PHYLOGENY OF *RHEUM* (POLYGONACEAE) BASED ON CPDNA *TRN*S-G SEQUENCES

WEIWEI LI AND AILAN WANG[*]

School of Life and Sciences, Ludong University, Yantai, 264025, Shandong, P. R. China
[*]Corresponding author's email: waltp98@126.com

Abstract

Rheum L. is a perennial herb of Polygonaceae, with about 60 species, most of which are distributed in the Qinghai-Tibet Plateau (QTP). Due to the unique habitats of the QTP, *Rheum* L. was one of the typical representatives for studying the species diversity of this region. To investigate the monophyletic origin of this genus and evaluate its infrageneric relationships, a total of 27 individuals distributed in seven of eight sections of *Rheum* (only excluding Sect. *Orbicularia*) were collected in the QTP and adjacent areas. Molecular phylogenetic trees were reconstructed based on cpDNA *trn*S-G sequences. The results showed that the monophyletic origin of Sect. *Palmata* was supported, while another six sections (Sect. *Rheum*, Sect. *Acuminata,* Sect. *Deserticola,* Sect. *Spiciformia,* Sect. *Globulosa* and Sect. *Nobilia*) were found to be paraphyletic origin. *Rh. kialense* of Sect. *Acuminata* clustered as a subclade with *Rh. Likiangense* 3 of Sect. *Rheum*, *Rh. pumilum* and *Rh. sublanceolatum* of Sect. *Deserticola. Rh. alexandrae* and *Rh.nobile*, the only two species in Sect. *Nobilia*, also scattered in different groups. The monophyletic origin of the monotypic Sect. (*Globulosum*) was also unsupported, and it was found to be clustered together with *Rh. nanum of Sect. Deserticola* in the ML tree. In conclusion, the phylogeny of *Rheum* based on cpDNA *trn*S-G sequences may serve as a reference for exploring specific patterns of rapid speciation in QTP and similar geographical environment.

Key words: *Rheum*, cpDNA *trn*S-G sequences, Phylogeny, Monophyletic origin.

Introduction

Multiple studies have shown that the species diversification on Qinghai-Tibet Plateau (QTP) is related to the uplifts after the late Tertiary period (Liu *et al*., 2006; Wang *et al*., 2009a; Xu *et al*., 2010; Zhang *et al*., 2012; Yu *et al*., 2014; Favre *et al*., 2015; Xing & Ree, 2017; Ren *et al*., 2018; Yang *et al*., 2019), and the climate oscillations of the Quaternary period (Myers *et al*., 2000; Sun *et al*., 2012; Ebersbach *et al*., 2018; Yang *et al*., 2019; Azani *et al*., 2019). The genus *Rheum* L. of the family Polygonaceae is one of the typical representatives for studying the species diversity in the QTP, which includes about 60 species, and is widely distributed at high latitudes regions of the QTP with a few species extending to central and western Asia and Europe (Losina-Losinskaya, 1936; Kao & Cheng, 1975). As a result, the QTP and its surrounding regions are speculated to be the centres of the origin and differentiation of *Rheum*. The special geographic location of the QTP made the morphological traits of plant appear to be characteristics of convergent evolution (Tsukaya & Tsuge, 2001; Wang *et al*., 2009b; Arakaki *et al*., 2011; Sun *et al*., 2012; Wen *et al*., 2014; Yang *et al*., 2019), such as indistinct stems, large translucent cream-colored bracts, and capitula, etc. In traditional taxonomy, the genus *Rheum* was divided into nine sections by Losina-Losinskaya (1936), seven sections by Kao & Cheng (1975) and eight sections by Li (1998), based on gross morphological traits. On account of the interference of convergent evolution in the traditional taxonomy of *Rheum*, some species might be incorrectly identified, resulting in controversy (Wang *et al*., 2005). Palynological research also has revealed inconsistent results based on diverse pollen exine sculpturing in *Rheum* (Yang *et al*., 2001).

