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Monophyly and infrageneric variation of *Corispermum* L. (Chenopodiaceae), evidence from sequence data *psb*B-*psb*H, *rbc*L and ITS

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Abstract: *Corispermum* is one of the most problematic taxonomic genera in Chenopodiaceae. To understand the phylogeny and infrageneric variation of *Corispermum*, we sequenced the nuclear ribosomal ITS region and two chloroplast DNA regions (*rbc*L and *psb*B-*psb*H) of 22 species and three varieties of *Corispermum* and the related genus *Agriophyllum*. Several representative species of *Salsola*, *Suaeda*, *Chenopodium*, *Kalidium* and *Camphorosma* served as outgroups. Our phylogenetic trees confirm that the tribe Corispermeae is monophyletic, *Corispermum* and *Agriophyllum* have a close relationship. *Corispermum* is demonstrated to be monophyletic, and contains at least four clades which, consequently, are served as the foundation of the infrageneric sectional variation of *Corispermum*, in terms of a combination of molecular data and morphological characters. The evolution of morphological characters for fruit wing and apex, two important characters in generic classification, is consistent with the sectional division of *Corispermum*, especially to the East Asian and Chinese taxa.

Keywords: Chenopodiaceae; classification; *Corispermum*; molecular phylogeny; infrageneric variation

Chenopodiaceae, comprising 110 genera and 1,700 species worldwide (Kadereit *et al*., 2003), is one of five largest families in the Central Asian flora, occupying extremely diverse ecological environments ranging from the most arid and hot submontane deserts to cold, high altitude deserts and the wet alpine belt. It is also one of the oldest and largest native and primarily desert families in Central Asian plants (Grubov, 1999). Thus, the family plays an important role, and is significant from the viewpoint of phytogeography and the evolution of flora (Grubov, 1999).

Chenopodiaceae was initially divided into two subfamilies, Spirolobeae, with a spiral embryo, and Cyclolobeae, with a peripheral embryo (Meyer, 1829). *Corispermum,* together with *Anthochlamys* and *Agriophyllum*, form the tribe Corispermeae, which belong to the subfamily Cyclolobeae (Moquin-Tandon, 1840). This tribe classification has been accepted by numerous authors. Corispermeae mainly occurs in Asia and Europe, except for *Corispermum*, which extended into North America. Ulbrich (1934) raised the number of subfamilies from two to eight, and his subfamily Corispermoideae included *Corispermum*, *Anthochlamys* and *Agriophyllum*. Kühn *et al*. (1993) set up four subfamilies, and tribe Corispermeae is included in subfamily Chenopodioideae. Anyway, as indicated by *rbc*L sequence evidence, the tribe Corispermeae is monophyletic (Kadereit *et al*., 2003).

Corispermum contains about 65 annual species in the world; of which 27 species occur in China and 12 are endemic (Zhu *et al*., 2003). Fenzl (1849) separated the first infrageneric division of *Corispermum* into two groups based on presence or absence of branched

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trichomes in the fruit, a very important taxonomic character in the genus. *Corispermum* was also divided into three sections (Mosyakin, 1994), namely sect. *Patellisperma* Mosyakin (monotypic), sect. *Declinata*, and sect. *Corispermum* which, in turn, was divided into at least seven subsections. Nevertheless, Mosyakin (1994) considered that questions still remained. Butnik (1981) presented the first study of fruit anatomy for the genus, with an examination of *C. lehmannianum*. Based on morphological and anatomic characters, Sukhorukov (2007) made a wider assessment of the diversity in fruit structure, and divided *Corispermum* into 13 groups. The fruit structure in the genus proved to be rather uniform, although several species from East Asia and China, such as *C. candelabrum*, *C. falcatum*, *C. grubovii*, *C. lepidocarpum*, *C. retortum*, were not sampled. Non-Kranz corispermoid leaf structure and C_3 photosynthesis have been reported for Corispermeae (Carolin *et al*., 1975; Carolin *et al*., 1978; Shomerilan *et al*., 1981; Akhani *et al*., 1997). There are a few studies of chromosome numbers in *Corispermum*, and all taxa reported are diploid with 2*n*=18 (Löve and Löve, 1961; Krasnikova *et al*., 2005).

There is a large elastic variation of the Chenopodiaceous stem under varying environmental conditions (Kühn *et al*., 1993). In *Corispermum*, certain characters used for segregation of a particular species and infraspecific entities were found to be unreliable and variable, e.g., plant size, branching habit, color, and shape of inflorescence, etc. Therefore, the currently accepted number of species in *Corispermum* is likely problematic; for instance, species numbers in China are probably exaggerated (Zhu *et al*., 2003).

Therefore, owing to the morphological plasticity, the anatomical uniformity, and the paucity of the molecular studies which focus on *Corispermum*, a more detailed study of molecular phylogeny is necessary. Here, we attempted to conduct a molecular phylogeny of *Corispermum* and its relatives, using *nr*DNA ITS sequences, *cp*DNA *psb*B-*psb*H spacer sequences, and the *rbc*L gene, with attention to: (1) test the monophyly of *Corispermum* and tribe Corispermeae; (2) research the phylogenetic relationship of the tribe Corispermeae; (3) and analyze the foundation of sectional classification within *Corispermum* from a phylogenetic aspect.

1 Materials and methods

1.1 Plant material

Our samples include 22 species and 3 subspecies of *Corispermum*, and 1 species of *Agriophyllum*. Seven representative species outside Corispermeae, *Salsola*, *Suaeda*, *Chenopodium*, *Kalidium* and *Camphorosma,* were included as outgroups (Table 1). Field collections and herbarium specimens served as sources of DNA materials. The herbaria in China were as follows: HNWP (Northwest Institute of Plateau Biology, Chinese Academy of Sciences (CAS), Xining, Qinghai); LZD (Cold and Arid Regions Environmental and Engineering Research Institute, CAS, Lanzhou, Gansu); and XJBI (Xinjiang Institute of Ecology and Geography, CAS, Urumqi, Xinjiang). The sequences of *Corispermum* reported in this paper are original, while outgroups were obtained from Genebank (Kadereit *et al*., 2003; Schutze *et al*., 2003; Kapralov *et al*., 2006; Akhani *et al*., 2007) (Table 1).

1.2 DNA isolation

Total DNA was isolated from the leaves of individual plants, either silica gel dried, or from the herbarium, using a modified cetyltrimrthyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987).

1.3 DNA amplification

Primer pairs was used for PCR amplification of the nuclear ribosomal ITS region, the chloroplast *rbc*L gene and the *psb*B-*psb*H region are as follows: primers for ITS: ITS1-f (5'-AGA AGT CGT AAC AAG GTT TCC GTA GC-3') (Kang *et al*., 2003) and ITS4-r (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al*., 1990); primers for *psb*B-*psb*H: *psb*B-*psb*H-f (5'-AGA TGT TTT TGC TGG TAT TGA-3') and *psb*B-*psb*H-r (5'-TTC AAC AGT TTG TGT AGC CA-3') (Xu *et al*., 2000; Schütze *et al*., 2003); and standard primers for *rbc*L: 1F (5'-ATG TCA CCA CAA ACA GAA ACT AAA GC-3'), 875F (5'-GCA GTT ATT GAT AGA CAG A-3'), 955F (5'-CGT CTA TCT GGT GGA GAT C-3') and 1460R (5'-CTT TTA GTA AAA GAT TGG GCC GAG-3') (Kadereit *et al*., 2003). The same primers that were used for PCR were also used for DNA sequencing.

