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Molecular phylogenetics of *Hippophae* L. (Elaeagnaceae) based on the internal transcribed spacer (ITS) sequences of nrDNA

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Abstract. The genus *Hippophae* comprises 7 species and 8 subspecies according to the latest classification, and has shown enormous ecological, nutrient and medicinal values. Here we analyzed the phylogenetic relationships among 15 taxa of the genus by comparing sequences of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA). ITS sequences in *Hippophae* varied in length from 651 bp to 666 bp. The aligned sequences were 690 bp in length and 269 (39.0%) were variable sites with 150 being parsimony-informative. The amount of polymorphism observed within a taxon was extremely low in most taxa except for two putative hybrid species. The aligned sequences were analyzed by maximum parsimony (MP) and neighbor-joining (NJ) methods. In the strict consensus trees of parsimony analysis, the monophyly of *Hippophae* was supported by 100% bootstrap value. *H. tibetana* was at the basal position of the genus, and the remaining taxa formed two clades with high bootstrap support. The first clade included subspecies of *H. rhamnoides* and the other one consisted of remaining species. Parsimony analysis also suggested that the species *H. tibetana*, *H. neurocarpa* and *H. salicifolia* were all distinct. Although the sequence divergence among subspecies of *H. rhamnoides* was also remarkably high, the molecular data supported the monophyly of *H. rhamnoides* when *H. rhamnoides* subsp. *gyantsensis* Rousi was excxluded. The NJ trees showed essentially the same topology. The taxonomical arrangement that divided the genus into two sections was not supported based on the ITS sequences. However, the hybrid origin of *H. goniocarpa* and *H. litangensis* proposed previously was supported by the present ITS data.

Key words: Elaeagnaceae, *Hippophae*, ITS (internal transcribed spacer) nucleotide sequences, phylogeny.

Hippophae L. is a small genus of Elaeagnaceae comprising seven species and eight subspecies according to the latest classification in Bartish et al. (2002). These species are all diploid of $2n = 24$ and are restricted to Qinghai-Xizang plateau and adjacent areas except *H. rhamnoides* L. which is distributed widely but fragmentally in Asia and Europe (Rousi 1971; Lu 1997; Lian et al. 2000; Bartish et al. 2000, 2002). During the last decades, many studies have been undertaken on this fascinating plant, concentrating on its agricultural, nutritional, medical and ornamental values (e.g. Tian 1985, Eliseev et al. 1989, Yao and Tigerstedt 1994, Singh et al. 1997). However, the taxonomic treatment and relationships between taxa in this genus remain in dispute

(Rousi 1971, Avdeyev 1983, Lian 1988, Hyvonen 1996, Lian et al. 2000, Bartish et al. 2002).

In his comprehensive study of the family Elaeagnaceae, Servettaz (1909) recognized one species with three subspecies in the genus *Hippophae*. Rousi (1971) raised these subspecies to species level, i.e. *H. rhamnoides* L., *H. tibetana* Schlecht and *H. salicifolia* D. Don, and described seven new subspecies of *H. rhamnoides*. On the basis of one brachyblast character and several quantitative characters, however, Avdeyev (1983) reduced the genus to single one species including two subspecies, *H. rhamnoides* subsp. *rhamnoides* and subsp. *salicifolia*. Hyvonen (1996) conducted a morphological cladistic analysis and found two distinct lineages which were recognized as two species. Based on fruit morphology, Lian (1988) established two sections in this genus, i.e. sect. *Hippophae* and sect. *Gyantsensis* Lian. After several extensive investigations on *Hippophae* in West China, a few of new taxa were described and one subspecies, *H. rhamnoides* subsp. *gyantsensis* Rousi, was raised to species status in recent taxonomical treatments (Liu and He 1978, Lian 1988, Lian et al. 1997). On these basis, Lian et al. (1997, 1998) recognized six species with eight subspecies in *H. rhamnoides*, two subspecies in *H. goniocarpa* and two subspecies in *H. neurocarpa*. As pointed out by Bartish et al. (2002), however, three subspecies used by Lian et al. (1997, 1998) have not been published or transferred validly. Although studies utilizing ultrastructure of leaf surface, isozymes and RAPD markers have been conducted (Yao and Tigerstedt 1993, Zhang and Gao 1992, Bartish et al. 2000, Lian et al. 2000), the circumscription of species in this genus remains unresolved, in particular some subspecies had been variously included within or excluded from *H. rhamnoides* by different authors. In addition, the phylogenetic relationships of *Hippophae* requires further clarification at species and subspecies levels.