Reconstruction of the molecular phylogeny is regarded as a reliable method for understanding rapid speciation caused by past geological events (Zhang *et al*., 2014; Hao

et al., 2015; Skourtanioti *et al*., 2016). DNA sequences are less influenced by the environment, and therefore they may provide reliable information to understand the systematics of *Rheum*, as morphological and palynological traits may do. Some gene sequences have been used to clarify phylogenetic relationships of *Rheum* species, such as the chloroplast genes *rbcL*, *matK*, *ndhF*, *accD*, *psaA*, *rbcL-accD*, intron of *trnK* and *trn*L-F (Yang *et al*., 2004; Wang *et al*., 2005; Sun *et al*., 2012). The results has shown that the rapid uplift of the QTP led to the rapid speciation and parallel evolution of *Rheum* species, resulting in the divergence between molecular phylogeny and morphological classification. The rapid speciation caused incomplete lineage sorting, which led to the deviation between the phylogenic trees and the species trees. This problem can be solved by using comprehensive gene trees to infer genome-wide species trees based on coalescent theory. Accordingly, more DNA sequences data were helpful to discern phylogenetic relationships although some of the data have been available. *Trn*L-F and *trn*S-G were both noncoding chloroplast DNA sequences and were usually used together to analyze phylogenetic relationships in plants due to their fast rate of evolution and great variation (Gao *et al*., 2017; Kamrani *et al*., 2017; Kocak *et al.*, 2018; Darbyshire *et al*., 2019; Paton *et al*., 2019）.

In previous studies, we have completed the reconstruction of the systematic trees based on *trn*L-F, but the *trn*S-G gene data was missing. In view of this, we considered using *trn*S-G sequences of chloroplast DNA to reconstruct the phylogenetic relationships of *Rheum* to obtain more valuable information. A 50% strict consensus tree (MP) and a maximum likelihood tree (ML) were reconstructed based on *trn*S-G sequences. Compared to our previous studies based on *trn*L-F, we found a similar topology, and also found some conflicts, for example, *Rh. golbulosum* of Sect. *Globulosa* was clustered with six species of Sect. *Rheum* in *trn*L-F dendogram but clustered

with *Rh. nanum* of Sect. *Deserticola* based on *trn*S-G sequences, and *Rh. spiciforme* of Sect. *Spiciformia* showed a different position in the phylogenetic tree of *trn*L-F compared to that in the *trn*S-G. These results are expected to be a supplement for resolving phylogenetic relationships in the closely related taxa of *Rheum*.

Materials and Methods

Sampling: A total of 27 samples in the QTP and surrounding areas were collected (Table 1), all these specimens belonged to seven of eight sections of *Rheum* (excluding *Sect. Orbicularia*) (Table 1) and represented most of the morphological range. The fresh leaves were placed in a sealed plastic bag and dried with silica gel for DNA extraction. Voucher specimens were deposited in the Herbarium of the Northwest Institute of Plateau Biology (HNWIPB), the Chinese Academy of Sciences, and School of Life Sciences, Lanzhou University.

Genomic processing: Total genomic DNA was extracted using the modified CTAB method (Doyle & Doyle, 1987) and then was isolated by gel electrophoresis. The isolated genome was diluted to 40 ng/µL and stored at -20℃ for later use. The *trn*S-G sequences were amplified with the primers of 'S' (5'-GCCGCTTTAGTCCACTCAGC) and 'G' (5'-GAACGAATCACACTTTTACCAC) (Hamilton, 1999). The PCR reactions were performed in a 25µL reaction system containing 40 ng template DNA, 18.75 µL sterile double-distilled water, 2.5µL of 10×Taq polymerase reaction buffer with 1.5 mM MgCl₂, 0.5 µL of 10 mM dNTPs, 1 µL 5 µM each of 'S' and 'G' primers, and 1 unit of Taq DNA polymerase. The PCR program was carried out under the following process: pre-denaturing for 4 min at 94°C, denaturing for 1 min at 94°C, annealing for 50 s at 51°C, and extending for 1.75 min at 72°C, with 38 cycles, plus a final extension for 8 min at 72°C. A CASpure PCR Purification Kit (CASARRAY) was used to purify the PCR products.