Table 1 List of sampled taxa with their respective vouchers and GenBank accession numbers

Species	Voucher	Source	GenBank		
			ITS	*psb*B-*psb*H	*rbc*L
***Corispermum* L.**					
C. candelabrum Iljin	Huang Z H, Zhang W 017 (LZD)	Azhen, NW Yiqi, Yimeng, Neimeng, China	JF792746	JF792771	JF792796
C. chinganicum Iljin	Huang Z H, Zhang W 068 (LZD)	Azhen, Yiqi, Yimeng, Neimeng, China	JF792743	JF792768	JF792793
C. confertum Bunge	Xue J J 2009014 (XJBI)	Changling, Songyuan, Jilin, China	JF792753	JF792778	JF792803
C. declinatum Steph. ex Stev	Zhang M L, Zhu G L, 082029 (XJBI)	Mosuowan, Xinjiang, China	JF792747	JF792772	JF792797
C. dilutum (Kitag.) Tsien et G.G. Ma	Liu Y X, Yang X L, 84145 (LZD)	Zhongwei, Ningxia, China	JF792744	JF792769	JF792794
C. dutreuilii Iljin	Sheng C Y, 129 (HNWP)	Saishenke, Wulan, Qinghai, China	JF792740	JF792764	JF792789
C. elongatum Bunge	Xue J J, 2009025 (XJBI)	Fuyu, Changchun, Jilin, China	JF792755	JF792780	JF7920805
C. heptapotamicum Bunge	Xi G S, 464 (HNWP)	Tanggeer, Gonghe, Qinghai, China	JF792733	JF792757	JF792782
C. lehmannianum Bunge	Yang X L, 870020 (LZD)	Mosuowan, Manasi, Xinjiang, China	JF792742	JF792766	JF792791
C. lepidocarpum Grub	Si G S, 1278 (HNWP)	Qinghaihu, Qinghai, China	JF792741	JF792765	JF792790
C. macrocarpum Bunge	Yang X L, 83187 (LZD)	Hongshixia, Yulin, Shanxi, China	----	JF792767	JF792792
C. macrocarpum var. *rubrum* Fuhet Wang-wei	Xue J J, 2009021 (XJBJ)	Changling, Songyuan, Jilin, China	JF792754	JF792779	JF792804
C. mongolicum Iljin	Liu S W, 3232 (HNWP)	Ashengong, Guide, Qinghai, China	JF792734	JF792758	JF792783
C. orientale Lam	Liu Y X, Yang X L, 82715 (LZD)	Eerqisihean, Buerjin, Xinjiang, China	JF792745	JF792770	JF792795
C. pamiricum Iljin	Guo B Z, 7434 (HNWP)	Dachaidan, Haixi,Qinghai, China	JF792739	JF792763	JF792788
C. patelliforme Iljin	Liu Y X, Yang X L, 81019 (LZD)	Shazhuyu, Qinghai, China	JF792736	JF792760	JF792785
C. platypterum Kitag	Xue J J, 2009010 (XJBI)	Changling, Songyuan, Jilin, China	JF792751	JF792776	JF792801
C. puberulum Iljin	Xue J J, 2009004 (XJBI)	Changling, Songyuan, Jilin, China	JF792749	JF792774	JF792799
C. puberulum var. *ellipsocarpum* Tsien et G.G. Ma	Xue J J, 2009012 (XJBI)	Changling, Songyuan, Jilin, China	JF792750	JF792775	JF792800
C. stauntonii Moq	Zhou L H, 3472 (HNWP)	Gangcha, Zhiduo, Qinghai, China	JF792732	JF792756	JF792781
C. stenolepis Kitag	Xue J J, 2009011 (XJBI)	Changling, Songyuan, Jilin, China	JF792752	JF792777	JF792802
C. tibeticum Iljin	H.B.G 1151 (HNWP)	Jianshe, Dari, Qinghai, China	JF792748	JF792773	JF792798
C. tibeticum var. pilocarcum R.F. Huang	Guo B Z, 6471 (HNWP)	Shanzhuyu, Gonghe, Qinghai, China	JF792737	JF792761	JF792786
C. tylocarpum Hance	Guo B Z, Wang W Y, 11699 (HNWP)	Delingha, Qinghai, China	JF792735	JF792759	JF792784
C. zaidamicum R.F. Huang	Du Q, 0558 (HNWP)	Xiangride, Qinghai,China	JF792738	JF792762	JF792787

Continuous 2

Species	Voucher	Source	Genbank		
			ITS	*psb*B-*psb*H	*rbc*L
***Agriophyllum* Bieb.**					
Ag. squarrosum (L.) Moq.	ITS, *psb*B-*psb*H: Zhang M L, Zhu G L, 082103 (XJBI) *rbc*L: Kadereit G, *et al*. 2003	ITS, *psb*B-*psb*H: Buerjin, Xinjiang, China *rbc*L: Astrakhan, Russia	JF792730	JF792731	AY270051
Outside Species					
***Camphorosma* L.**					
Ca. a monspeliaca L.	ITS: Akhani H, *et al*. 2007 *psb*B-*psb*H: Akhani H, *et al*. 2007 *rbc*L: Kadereit G, *et al*. 2003	ITS, *psb*B-*psb*H: Aksaray, Turkey *rbc*L: Atyrau, Kazakhstan	EF453393	EF453530	AY270071
***Chenopodium* L.**					
Ch. botrys L.	ITS: Kapralov M V, *et al*. 2006 *psb*B-*psb*H: Kapralov M V, *et al*. 2006 *rbc*L: Kadereit G, *et al*. 2003	*rbc*L: Konya, Turkey	DQ499336	DQ499420	AY270080
***Kalidium* Moq.**					
Ka. caspicum (L.) Ung.-Sternb	ITS: Akhani H, *et al*. 2007 *psb*B-*psb*H: Kapralov M V, *et al*. 2006 *rbc*L: Kadereit G, *et al*. 2003	ITS: Damghan, Semnan, Iran *psbB-psbH*: Semnan, Iran *rbc*L: Tashkent, Uzbekistan	EF453444	DQ499423	AY270102
***Salsola* L.**					
Sa. canescens Boiss.	ITS: Kapralov M V, *et al*. 2006 *psb*B-*psb*H: Akhani H, *et al*. 2007 *rbc*L: Kadereit G, *et al*. 2003	ITS: Tehran, Iran *psb*B-*psb*H: Tehran, Iran *rbc*L: Aksaray , Turkey	DQ499346	EF453623	AY270127
Sa. vermiculata L.	ITS: Akhani H, *et al*. 2007 *psb*B-*psb*H: Akhani H, *et al*. 2007 *rbc*L: Kadereit G, *et al*. 2003	ITS, *psb*B-*psb*H: Kermanshah, Iran *rbc*L: Campo de Nijar, Spain	EF453501	EF453622	AY270131
***Suaeda* Forssk.**					
Su. crassifolia Pall.	ITS: Schuetze P, *et al*. 2003 *psb*B-*psb*H: Schuetze P, *et al*. 2003 *rbc*L: Kadereit G, *et al*. 2003	ITS, *psb*B-*psb*H: Aidar-Kul, Uzbekistan *rbc*L: Gulistan, Tashkent, Uzbekistan	AY181820	AY181885	AY270136
Su. maritima (L.) Dumort	ITS: Akhani H, *et al*. 2007 *psb*B-*psb*H: Akhani H, *et al*. 2007 *rbc*L: Kadereit G, *et al*. 2003	ITS, *psb*B-*psb*H: Meyghan, Arak, Iran *rbc*L: North Sea coast	EF453508	EF453628	AY270137

HNWP (Herbarium, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining, Qinghai);
LZD (Herbarium, Cold and Arid Regions Environmental and Engineering Research Institute, Chinese Academy of Sciences, Lanzhou, Gansu);
XJBI (Herbarium, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Urumqi, Xinjiang).