Most recently, Bartish et al. (2002) summarized literature and recognized 15 taxa in *Hippophae* including seven species and eight subspecies after they validated two species and one subspecies that were previously published as nomina nuda. At the same time, on the basis of phylogenetic analyses of combining cpDNA and morphological characters, Bartish et al. (2002) found that *Hippophae* is a strongly supported monophyly, and two independent hybridizations were suggested. However, the subspecies of *H. rhamnoides*, the most widespread species, got weak support as a monophylum, and subdivision or recognizing sections within the genus was not proposed because of the weak internal support in the genus (Bartish et al. 2002).

Nuclear DNA data provide valuable information in phylogenetic study of plants, and the internal transcribed spacer (ITS) regions of the nrDNA have been shown to be a valuable source of evidence to resolve phylogenetic relationships at different taxonomic levels (e.g. Baldwin 1993, Baldwin et al. 1995, Sang et al. 1995, Wendel et al. 1995, Becerra and Venable 1999), in particular at intraspecific level because of the relatively rapid evolutionary rates of the ITS fragment. Up to date, sequence data of nuclear DNA have not been used in phylogenetic study of *Hippophae*. In the present study, we analyzed the nucleotide sequences of ITS region of the nuclear ribosomal DNA from 15 taxa of *Hippophae* and three outgroups to 1) address the circumscription of the genus and species within the genus; 2) reconstruct the phylogeny within the genus. This information should contribute to developing a reasonable classification system and to a better understanding of the evolution of *Hippophae*.

Materials and methods

Source of materials. Seven species and all subspecies of *H. rhamnoides* except for *H. rhamnoides* subsp. *caucasia* Rousi were sampled in this study (Table 1). One undescribed taxa, *H. rhamnoides* subsp. *wolongensis* Lian, Sun et Chen (Lian et al. unpublished), was included in the analysis. Three species from the genus *Elaeagnus* L. were also included in the study as outgroups. All materials were collected from natural populations or from

Table 1. Accessions of the genus *Hippophae* and the outgroups used in this study

Taxon	Locality	Voucher	GenBank accession no.
Hippophae gyantsensis (Rousi) Lian	Basu, Xizang	Lian et al. 920105	AF440247
H. rhamnoides L.			
ssp. *sinensis* Rousi	Songpan, Sichuan	Sun et al. S711	AF440241
ssp. *turkestanica* Rousi	Kashi, Xinjiang (cultivated in Beijing)	Ma et al. 18	AF440243
ssp. *rhamnoides*	Berlin, Germany (cultivated in Beijing)	Ma et al. 8	AF440242
ssp. *mongolica* Rousi	Altai, Russia (cultivated in Beijing)	Ma et al. 19	AF440244
ssp. *carpatica* Rousi	Berlin, Germany (cultivated in Beijing)	Ma et al. 4	AF440245
ssp. *fluviatilis* (Soest) Rousi	Munchen, Germany (cultivated in Beijing)	Ma et al. 7	AF440248
ssp. *yunnanensis* Rousi	Litang, Sichuan	Sun et al. Y22	AF440250
ssp. *wolongensis* Lian et al.	Wenchuan, Sichuan	Sun et al. W110	AF440252
H. salicifolia D. Don	Chuona, Tibet (cultivated in Sichuan)	Ma et al. 3	AF440246
H. tibetana Schlecht.	Qilian, Qinghai	Chen & Sun, T6	AF440249
H. goniocarpa Y.S. Lian et al. ex Swenson et Bartish	Songpan, Sichuan	Sun et al. G11	AF440255
H. litangensis Y.S. Lian et X.L. Chen ex Swenson et Bartish	Litang, Sichuan	Sun et al. L17	AF440251
H. neurocarpa S.W. Liu et T.N. He			
ssp. *neurocarpa*	Songpan, Sichuan	Sun et al. N57	AF440253
ssp. *stellatopilosa* Y.S. Lian et al. ex Swenson et Bartish	Litang, Sichuan	Sun et al. E17	AF440254
Elaeagnus umbellata Thunb.	Botanical garden of Beijing (cultivated)	Ma et al. 1	AF440257
E. bockii Diels	Wolong, Sichuan	Sun et al. WF	AF440258
E. angustifolia L.	Botanical garden of Beijing (cultivated)	Ma et al. 2	AF440256s