An automated DNA analysis was done using dye-terminator chemistry according to the manufacturer's protocols and carried out the sequencing works using amplified 'S' and 'G' primers by BGI. All DNA sequences submitted to GenBank (Table 1.)

Data analysis: Sequences alignment were performed by CLUSTAL X software (Thompson *et al.*, 1997), and then manual correction and shearing were carried out by BioEdit software. PAUP 4.010b (Swofford, 2000) were executed to construct the phylogenetic tree of maximum parsimony (MP), maximum likelihood (ML) and Bayesian analysis. Gaps were treated as missing characters and all characters were unweighted.

For the maximum parsimony (MP) analysis, search parameters with 100 replicates of random addition of sequences, ACCTRAN character optimization, TBR branch-swapping and STEEPEST DESCENT options were chosen to perform Heuristic parsimony searches. The 50% majority rule consensus tree calculated from 20,000 trees was selected for analysis. Bootstrap values (BS) revealing the relative support of the branches were calculated from 1,000 replicates using a heuristic search with a simple addition of sequences and TBR branch-swapping options.

Modeltest 3.06 (Guindon & Gascuel, 2003) has been used to estimate the best-fitting model of molecular evolution for maximum likelihood (ML) and Bayesian analysis.The ML tree was constructed in the selected model using PAUP 4.010b with simple addition, TBR branch-swapping, and MULTREES options.

For the Bayesian analysis, four simultaneous Monte Carlo Markov chains were run for 2,000,000 generations, saving a tree every 1000 generations. Posterior probability (PS) for Bayesian analysis assessed supporting value for phylogenetic groups (Huelsenbeck & Ronquist, 2001).

Table 1. List of taxa and sources of plant material analyzed and accessions in GenBank.

Section	Species	Locations	Accession number in GenBank
Sect. I *Rheum* (RHEU)	R. australe D. Don	Deqing, Xizang, Liu JQ 1101	AY920236
	R. webbianum Royle	Cuori, Xizang, Sn31	AY920235
	R. hotaoense C. Y. Cheng et Kao	Ledu, Qinghai, Yang MH 99130-1	AY920220
	R. wittrockii Lundstr.	Yili, Xingjiang, Yang MH 99059	AY920221
		Yushu, Qinghai, Q99147 1234	AY920232
	R. likiangense (L.) Sam.	Banma, Qinghai ,820	AY920233
		Seda, Sichuan, Liu JQ 2022	AY920239
	R. lhasaense A. J. Li et P. K. Hsiao	Sangri, Xizang, Liu JQ 1133	AY920231
	R. rhaponticuml L.	Botanical Garden, GG001	AY920219
Sect. Ⅱ *Palmata* A. Los. (PALM)	R. officinale Ball.	Nanchuan, Chongqing, M991013	AY920227
		Chuxiong, Yunnan,Liu JQ 2147	AY920226
		Huzhu, Qinghai, Liu JQ 1075	AY920223
	R. palmatum L.	Changdu,Xizang, Liu JQ 1245	AY920225
		Kangding, Sichuan, Liu JQ 2082	AY920228
	R. tanguticum var. liupanshanense C. Y. Cheng et Kao	Gande, Qinghai, Liu JQ 1773	AY920224
		Kangding, Sichuan, Liu JQ 2060	AY920229
Sect. III. Acuminata C. Y. Cheng et Kao (ACUM)	R. kialense Franch.	Kangding, Sichuan, Liu JQ 2050	AY920240
Sect. Ⅳ *Deserticola Maxim* (DESE)	R. sublanceolatum C Y. Cheng et Kao	Chenduo, Qinghai, Liu JQ 847	AY920242
	R. pumilum Maxim.	Chenduo, Qinghai, Yang MH 99145	AY920241
	R. nanum Siev. ex Pall.	Balikun, Xinjiang, Yang MH 99129-1	AY920243
Sect. VI*Spiciformia A. Los.* (SPIC)	R. spiciforme Royle	Yeduo, Qinghai, Liu JQ 689	AY920238
	R. moocroftianum Royle	Yeduo, Qinghai, Liu JQ 688	AY920244
	R. przewalskyi A. Los.	Huzhu, Qinghai, Q99136	AY920222
	R. reticulatum A. Los.	Maduo, Qinghai, Liu JQ 820	AY920245
Sect. Ⅶ*Globulosa C. Y. Cheng et Kao* (GLOB)	R. globulosum Gage	Dazi, Xizang, SN221	AY920234
Sect. Ⅷ *Nobilia A. Los.* (NOBI)	R. nobile Hook .f .et Thoms	Linzhi, Qinghai, Liu JQ 1206	AY920237
	R. alexandrae Batal.	Kangding, Sichuan, Liu JQ 2051	AY920230
Out group	Oxyria digyna	Kangding, Sichuan, Liu JQ 2087	AY920246