PCR was carried out using 20 μl of reaction volumes containing the following: 10×PCR buffer with Mg^{2+}, 2 μl; 5 μM forward and reverse primers, 1.6 μl each; 5 U/μl Taq polymerase, 0.2 μl; 2.5 mM of dNTP mixture, 2 μl; with DNA and ddH_2O added to 20 μl. The PCR was programmed as follows: denaturation at 95ºC for 2 min; 30 cycles consisting of 94ºC for 30 s, 52ºC or 54ºC for 30 s, 72ºC for 90 s; 72ºC for 10 min; 4ºC hold. PCR products were electrophoresed using 0.1% agarose gel in 1×TAE (pH 8.3) buffer.

1.4 DNA sequencing

Purification and sequencing were carried out by the Beijing Sun Biotechnology Corporation (Beijng, China), and sequencing was performed with ABI 3730 DNA Analyzer (Applied Biosystems, CA, USA).

1.5 DNA analysis

Forward and reverse sequences were analyzed and edited by DNASTAR Lasergene 7.1 and aligned using Clustal X (Thompson *et al*., 1997). The alignment of the ITS, *psb*B-*psb*H and *rbc*L was also carried out manual correction. Ambiguous nucleotide positions were excluded from the analysis (Kadereit *et al*., 2006), and gaps were treated as missing data. The starting and ending positions of ITS, *psb*B-*psb*H, and *rbc*L were determined by comparison with the Genbank entries that served as outgroups (Table 1). Finally, three datasets consisting of ITS, *psb*B-*psb*H+*rbc*L (*cp*DNA) and ITS+*psb*B-*psb*H+*rbc*L (three-gene combined) were assembled.

1.6 Phylogenetic analysis

All three datasets were analyzed by Maximum parsimony (MP), Maximum likelihood (ML) and the analyses of Bayesian inference (BI), MP and ML were executed in PAUP* 4.0b1.0 (Swofford, 2002). Bayesian inference analysis was conducted using MrBayes, version 3.0b4 (Huelsenbeck and Ronquist, 2001; Huelsenbeck and Rannala, 2004).

1.6.1 MP analysis

Heuristic searches were employed for the MP analysis, with a random stepwise addition of taxa, and tree bisection reconnection (TBR) branch swapping. Clade support values were estimated by bootstrap analysis with 100 bootstrap replicates (Felsenstein, 1985; Hillis and Bull, 1993).

1.6.2 ML analysis

Heuristic searches were also used for the ML analysis, and clade support values were estimated using bootstrap analysis, with 100 bootstrap replicates.

1.6.3 BI analysis

The BI analysis of the phylogenetic trees was performed using MrBayes version 3.0b4 (Huelsenbeck and Ronquist, 2001; Huelsenbeck and Rannala, 2004). Some parameters from the Modeltest were also included in the analysis. The option was set up using 2,000,000 generations of Markov Chain Monte Carlo (MCMC) and a sample frequency of 1,000. Saturation was reached after a burn-in of 1,000 generations. Clade support was assessed using Bayesian posterior probability (Huelsenbeck and Rannala, 2004); these probabilities were estimated as the proportion of trees sampled after burn-in that contained each of the observed bipartitions.

1.6.4 Modeltest

For Maximum likelihood and Bayesian inference analyses, the appropriate model of DNA substitution for elucidation of phylogenetic relationships under ML was estimated using Modeltest 3.06 (Posada and Crandall, 1998). The Akaike information criterion (AIC) and hierarchical likelihood ratio test (hLRT) were calculated, but, in general, AIC was chosen (Posada and Buckley, 2004).

For the ITS dataset, the GTR+I+G model was chosen by AIC with a gamma distribution shape parameter of 1.4035 and an assumed proportion of invariable sites of 0.3113. Base frequencies were set to A=0.2202, C=0.2581, G=0.2810, and T=0.2406. The rate matrix was set to AC=0.7035, AG=2.0950, AT=2.8214, CG=0.2310, CT=3.2011, and GT=1.0000. For *psb*B-*psb*H + *rbc*L dataset, the most appropriate model was TIM + I with equal rates for all sites, and an assumed proportion of invariable sites of 0.7006. Estimated base frequencies were set to A=0.2892, C=0.1800, G=0.2155, and T=0.3153. The rate matrix was set to AC=1.0000, AG=1.7244, AT=0.3215, CG=0.3215, CT=2.7207, and GT=1.0000. For the three-gene com-

bined sequences dataset, the GTR+G+I model was chosen by AIC with the gamma distribution shape parameter set to 0.7217 and proportion of invariable sites set to 0.5523. Base frequencies were set to A=0.2734, C=0.2027, G=0.2322, and T=0.2917. The rate matrix was set to AC= 0.8187, AG=1.6895, AT=0.8695, CG= 0.3529, CT=2.5486, and GT=1.0000.

1.7 Morphological character evolution

Published morphological data (Zhu *et al*., 2003) and the herbarium materials were used to assign character to species of *Corispermum* and outgroups. The assigned 15 characters in Tables 2 and 3, and two characters, broad or narrow fruit wing and emarginate or round fruit apex were analyzed on the basis of three-gene combined phylogenetic tree, and conducted by MacClade (Maddison and Maddison, 1992).

Table 2 The 15 morphological characters of *Corispermum* and outgroup taxa

No.	Morphological character
1	Habit. Herb annual (0); subshrubs, shrubs (1).
2	Leaf. Petiolate (0); sessile (1).
3	Leaf shape. Flattened (0); terete (1); undeveloped (2).
4	Bract. With bract (0); absent (1).
5	Bracteole. With bracteole (0); absent (1).
6	Inflorescences. Spike (0); panide (1); glonerule (2).
7	Fruite shape. Globose (0); compressed (1).
8	Fruit hairs. Yes (0); no (1).
9	Fruit wing. Broad (0); narrow (1); absent (2).
10	Rostrum. Yes (0); no (1).
11	Fruit apex emarginate. Yes (0); no (1).
12	Pericarp membranous. Yes (0); no (1).
13	Feed. Vertical (0); horizontal (1).
14	Feed shape. Orbicular (0); oblong (1).
15	Embryo. Circularity (0); spiral (1).