cultivated plants (Table 1). For those sampled from natural populations, fresh leaves were collected individually and dried with silica-gel in the fields. Plants of *H. salicifolia* was grown in Maoxian, Sichuan and was introduced from its original location in Chuona, Tibet. Fresh leaves of *E. angustifolia* and *E. umbellata* were directly collected from plants cultivated in Botanical Garden of Beijing. For those taxa involving hybridazation, such as *H. rhamnoides* subsp. *sinensis* Rousi, *H. goniocarpa* Y. S. Lian et al. ex Swenson et Bartish, and *H. neurocarpa* Liu et T. N. He, several individuals were sampled from different populations across their distribution. Voucher specimens are deposited in NWNU (Table 1).

Total DNA extraction and amplification of ITS region. Total genomic DNAs from individual plants were extracted using the CTAB method as described in the following. Dried leaf materials were ground to fine powder in a 2 mL Eppendorf tube, and then mixed with 900 μL of preheated 2×CTAB extraction buffer containing 0.3% mercaptoethanol. The homogenate was incubated at 65 °C for 45 min prior to adding 900 μL of chloroform:isoamyl alcohol (24:1, v/v). After mixing by inversion for 10 min the mixture was centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatant reserved and mixed with 2/3 vol ice-cold isopropanol. The DNA was recovered as a pellet by centrifugation at 8,000 rpm for 10 min at 4 °C, washed twice with 500 μL of 70% ethanol, dried, and dissolved in 200 μL of 1×TE buffer. DNA quality and quantity were determined in 0.8% agrose gels (Ge et al. 1999).

Double-stranded DNA of the complete ITS region (including ITS1, 5.8S and ITS2) was amplified with the primers P_1 (5′-AGAAGTCGTAAC AAGGTTTCCGTAGG-3′) and P_4 (5′-TCCTCC GCTTATTGATATGC-3′). Amplifications were carried out in 20 μl reaction volume with 2 μl of 10×Tris-HCl reaction buffer (pH 8.3), 2 μl of dNTP mixtures (10 mmol/L), 1 μl DMSO, 2 μl of each primer (2 μmol/L), 0.15 μl of *Taq* polymerase (Unit/μl), and 2 μl (10–20 ng) of total DNA. PCRs were performed on PCR System 9600 (Pe Biosystems), programmed for 4 min at 70 °C, 2 cycles of 1 min at 94 °C, 20 s at 48 °C, 50 s at 72 °C, followed by 38 cycles of 20 s at 94 °C, 20 s at 48 °C, 50 s at 72 °C, and then 4 min at 72 °C. PCR products were purified using Wizard PCR Preps DNA Purification system (Promega).

Sequencing of ITS region and sequence alignment. Double stranded sequencing of the purified DNAs was performed on an ABI 377 DNA Sequencing System with the ABI Prism Bigdye^{TM} terminator Cycle Sequencing Reading Reaction Kit. The two PCR primers were used as sequencing primers. Sequencing reactions were carried out in 10 μl reaction volume with 1.5 μl of Bigdye Mix, 1 μl of primer, 2.5 μl of purified template DNA, and programmed for 25 cycles of 10 s at 94 °C, 5 s at 52 °C, 4 min at 60 °C. DNA sequences were read for both strands. The sequence boundaries of two ITS regions and the coding regions of nuclear rDNA were determined by comparison with the published sequences in GenBank. All ITS sequences, including taxa of *Hippophae* and outgroups, were aligned manually using sequential pairwise comparisons. The presence of several insertions and deletions was not a significant factor in aligning the sequences.

Phylogenetic analyses. All sequences were deposited in GenBank (see Table 1 for accession numbers). Parsimony analyses were conducted using PAUP4.0. The Branch-and-Bound algorithm was used to find the most parsimonious trees. Gaps were coded as missing and any polymorphic site was treated as two possible nucleotides at the same site following IUB base genetic code. Strict and 50% majority rule consensus trees were calculated from all most parsimonious tress. The consistency (CI) and retention indices (RI) were calculated. Bootstrap analyses were performed using 1000 replicates and the heuristic search algorithm. Parsimony analyses were also performed after excluding the sequences of the two putative hybrids, *H. goniocarpa* and *H. litangensis* Y.S. Lian et X.L. Chen ex Swenson et Bartish. The sequences data were also analyzed with a neighbor-joining (NJ) method using the Kimura two-parameter distance estimates (Saitou and Nei 1987, Kimura 1980).