Results

Traits of DNA sequences: The length of all *trn*s-G sequences ranged from 953 to 1041bp. A matirx of 1153 bp was obtained after alignment, of which 794 positions were consistent, 289 positions were variable but parsimoniously uninformative, and 70 positions were informative with indels excluded, which accounted for 6.07% of the total length. The pairwise distance between *Rheum* species ranged from 0 to 8.76%. The maximum distance existed between *Rh. pumilum* and *Rh. nanum*. The minimum pairwise distances existed between, *Rh. palmatum*3 vs. *Rh. tanguticum*2, and *Rh. hotaoense* vs. *Rh. alexandrae*, which had not completely consistent sequences, because of variable but parsimoniously uninformative positions. The pairwise distance between the ingroup *Rheum* species and the outgroup *Oxyria digyna* varied from 19.68% to 24.13%, and the maximum distance existed between *Rh. pumium* and *Ox. digyna.*

Phylogenetic analysis: We obtained a 50% strict consensus tree (MP tree, Fig. 1) (length=449 steps, RI=0.716, CI=0.884), and branch lengths were noted on the branches and bootstrap values were noted below the branches. Six of seven sections (excluding Sect. *Palmata*) in *Rheum* delimitated according to morphological characters were not identified on all trees. Within the *Rheum* genus, there appeared to be four major tentative groups (Group A, B, C and D) with moderate bootstrap values (BS > 50). Three species (including seven samples), *Rh. officinale*, *Rh. palmatum* and *Rh. tanguticum*, all belonged to Sect. *Palmata*, clustered together in Group A, indicating a single origin of this section with moderate support (BS=59), however, other sections didn't form monophyletic groups. Group A containing the three species of Sect. *Palmata* clustered as a parallel subclade, sister to *Rh. rhaponticum*, *Rh. hotaoense*, *Rh. wittrockii* of Sect. *Rheum*, *Rh. przewalskyi* of Sect. *Spiciformia* and *Rh. alexandrae* of Sect. *Nobila*, with moderate support (BS=62). Group B comprised of two species including *Rh. lhasaense*, *Rh. likiangense* 1 and *Rh. likiangense* 2 of Sect. *Rheum*. Group C comprised four species root in three sections, in which, *Rh. likiangense* 3 of Sect. *Rheum* and *Rh. kialense* of Sect. *Acuminata* clustered as a subclade, sister to the other subclade containing *Rh. pumilum* and *Rh. sublanceolatum* of Sect. *Deserticola*, and received moderate support (BS=64). Group D comprised three species of Sect. *Spiciforma*, including *Rh. moorcroftianum*, *Rh. reticulatum* and *Rh. spiciforme*. In addation, *Rh. webbianum* and *Rh. australe* of Sect. *Rheum* clustered with Group C and Group D with a low support (BS<50), and the phylogenetic positions of *Rh. nobile* of Sect. *Nobilia* and *Rh. golbulosum* of Sect. *Globulosa* weren't identified.