Table 3 Morphological character matrix of *Corispermum* and outgroup taxa

Species	Morphological character														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Corispermum candelabrum	0	1	0	0	1	0	1	0	0	0	1	1	0	1	0
C. chinganicum	0	1	0	0	1	0	1	1	0	0	1	1	0	1	0
C. confertum	0	1	0	0	1	0	1	1	0	0	0	1	0	0	0
C. declinatum	0	1	0	0	1	0	1	1	1	0	1	1	0	1	0
C. dilutum	0	1	0	0	1	0	1	1	0	0	0	1	0	1	0
C. dutreuilii	0	1	0	0	1	0	1	1	0	0	1	1	0	1	0
C .elongatum	0	1	0	0	1	0	1	1	1	0	0	1	0	1	0
C. heptapotamicum	0	1	0	0	1	0	1	1	1	0	1	1	0	1	0
C. lehmannianum	0	1	0	0	1	0	1	1	0	0	1	1	0	1	0
C. lepidocarpum	0	1	0	0	1	0	1	0	0	0	0	1	0	1	0
C. macrocarpum	0	1	0	0	1	0	1	1	0	0	0	1	0	1	0
C. macrocarpum var. *rubrum*	0	1	0	0	1	0	1	0	0	0	0	1	0	1	0
C. mongolicum	0	1	0	0	1	0	1	1	1	0	1	1	0	1	0
C. orientale	0	1	0	0	1	0	1	1	0	0	1	1	0	1	0
C. pamiricum	0	1	0	0	1	0	1	1	1	0	1	1	0	1	0
C. patelliforme	0	1	0	0	1	0	1	1	1	0	1	1	0	0	0
C. platypterum	0	1	0	0	1	0	1	1	0	0	0	1	0	0	0
C. puberulum	0	1	0	0	1	0	1	0	0	0	0	1	0	1	0
C. puberulum var. *ellipsocarpum*	0	1	0	0	1	0	1	1	0	0	0	1	0	1	0
C. stauntonii	0	1	0	0	1	0	1	1	0	0	1	1	0	1	0
C. stenolepis	0	1	0	0	1	0	1	1	0	0	0	1	0	0	0
C. tibeticum	0	1	0	0	1	0	1	1	0	0	1	1	0	1	0
C. tibeticum var. *pilocarpum*	0	1	0	0	1	0	1	1	0	0	1	1	0	1	0
C. tylocarpum	0	1	0	0	1	0	1	0	1	0	1	1	0	1	0
Agriophyllum squarrosum	0	1	0	0	1	0	1	1	1	0	1	1	0	1	0
Camphorosma monspeliaca	1	1	?	0	1	0	1	1	2	1	1	0	0	1	0
Chenopodium botrys	0	0	0	1	1	1	0	1	2	1	1	0	1	0	0
Kalidium caspicum	1	1	2	0	1	0	0	1	2	1	1	?	0	0	0
Salsola canescens	1	1	1	1	0	0	0	1	2	1	1	?	1	0	1
Suaeda crassifolia	0	1	1	1	0	2	1	1	2	1	1	0	1	1	1

Table 4 Data set and tree statistics from separated MP analyses of ITS, *psb*B-*psb*H + *rbc*L and three-gene combined for *Corispermum*

Data set statistics					Tree statistics				
Genic region	Taxa	Aligned length (bp)	Number and percentage of variable characters	Number and percentage of parsimony informative characters	Number of shortest trees	Length	CI	RI	RC
ITS	33	661	294 (44.5%)	212 (33.4%)	80	607	0.7298	0.7481	0.5460
*psb*B-*psb*H + *rbc*L	33	1,979	257 (13.0%)	149 (7.5%)	2,000	326	0.8497	0.8951	0.7605
Three-gene combined	33	2,640	551 (20.9%)	361 (13.7%)	1,000	918	0.7843	0.8229	0.6454

Note: CI, Consistency index, RI, Retention index, RC, Rescaled consistency.

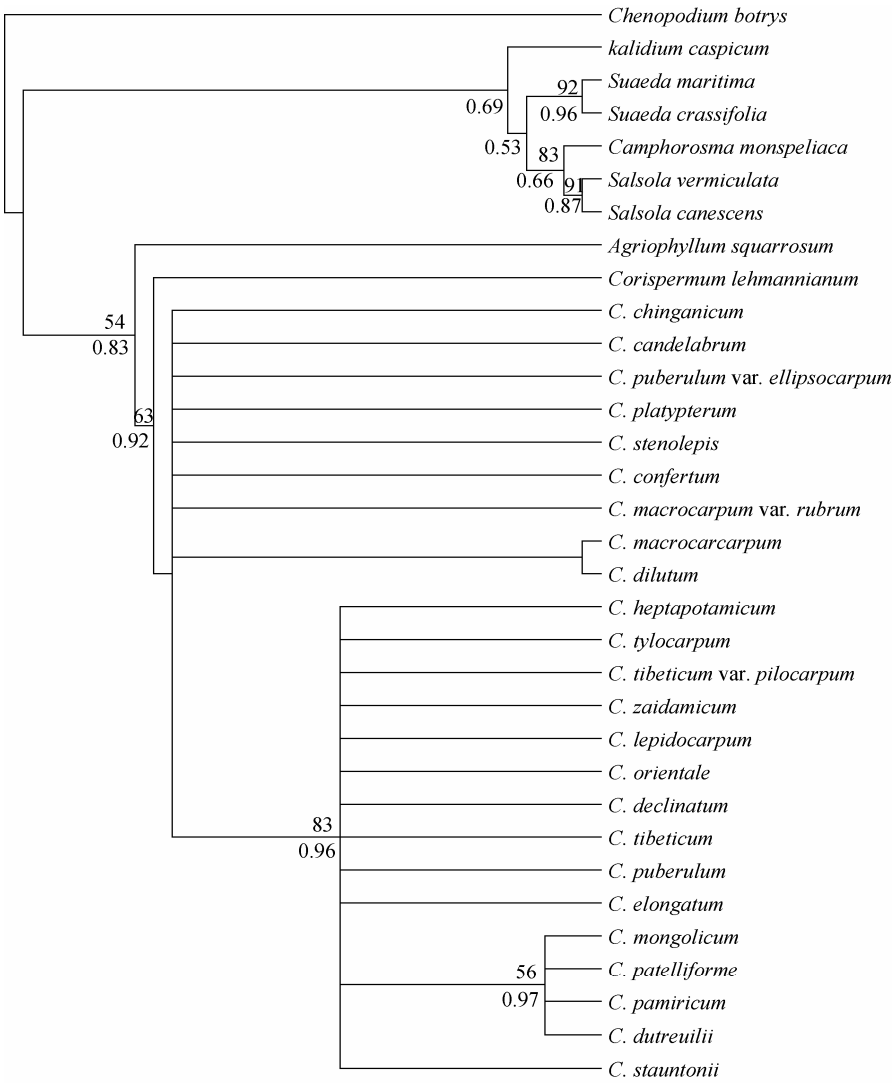

Fig. 1 Maximum likelihood tree derived from ITS sequences (Maximum parsimony bootstrap values are shown above branches, Bayesian posterior probabilities below)

2 Results

The results of the MP analysis of the ITS sequence, two chloroplast DNA sequences, and combined 3-gene dataset are listed in Table 4. The topologies of the three trees from each of the MP, ML and BI analyses were essentially similar. Therefore, we only showed the trees of the ML analysis.