Results

Length and variation of ITS regions. Complete sequences of the ITS regions were generated from all materials, including 15 taxa of *Hippophae* and three outgroups. The boundaries of ITS1, ITS2, and adjacent coding regions were determined by comparing the published sequences from Genebank. Among 15 taxa of the

genus, ITS sequences varied in length from 651 bp to 666 bp. The ITS sequences of three outgroups were between 646 bp and 651 bp in length. The length of ITS1 of *Hippophae* ranged from 270 bp to 282 bp, while ITS2 ranged from 218 bp to 220 bp. The 5.8S rDNA was 163 bp in length with the exception of *H. tibetana* and *H. salicifolia* (164 bp) (Table 2).

The amplified products were 710 bp (690 bp belong to ITS sequence) in length after the alignment. Of 269 (39.0%) variable sites, 150 were parsimony-informative. 61.0 % of the variable sites (164) were found in ITS1, of which 98 were potentially phylogenetically informative. In ITS2, 88 (32.7%) sites were variable, and 46 were potentially phylogenetically informative. In 5.8S, 17 variable sites (6.3%) were found with 6 being parsimony-informative (Table 2). The amount of polymorphism observed within one taxon was extremely low in most taxa except for the two putative hybrids (*H. goniocarpa* and *H. litangensis* Y.S Lian et X.L. Chen ex Swenson et Bartish) which maintained high level of polymorphic sites in ITS region. For example, 52 to 58 polymorphic sites are detected in different individuals of *H. goniocarpa* on which the putative parents have different basepairs (data not shown). The high polymorphism in *H. goniocarpa* and *H. litangensis* imply their hybrid origin. Several individuals from different populations of *H. rhamnoides* subsp. *sinensis*, *H. goniocarpa* and *H. neurocarpa* were sequenced, and only one polymorphic site occurred in *H. rhamnoides* subsp. *sinensis*, *H. neurocarpa*. Very low levels of sequence divergence (*H. goniocarpa*) within one taxon are observed. Pairwise Kimura one 2-parameter distances within *Hippophae* ranged from 0.15% to 11.88% (Table 2). Divergence values between subspecies within *H. rhamnoides* also vary significantly from 0.15% to 6.22%.

ITS phylogeny. Parsimony analysis of ITS sequences yielded 21 equally most parsimonious trees of 351 steps including all taxa (CI = 0.846; RI = 0.862, including uninformative characters). In addition to a highly supported monophyly of the genus *Hippophae* (100% bootstrap value), the strict consensus tree shows that *H. tibetana* is basal within the genus, and the remaining taxa form a clade with 72% bootstrap support (Fig. 1a). This clade consists of two well-defined subclades. The first one was supported by the high bootstrap value (97%) and includes all subspecies of *H. rhamnoides*, in which the undescribed subspecies (*H. rhamnoides* subsp. *wolongensis*) and subsp. *yunnanensis* Rousi are basal to the remaining subspecies. The other subclade (supported by 91% bootstrap value) includes two putative hybrids (*H. goniocarpa* and *H. litangensis* Y.S. Lian et X.L. Chen ex Swenson et Bartish) and two subspecies of *H. neurocarpa* (*H. neurocarpa* subsp. *neurocarpa* and *H. neurocarpa* subsp. *stellatopilosa* Y.S. Lian et al. ex Swenson et Bartish) as well as *H. gyantsensis* (Rousi) Lian and *H. salicifolia*. However, four species,

Table 2. Length and G + C content in ITS regions of *Hippophae* and the outgroups

ITS regions		Length before alignment (bp)	Length after alignment (bp)	Variable sites (bp)	Parsimony informative sites (bp)	Mean G + C content (%)
ITS1	*Hippophae*	270–282	301	164	98	47.37
	outgroup	265, 269				
5.8S	*Hippophae*	163, 164	165	17	6	54.07
	outgroup	163				
ITS2	*Hippophae*	218–220	224	88	46	53.64
	outgroup	218, 219				
Total	*Hippophae*	651–666	690	269	150	51.18
	outgroup	646–651				