The maximum likelihood tree (ML) was reconstructed based on the best-fit model (HKY+G by HLRT, -lnL=3607.2998), with PS values provided by Bayesian analysis labeled in the tree (Fig. 2). Five groups (Group A, B, C, D and E) were obtained, in which, the first four groups have the same composition as the MP tree but different support rates. Group A and Group C acquired a complete support of PS=100 (BS value of Group A=59, BS

value of Group C=64), Group D acquired a high support of PS=98 (BS=88), whereas Group B acquired a relatively low support value of PS=52 (BS=59). In addition, the monophyletic species *Rh. globulosum* of Sect. *Globulosa*, clustered with *Rh. nanum* of Sect. *Deserticola* with a moderate support value of PS=66 (Group E) in the ML tree. Whereas in the MP tree, *Rh. globulosum* was not clustered with any other species. Because of the relatively high PS values provided by the Bayesian analysis, we have confidence in the phylogenetic implications of this analysis.

Discussion

Monophyletic origin and paraphyletic origin of different sections: The cpDNA *trn*S-G sequences were relatively variable (289 bp) in *Rheum*, but most variations were uninformative (219 bp) with numerous indels, resulting in short branch lengths and low resolution of the lineages in the phylogenetic tree. The MP and ML trees (Figs. 1 & 2) revealed similar topology. All the species of Sect. *Palmata* clustered in one clade (Group A, BS=59, PS=100), which was consistent with the taxonomy based on the morphology supported the monophyletic origin of the section. However, the other six sections of *Rheum* according to morphological characteristics were not identified on all trees, such as, six species of Sect. *Rheum viz. Rh. rhaponticum*, *Rh. hotaoense*, *Rh. lhasaense*, *Rh. likiangense*, *Rh. webbianum* and *Rh. australe*, were scattered in four branches, revealing their paraphyletic traits. Three species of of Sect. *Deserticola,* namely *Rh. nanum, Rh. pumilum* and *Rh. sublanceolatum* with specialized features of indistinct stems, were scattered in two branches, showing a paraphyletic taxa, as well as, four species of of Sect. *Spiciformia*, including *Rh. moorcroftianum*, *Rh. reticulatum*, *Rh. spiciforme* and *Rh. przewalskyi* with stunt body, indistinct stems and spike inflorescence, also showed a paraphyletic taxa. This phenomenon can be explained by convergent evolution. In the late tertiary periods, the QTP experienced a rapid uplifting and formed stable cold and dry habitats (Shi *et al.*, 1998; Liu *et al.*, 2006; Wang *et al.*, 2009a). To adapt to the stable aridity, severe cold and other extreme environments, different species may have experienced similar environmental challenges and then gradually formed similar phenotype. For example, the species of Sect. *Deserticola* and Sect. *Spiciformia* developed indistinct stems to resist the arid and windy environment of the plateau, and the two species of Sect. *Nobilia* had colourful bracts to reduce damage to reproductive organs from ultraviolet and cold radiation at high altitudes (Omori & Ohba, 1996; Omori *et al.*, 2000). These features of convergent evolution affected traditional taxonomy of Sect. *Nobilia,* leading to paraphyletic traits in the phylogenetic trees. One view is that convergent evolution may lead to what is called "random fixation of unique morphological characters" (Kadereit, 1994), such as the capitulum of *Rh. globulosum* in the monotypic Sect. *Globulosa.* Monotypic traits of Sect. *Globulosa* based on traditional taxonomy was not supported in the phylogenetic analysis, *Rh. globulosum* and *Rh. nanum* of Sect. *Deserticola* were clustered in Group E in the ML tree.
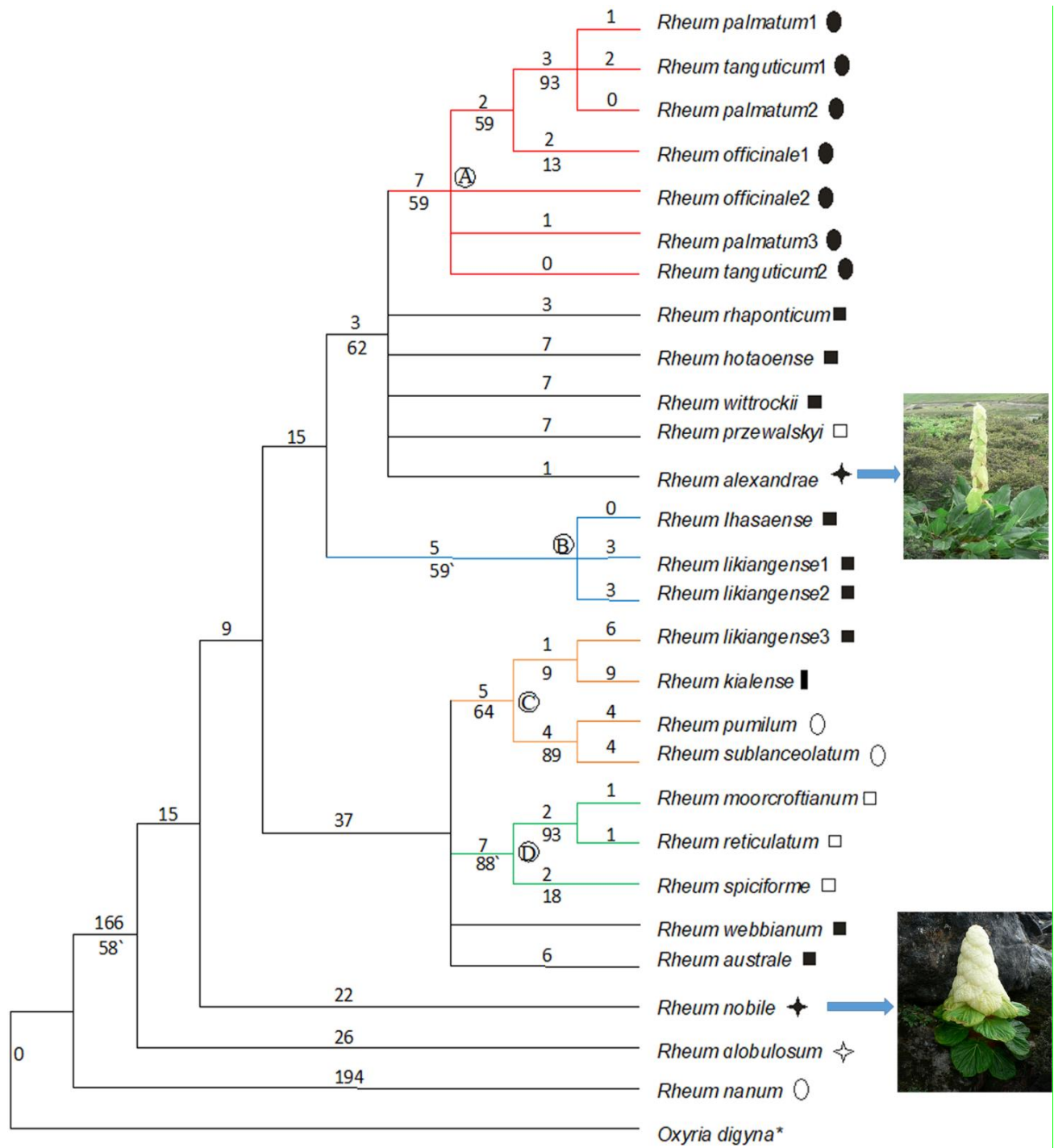
Fig. 1. The strict consensus tree from parsimony analysis of *trn*S-G regions (gaps coded as missing values)
Tree-length=449 steps; information characters=70; CI=0.884, RI=0.716. Numbers above the branches are branch lengths. Numbers below are bootstrap values (above 50%)
The symbols following species names indicate the corresponding sections: Sect. Rheum (▦), Sect. Palmata (▦), Sect. Deserticola (○), Sect. Spiciforma (□), Sect. Nobila (✦), Sect. Globulosa(✧), Sect. Acuminata (▮). Asterisks (*) denote one outgroup; brackets on the right indicate different subclades. The four groups containing all species of *Rheum* are shown as A, B, C, and D.