2.1 ITS sequence analysis

The ITS data matrix is comprised of 33 taxa and 661 bp of characters, with *Chenopodium botrys* as the defined outgroup. The ITS tree was basically divided into two parts, one comprising *Corispermum* and *Agriophyllum* and another consisting of the outgroup

genera (Fig. 1). A clade of 14 species was resolved within *Corispermum*. The ML analysis supported that *Corispermum* is monophyletic (bootstrap value (bt) = 63%, posterior probability (pP) = 92%) (Fig. 1), and *Agriophyllum* is sister to *Corispermum*.

2.2 Chloroplast DNA sequences analysis

The chloroplast DNA sequence data matrix is comprised of 33 taxa and 1,979 bp of characters, with *Chenopodium botrys* defined as outgroup. Monophyly of the genus *Corispermum* is strongly supported (bt=100%, pP= 100%) (Fig. 2). In the *cp*DNA tree, three clades are resolved within *Corispermum* (Fig. 2), clade 1 (bt=83%, pP=100%), clade 3 (bt=94%, pP=100%), and clade 4 (bt=51%, pP=74%) (Fig. 2). *Agriophyllum* is sister to *Corispermum* (bt=100%, pP=100%) (Fig. 2).

2.3 Three-gene combined analysis

The three-gene combined data matrix is comprised of 33 taxa and 2,640 bp of sequence characters, with *Chenopodium botrys* defined as outgroup. *Corispermum* forms a monophyly and includes four clades: clade 1 (bt=91%, pP=100%), clade 2, clade 3 (bt=53%, pP=81%), clade 4 (bt=100%, pP=100%) (Fig. 3). The sister relationship between *Agriophyllum* and *Corispermum* is strongly supported (bt=100%, pP=100%) (Fig. 3). Clade 2 is sister to clade 3, clade 1 to clade 2 + clade 3, and clade 4 to clade 1 + clade 2 + clade 3 (Fig. 3). There are two well-supported groups, one in clade 2, *Corispermum* patelliforme +*Corispermum pamiricum* +*Corispermum dutreuill* (pP=98%) (Fig. 3), another in

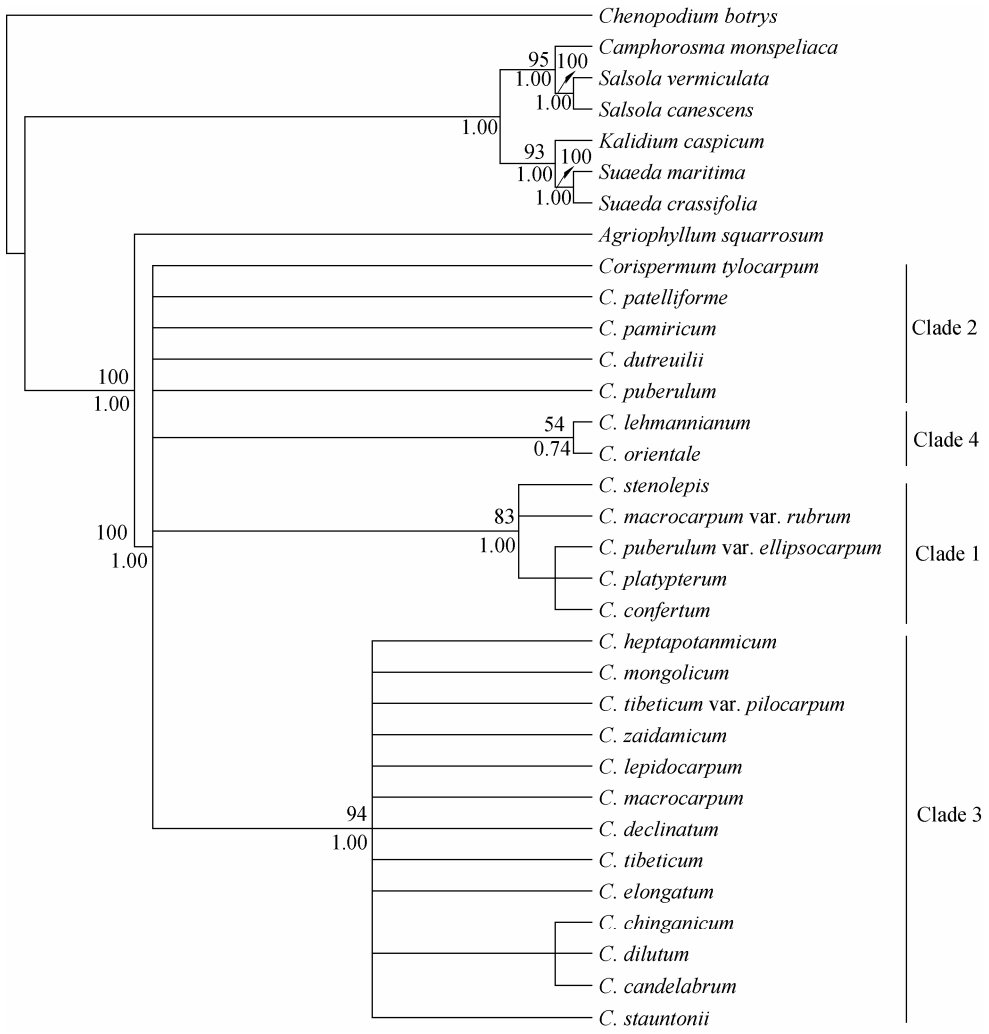

Fig. 2 Maximum likelihood tree derived from *psb*B-*psb*H and *rbc*L sequences (Maximum parsimony bootstrap values are shown above branches, Bayesian posterior probabilities below)

