum* formed a monophyletic group in the nrDNA ITS and combined trees. These three species share common morphological characteristics, including solitary flowers, a hypanthium at least 3 mm long, and a cylindrical shape at the fruiting stage (Polatschek & Rechinger 1968, Yousef Naanaie 2010). Their close relationship is further supported by leaf micromorphological features, such as a raised outer periclinal layer, overlapping-stout pristomata, and sinuolate inner stomata rim, as well as palynological characters, including micro-verrucate to micro-baculate colpi and pseudocolpi membrane) (Mahmoodi *et al.* 2022a, b). Among these, *L. hyssopifolia* and *L. junceum* also formed a monophyletic group in the previous phylogenetic study by Morris (2007). The morphological and cytological findings align with the molecular data. Koehne (1903) placed these two species close to Eurasian *Lythrum* species due to their characteristic one or two flowers per axis, a thick nectar ring in the ovary, and a relatively long style.

Lythrum hyssopifolia (n = 10) is an annual herb with an erect ascending stem, homomorphic flowers, 2–6 stamens, and a capsule shorter than the hypanthium (Koehne 1903, Webb 1967, Yousef Naanaie 2010). This species, which is widespread in seasonally wet habitats in southern Eurasia, has also become invasive in several regions of the world. *Lythrum junceum* (n = 5) is a biennial to perennial species characterized by a decumbent stem, tristylous flowers, 12 stamens, and a corolla tube length of 5–6 mm. It is distributed in southern Europe and Central Asia (Polatschek & Rechinger 1968, Morris 2007). *Lythrum thymifolia*, an annual species, features homomorphic flowers, 2–6 stamens, and a short corolla length. It is distinguished from *L. hyssopifolia* by differences in hypanthium and corolla length (Polatschek & Rechinger 1968).

Based on the present findings, *L. portula* formed an independent clade and exhibited a sister group relationship with other *Lythrum* species (*L. salicaria*, *L. virgatum*, *L. thymifolia*, *L. junceum*, *L. hyssopifolia*, and *L. tribracteatum*) in the tree derived from ITS dataset. *Lythrum portula* is an annual, hairless herbaceous plant with a creeping stem and roots at the nodes, opposite leaves, short and weak petioles, an oval or almost round shape, and a rounded apex. Although the species is native to Europe, it also occurs in western Asia, often growing in wet habitats such as marshes (Webb *et al.* 1988, Johnson & Brooke 1989).

The species of *Ammannia* formed a monophyletic group in both trees. In cpDNA trees, *A. auriculata* Willd., *A. coccinea*, and *A. multiflora* clustered together, while in ITS trees, *A. auriculata* grouped with *A. baccifera* and *A. multiflora* with *A. coccinea* was united in smaller clades. According to Graham's classification (1985), *Ammannia* species were primarily divided into two subgenera, *Ammannia* and *Cryptotheca* (Blume) Koehne. These subgenera were further categorized into two sections and four series based on style length, style to carpel length ratio, petal presence or absence, and petal color. For instance, *A. baccifera* is grouped under the section *Ammannia*, identified by styles that are nearly 0.5 mm or shorter, while *A. auriculata* and *A. coccinea* are classified in the *Eustylia* section, which comprises species with styles ranging from 1 mm in length or longer.

In the Flora Iranica (Polatschek & Rechinger 1968) and the Flora of Iran (Yousef Naanaie 2010), species classification based on style length and inflorescence type placed *A. auriculata* with *A. multiflora* (both having a style length of 1.0–2.0 mm) and *A. baccifera* with *A. verticillata* (Ard.) Lam. (characterized by either a shorter style (to 0.25 mm) or the complete absence of a style). However, in the current research, *A. baccifera* grouped with *A. auriculata*, while *A. multiflora* clustered with *A. coccinea* (Fig. 2), forming a monophyletic group. This inconsistency means that, the phylogenetic reconstruction of *Ammannia* species is in conflict with classifications based on morphological features. A revision of the species classification within the genus is, therefore, necessary. The close relationship between *A. baccifera* and *A. auriculata* is further supported by common morphological, anatomical, and palynological characteristics. These two annual herbaceous species share several distinguishing characteristics, including an erect, glabrous stem; opposite, acute leaves; cyme inflorescence; campanulate hypanthium during flowering, four triangular sepals, bracteoles shorter than the floral tube; stamens equal in length to the hypanthium, spherical ovaries, capitate stigma, and spherical capsules (Haining *et al.* 2007, Yousef Naanaie 2010). They also exhibit similarities in leaf micromorphological features, such as stomatal size and inner stomata rim shape (Mahmoodi *et al.* 2022b), as well as in pollen morphology, including the equatorial outline of pollen grain, similar colpi and pseudocolpi widths, and colpi longer than pseudocolpi (Mahmoodi *et al.* 2022a). Furthermore, the close affinity between *A. multiflora* and *A. coccinea*, is supported by their morphological similarities, as both are herbaceous annuals with opposite, linear or lanceolate leaves, cyme inflorescence, and four stamens.

The phylogenetic tree reconstructed in this study also indicates a sister relationship between *La. inermis* and the species of *Ammannia*. *Lawsonia inermis* is a glabrous shrub or small tree, reaching 2–6 m in height, and is native to North Africa and Southwest Asia (Yadav *et al.* 2013). It is both cultivated and naturally occurring in western and southern Iran. In all reconstructed trees, the species of *Rotala*, including *R. indica* and *R. rotundifolia*, formed a monophyletic group. This relationship is supported by several morphological features, such as habit, opposite, ovoid leaves, and four sepals and petals (Haining *et al.* 2007). Previous molecular study also supports this grouping (Graham *et al.* 2005).

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Appendix 1. List of taxa and their accession numbers which were retrieved from GenBank

Taxon	GenBank accession No.	
	nrDNA ITS	*trn*H-*psb*A
Ammannia auriculata Willd.	MH808727	-
A. baccifera L.	MH173266	-
A. coccinea Rottb.	MG237340	DQ006199
A. multiflora Roxb.	MF599386	-
Duabanga grandiflora (Roxb. ex DC.) Walp.	AF163695	-
Lawsonia inermis L.	KF850586	GQ435226
Lythrum junceum Banks & Sol.	MG975401	-
L. hyssopifolia L.	MG975399	-
L. portula (L.) D. A. Webb.	MG237615	-
L. salicaria L.	MK895653	KC584962
L. thesioides M. Bieb.	MH245820	-
L. thymifolia L.	MG975400	-
L. tribracteatum Salzm. ex Spreng.	MG975398	-
L. virgatum L.	MG975397	-
Punica granatum L.	AY035761	GQ435338
Rotala indica (Willd.) Koehne	MG991825	-
R. rotundifolia (Buch.-Ham. ex Roxb.) Koehne	MH071602	GU135292
Sonneratia alba Sm.	AF163701	MH583031
Trapa natans L.	KX098577	KP280472
Ludwigia peruviana (L.) H. Hara	KX168366	GU135322
L. grandiflora (Michx.) Greuter & Burdet	KX168323	KC996841