Adaptive radiation: Five species distributed in Sect. *Rheum*, Sect. *Spiciforma*, and Sect. *Nobilia* sister to Group A as parallel short branches with high support (BS=62, PS=84). According to previous studies, the branch length and strength of bootstrap support are generally correlated with rapid speciation (Richardson *et al*., 2001). The short branches and moderate support in the MP tree indicated that rapid radiation and explosive diversification occurred in *Rheum*. It is considered that rapid radiations are related to the large-scale uplifts in the late Tertiary period, and the climate oscillations in the Quaternary period of the QTP, in the past 10 million years (Harrison *et al*., 1992; Li *et al*., 1995; Shi *et al*., 1998). The rapid uplifts and multiple alternation of glacial epoch and interglacial epoch may promote the formation of fragmented little habitats, and then lead to rapid speciation of *Rheum*.
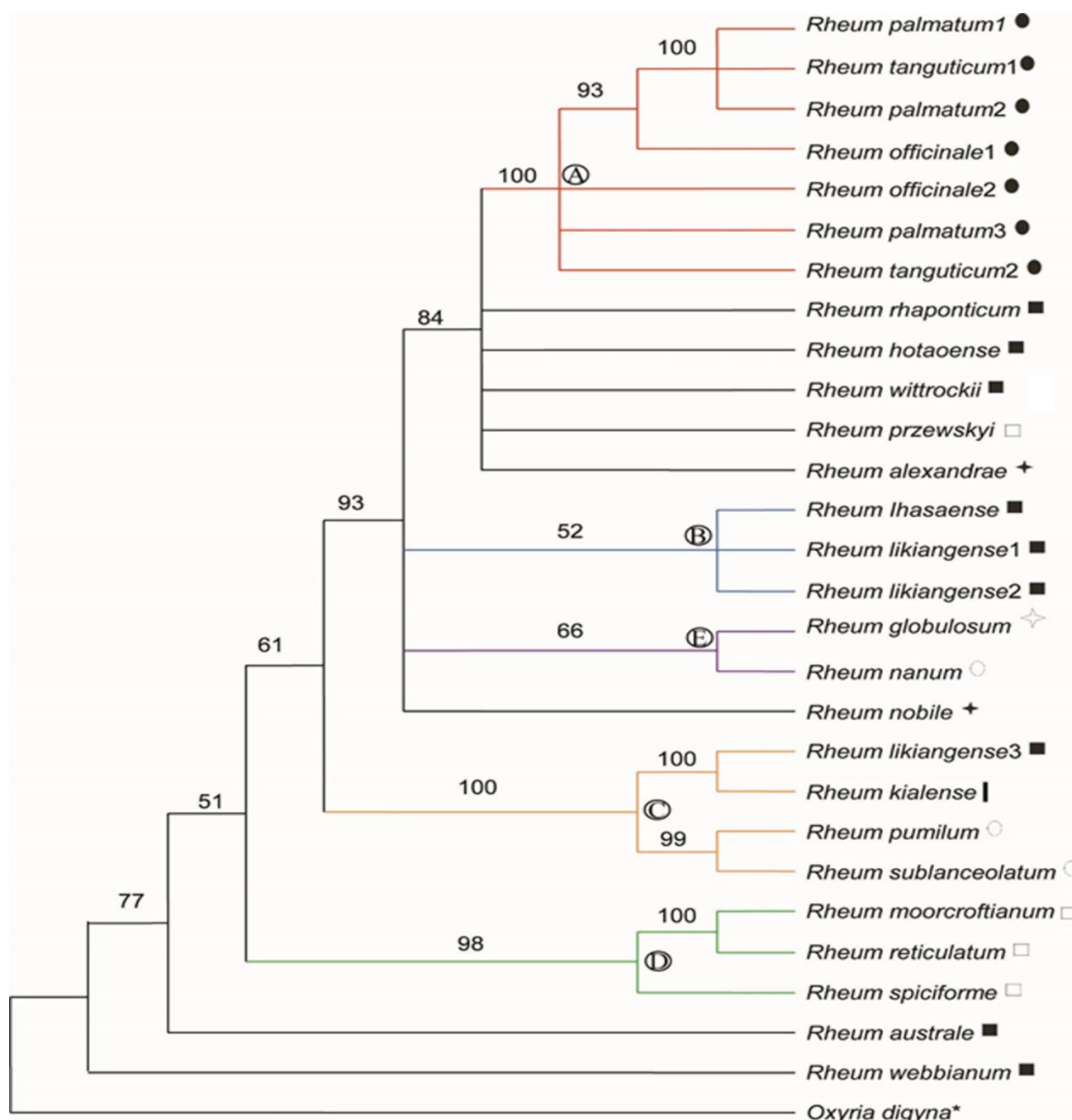
Fig. 2. Maximum likelihood tree of *trn*S-G genes.
Support values above 50% are shown as nodes in the Bayesian consensus tree. Names are the same as in Fig. 1. (Five groups are shown.)