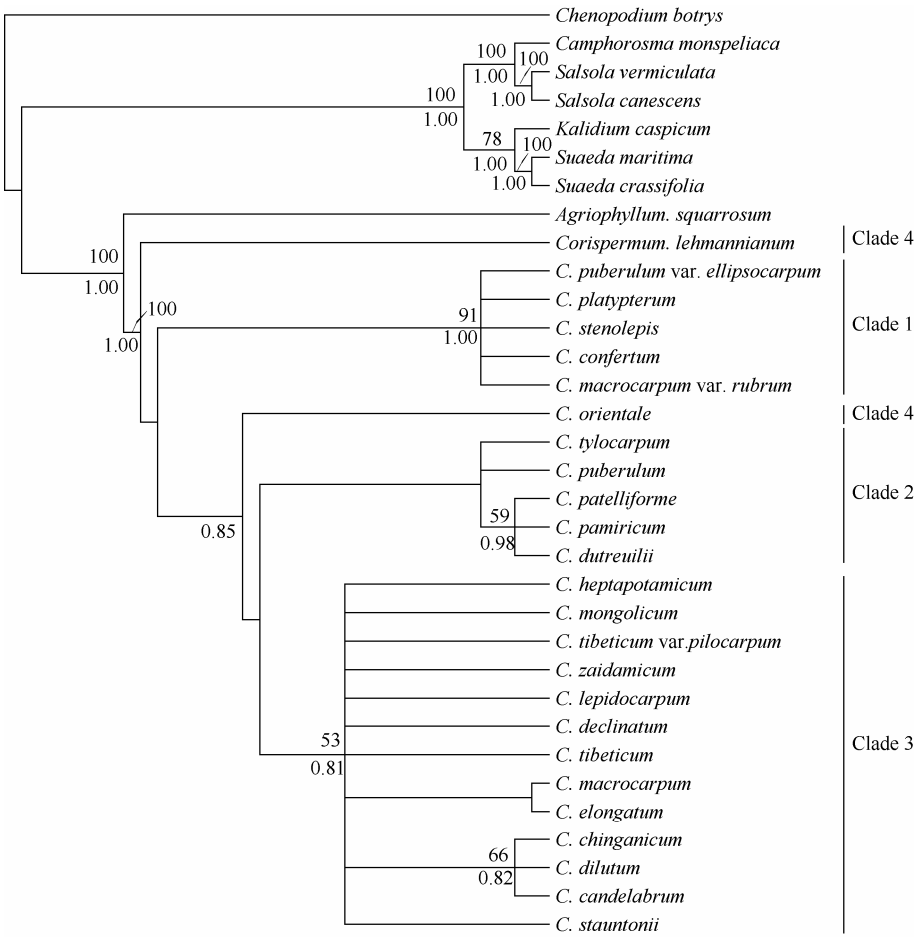

Fig. 3 Maximum likelihood tree derived from ITS, *psb*B-*psb*H, *rbc*L sequences (Maximum parsimony bootstrap values are shown above branches, Bayesian posterior probabilities below)

clade 3, *Corispermum chinganicum* + *Corispermum dilutum* + *Corispermum candelabrum* (pP=82%) (Fig. 3).

2.4 Morphological character evolution

The evolution of two characters, fruit wing and fruit apex in *Corispermum* and outgroups were examined. These two characters are the key characters for the classification, especially at section level. The fruit wing evolution is illustrated in Fig. 4, and in terms of the character state, the narrow fruit wing is shown in clade 2 and subclade of clade 3. The emargination of fruit apex appears in clade 1 and subclade 2 of clade 3 in Fig. 5.

3 Discussion and conclusion

3.1 The monophyly of Corispermeae Moq.

The present molecular phylogeny clearly indicates the monophyly of Corispermeae. This tribe comprises of only three genera, *Corispermum* (sixty species), *Agriophyllum* (six species), and *Anthochlamys* (two species) (Kühn *et al*., 1993; Kadereit *et al*., 2003). In the previous molecular phylogeny (Kadereit *et al*., 2003), only three species were sampled from the tribe. Since the attention was at the family level. In comparison, the present phylogenetic results, with a sufficient number of species (27 species) and strong support (bt=100%, pP=100%) (Figs. 2 and 3), confirm monophyly of the tribe and are in agreement with previous phylogeny (Kadereit *et al*., 2003).

The molecular results also coincide with morphology and anatomy. Morphologically, in contrast to other groups, the leaves of this tribe are laminated, and the seeds are usually covered with dendroid and stellate hairs, and the tepals are not persistent (Zhu *et al*., 2003). As mentioned, non-Kranz corispermoid leaf structure and C_3 photosynthesis have been reported for Corispermeae (or Corispermoideae)

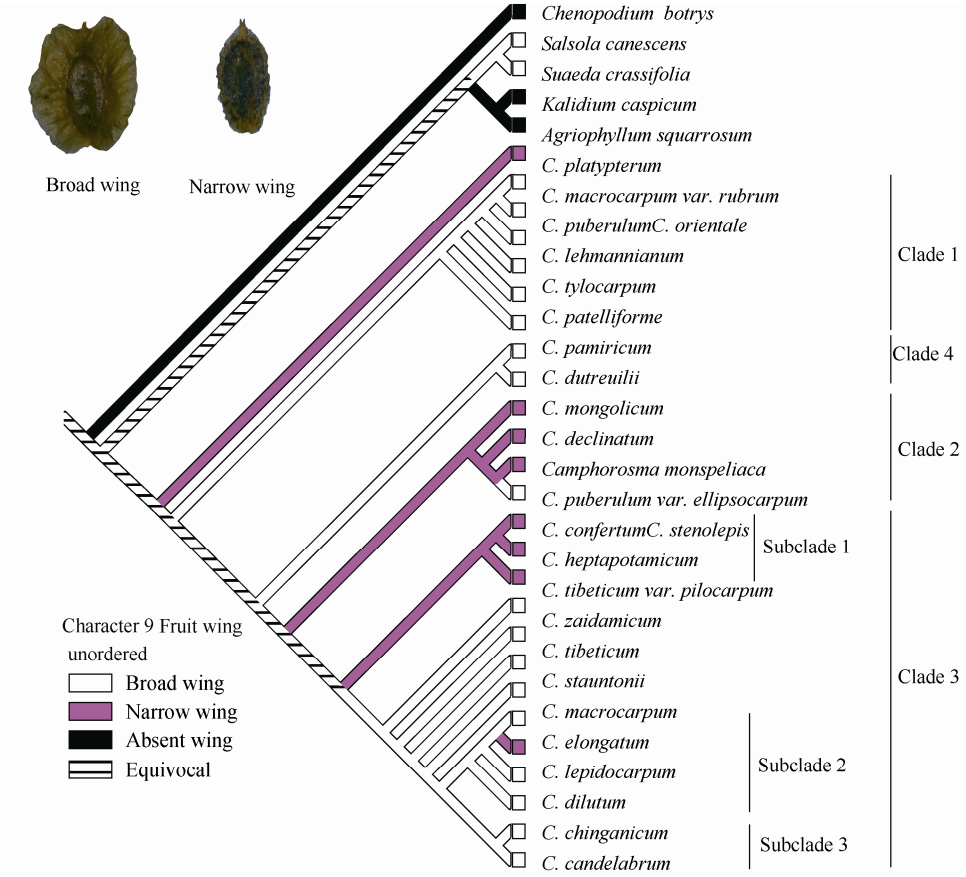

Fig. 4 The character evolution cladogram of the fruit wing and the character distribution of having broad or narrow wings

(Carolin *et al*., 1975; Shomerilan *et al*., 1981; Akhani *et al*., 1997).

The fruits of all Corispermeae species possess supporting tissue which consists of macrosclereids, a tissue usually absent in other subfamilies, or consisting of brachysclereids in some Salsoloideae genera (Sukhorukov, 2007). Therefore, in terms of morphological, anatomical and molecular evidence, the placement of the three genera within the tribe should withstand scrutiny.

3.2 The relationship between *Corispermum* and *Agriophyllum*

In Figs. 2 and 3, *Corispermum* is closely related to *Agriophyllum* (bt=100%, pP=100%), which is in agreement with the results of the single gene *rbc*L study (bt=94%) (Kadereit *et al*., 2003). In fact, according to the taxonomic classification, both genera share the similar aspects of having annual herbs, covered with hairs, having laminated leaves, fruit with two beaks, and often covered with dendroid and stellate hairs. Therefore, through the combination of morphological characteristics and molecular phylogeny, a close relationship between *Corispermum* and *Agriophyllum* can be ascertained.

3.3 Monophyly of *Corispermum*

The monophyly of *Corispermum* was revealed with high support (bt=100%, pP=100%) in this paper (Figs. 2 and 3). As a core group of the tribe, *Corispermum* has numerous species and a broad Northern Hemisphere distribution in Eurasia and North America. Through the history of classification (Ulbrich, 1934; Zhu *et al*., 2003), *Corispermum* has never been doubted because of its distinct morphological characters, such as the annual herbs, often covered with dendroid and stellate hairs, spikelike inflorescences, absent bractlets, compressed utricled fruit, winged margin, and fruit apex is emarginated or rounded to acute, and the beak is with a two-fid tip formed from the style bases. Based on sufficient sampling of species, our molecular results coincide with the morphological classification. Thus, *Corispermum* is verified to be a natural taxon in Chenopodiaceae.

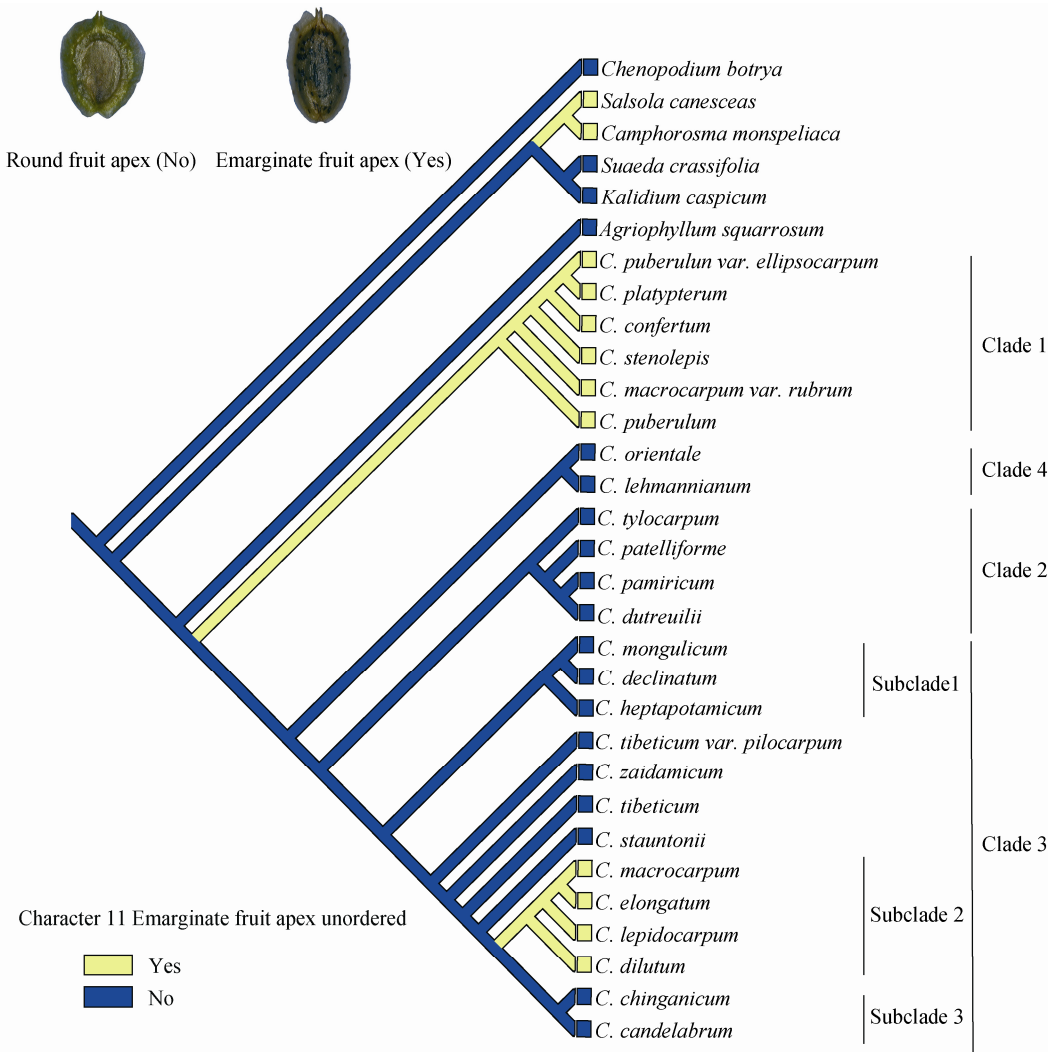

Fig. 5 Character evolution cladogram of fruit apex and character distribution of round apex and emarginate apex

3.4 Phylogenetic clades and relationship within *Corispermum*

Our molecular analysis identified four clades among the sampled *Corispermum* species. Vegetative and floral characters in *Corispermum* have considerable plasticity and instability in different habitats, so the characters of fruit morphology have been regarded as the most important taxonomic characters (Ulbrich, 1934; Zhu *et al*., 2003). However, fruit variation is plastic as well, in regards to shape, size, color and pubescence. Thus, there is, at present, no perfect infrageneric classification for *Corispermum*. As mentioned above, Mosyakin (1994) divided *Corispermum* into three sections based on the morphological characters of Central and East Asian species. At the same time, he also admitted that his subdivisions might be not suitable for the whole genus and distribution. Later, based on anatomical and morphological traits, *Corispermum* was divided into 13 groups (Sukhorukov, 2007). However, our analysis seemed to not support the two classifications within *Corispermum*.

In our three-gene combined trees (Fig. 3), *Corispermum* is illustrated to form four major clades. These four clades could be regarded as the foundation of four sections within *Corispermum*.

Clade 1 includes three species and two varieties: *C. platypterum*, *C. stenolepis*, *C. confertum*, *C. puberulum* var. *ellipsocarpum* and *C. macrocarpum* var. *rubrum*. The monophyly of the clade 1 is well supported (bt=83%, pP=100%, *cp*DNA tree, Fig. 2; bt=91%, pP=100%, three-gene combined tree, Fig. 3). These species occur in Northeastern China, and have the

characteristics of a broadly elliptic or suborbicular fruit, distinctly emarginated fruit apex, and a broad fruit wing. Mosyakin (1994) treated *C. platypterum* and *C. stenolepis* in *Corispermum* sect. *Declinata* Mosyakin. Therefore, we suggest that *C. platypterum, C. stenolepis, C. confertum, C. puberulum* var. *ellipsocarpum* and *C. macrocarpum* var. *rubrum* should be set apart from *Corispermum* sect. *Declinata* and classified as separate section.

Clade 2 is composed of *C. tylocarpum, C. puberulum, C. pamiricum, C. patelliforme,* and *C. dutreuilii.* All of these species, except for *C. puberulum*, possess the character of a non-emarginated fruit apex. *C. tylocarpum* was treated as a member of *Corispermum* sect. *Declinata* Mosyakin which includes *C. tylocarpum* and *C. declinatum* (Mosyakin, 1994). *C. tylocarpum* and *C. declinatum* were also put into one group by Sukhorukov (2007). From our molecular data, *C. tylocarpum* and *C. declinatum* pertain to two different clades. Mosyakin (1994) separated *C. patelliforme* as the only species of the monotypic section *Patellisperma*. Consequently, the present treatment, based on the molecular data is neither congruent with Mosyakin (1994) nor Sukhorukov (1997). Meanwhile, in the *cp*DNA tree, the five species were placed in separated locations, whereas in the three-gene combined tree, they are combined into a clade without support. Therefore, this clade needs further research.