Hybridization and introgression: In the phylogenetic trees of *Rheum*, we found that Group C included four species of three different sections, Sect. *Rheum,* Sect. *Acuminata* and Sect. *Deserticola*. Two species of Sect. *Rheum* clustered in Group B, and three species of Sect. *Spiciformia* were grouped in Group D, with other individuals of the two sections scattered across different branches. This may be explained by hybridization and introgression. It has been reported that hybridization would cause the introgression of different individuals, leading to low sequence divergence and causing different morphological species to cluster together in phylogenetic trees (Sang *et al*., 1997; Tsukaya *et al*., 2003). To demonstrate the hybrid origins of this species, low copy

nuclear genes might be promising candidates because of their parents' genetic characteristics (Wan *et al*., 2011).

The gene trees conflict: We have constructed molecular phylogenetic trees based on other chloroplast DNA sequences (Wang *et al*., 2005; Sun *et al*., 2012). Compared with our previous studies, similar topology was observed in the *trn*S-G phylogenetic trees, also implied rapid radiation and paraphyletic origin of the most sections (except Sect. *Palmata*.) in the *Rheum* genus (Figs. 1 & 2). The monophyletic origin of Sect. *Palmata* was confirmed in the parsimonious trees based on *trn*S-G (Figs. 1 & 2, Group A) and *trn*L-F sequences, but in the trees based on other cpDNA sequences, the species of Sect. *Palmata* clustered with *Rh*.

Acuminatum of Sect. Acuminata, indicated a paraphyletic origin (Sun et al., 2012). Similarly, Rh. globulosum of the monotypic Sect. Globulosa, grouped with species of Sect. Rheum in the trnL-F topology, whereas it grouped with Rh. nanum of Sect. Deserticola in the tree based on trnS-G (Fig. 2, Group E) and other cpDNA sequences (Wang et al., 2005; Sun et al., 2012). The gene trees conflicts maybe due to the following three aspects: 1) The difference of sample size leads to the formation of inconsistent phylogenetic trees, and the increase of individuals may avoid these differences. 2) The DNA fragments we used, may not represent the total genomics accurately, which caused the branching pattern of the genetic tree to be different from that of the species tree. Therefore to the selection of suitable gene fragments for phylogenetic relationship reconstruction has become a vital prerequisite. 3) The reticular evolutionary relationship caused by hybridization and introgression is expressed in the form of genetic tree conflict. Some studies have shown that gene trees conflict is particularly likely to occur between chloroplast genes or mitochondrial genes inherited by a single parent and nuclear genes inherited from both parents (Soltis & Kuzoff, 1995; Doyle et al., 1999; Ge et al., 1999; Guo & Ge, 2005; Zou & Ge, 2008; Walker et al., 2019). To obtain a more reliable result, a larger DNA dataset with more informative sites is necessary.

Acknowledgments

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