Clade 3 had ample support (bt=94%, pP=100%, *cp*DNA tree; bt=53%, pP=81% three-gene combined tree). In clade 3, there are twelve species and one variety. The morphological variation in this clade is large. For example, the fruits of some species are glabrous, while other species are covered with stellate hairs; some wings are broad and others narrow, and other fruit apexes are rounded or acute and some are emarginated. A detailed list of morphological variation among the species of this clade is presented in Table 5. Sukhorukov (2007) placed *C. chinganicum*, *C. heptapotamicum*, and *C. mongolicum* into a single group named *Heptapotamicum*, and *C. macrocarpum* into the *Macrocarpum* group. However, he didn't deal with the other species in clade 3. Considering the molecular trees and the morphological characters together, we

Table 5 The fruits morphological characters and distribution of *Corispermum*

Section		Taxa	Fruit				Distribution in China			
			Shape	Hairs	Wing	Apex	NE	N	NW	Tibet
Section 1		*C. puberulum* var. *ellipsocarpum*	broadly elliptic	yes	broad	emarginate	+			
		C. macrocarpum var. *rubrum*	broadly elliptic	yes	broad	emarginate	+			
		C. platypterum	suborbicular	no	broad	emarginate	+			
		C. stenolepis	suborbicular	no	broad	emarginate	+			
		C. confertum	suborbicular	no	broad	emarginate	+			
		C. puberulum	obovate-oblong	yes	broad	emarginate	+			
Section 2		*C. tylocarpum*	obovate-oblong	yes	narrow	acute	+	+	+	
		C. pamiricum	obovate-elliptic	no	narrow	erect				+
		C. dutreuilii	oblong-obovate	no	broad	emarginate			+	+
		C. patelliforme	discoid	no	narrow	round			+	
Section 3		*C. declinatum*	obovate-oblong	no	narrow	acute	+	+	+	+
	Subsection 1	*C. mogolicum*	broadly elliptic	no	narrow	round			+	
		C. heptapotamicum	elliptic	no	narrow	round			+	
		C. lepidocarpum	ovate	yes	broad	emarginate				+
	Subsection 2	*C. macrocarpum*	broadly elliptic	no	broad	emarginate	+			
		C. elongatum	oblong-elliptic	no	narrow	emarginate	+			
		C. dilutum	obovate	no	broad	emarginate	+			
	Subsection 3	*C. candelabrum*	oblong-obovate	yes	broad	round	+	+		
		C. chinganicum	sublustrous	no	broad	round	+		+	
		C. zaidamicum	–	no	broad	?				

suggest that clade 3 should be incorporated into one taxonomical unit, but subdivision of this clade needs further study. Clade 4 is comprised of *C. lehmannianum* and *C. orientale*. Although the two species share similar morphological characters, such as broadly elliptic fruits without hairs, a broad wing, and non-emarginated apex, the present phylogenetic trees have not unequivocally shown as a clade. In the *cp*DNA tree, *C. lehmannianum* and *C. orientale* are placed together (bt=54%, pP=74%) (Fig. 2), but they are not united in the three-gene combined tree (Fig. 3). Even though the weak support of this clade comes from the phylogenetic trees, we conditionally place the two species in their own sections, due to the undoubted similarity of morphological characters of the fruits.

3.5 Taxonomic treatment

Combining the molecular evidence and morphological characters of the fruits, we attempt to bring out a foundation of infrageneric classification in *Corispermum*. Four sections can be discerned among the sampled species from East Asia and China. Section 1 includes all the species of clade 1 and *C. puberulum* from clade 2; section 2 is composed of species of clade 2 except for *C. puberulum*. Section 3 consists of the same species as clade 3, and the same applies to section 4 and clade 4 (Table 5).

Species in section 1 share the following morphological characters: the stem is erect; the leaves are linear, 1-veined, glabrous, or covered with stellate hairs; the fruit base is cordate; the beak of utricle is 3–6 mm long; the teeth is 2.8–5 mm long; the fruit apex is emarginated, and the wing is broad. This section mainly occurs in East Asia (Table 5). This division is also congruent with the evolution of fruit apex. The fruit apex evolution in Fig. 5 shows that section 1, with the apex emarginated fruit, is isolated from other sections in *Corispermum* and section 1 is an advanced group, while others are primitive.

The morphological characters of section 2 are included: erect stem, linear or oblanceolate, 1-(3)-veined, and glabrous utricle leaves (except for *C. tylocarpum*, which is covered with dendroid or stellate hairs), apex acute or rounded fruit without emargination, and narrow fruit wing (except for *C. dutreuilii*). The species of section 2 are mainly distributed in Northwestern China (Table 5). In Fig. 4 the fruit wing evolution illustrates that clade 2 and subclade 1 of clade 3 with a narrow wing is primitive, and section 2 could form a group including clade 2.

Section 3 has the characters of erect stem, linear and 1-veined leaves. The characters of the utricle are complex and described in detail in Table 5. In Fig. 3 section 3 (clade 3) has an ample support (bt=94%, pP=100%, *cp*DNA tree; bt=53%, pP=81% three-gene combined tree), whereas Fig. 4 illustrates that section 3 (clade 3) could be divided into two parts according to broad or narrow fruit wing except for *C. elongatum*, namely subclade 1 and others. Using the three-gene combined phylogenetic tree (Fig. 3) and the fruit wing and fruit apex evolution (Figs. 4 and 5), we divided this section into three subsections. Currently, four species could not be treated currently because of the complicated morphological variation (Table 5). Of course, the subdivision of section 3 could be improved if more samples are analyzed.

Two species of section 4 could be recognized from other sections from Figs. 4 and 5, by their corresponding characters of an erect stem, the linear or oblanceolate, 1-veined leaves with a broadly elliptic and glabrous utricle, having a rounded fruit apex, and a broad fruit wing. Among them two species occur in northern Xinjiang (Table 5).

In short, since we only sampled about half of the species in the genus, mainly from East Asia and China, our sectional designations may be lack a balanced and valid foundation. However, these treatments, on the basis of the phylogeny and morphological evolution, should be a contribution for whole generic divisions especially lacking of valid sectional circumscription within genus.

3.6 Infraspecific variation

In this paper, the variability of the infraspecific rank in *Corispermum* appears to be excessively large. Among our sampling species, there are three varieties, i.e. *C. puberulum* var. *ellipsocarpum*, *C. macrocarpun* var. *rubrum* and *C. tibeticum* var. *pilocarpum*. *C. tibeticum* and its variety, var. *pilocarpum*, are clustered in the same clade (Figs. 2 and 3). However, *C. puberulum* and its variety, var. *ellipsocarpum*, were placed into different clades (Figs. 2 and 3), and the same thing occured with *C. macrocarpun* and *C. macrocarpun* var. *rubrum*. Clearly, according to current treatments, the variation at the varietal level sometimes exceeded that at the species level in *Corispermum*. Our phylogenetic trees could not yet provide enough evidence to say anything about the status of these varieties with our limited number of samples. Therefore, the treatment

of the taxonomical ranks of these species and their varieties should depend upon future findings of phylogeny and taxonomy in the future.

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