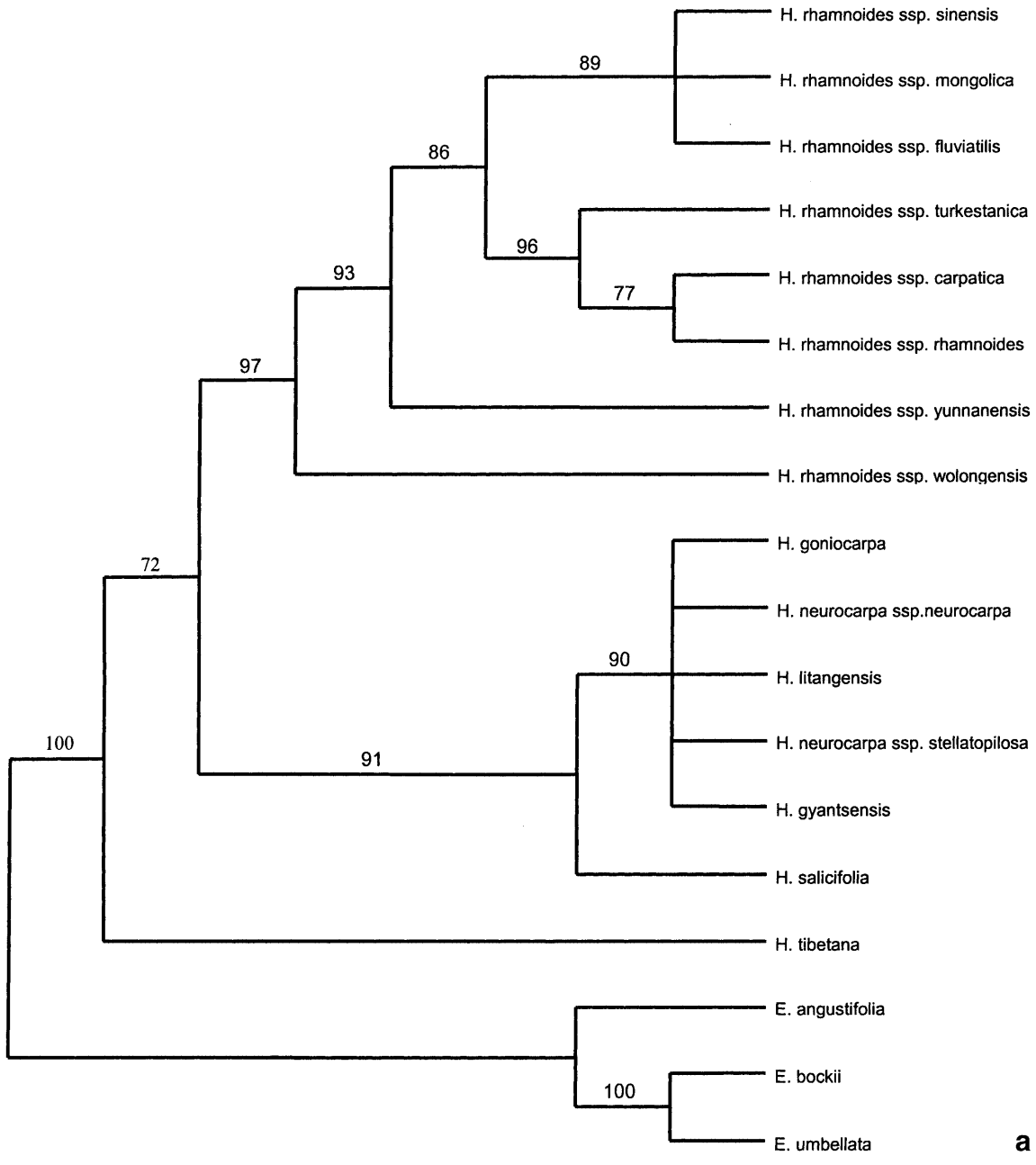

Fig. 1. Phylogenetic relationships among *Hippophae* species based on ITS sequences with two hybrids included. **a** Strict consensus of 21 equally most parsimonious trees (tree length = 351, CI = 0.846, RI = 0.862). **b** Neighbor-Joining tree using the Kimura two-parameter distance estimates. Bootstrap percentages more than 50% are shown above each branch

H. goniocarpa, *H. litangensis*, *H. neurocarpa* and *H. gyantsensis*, form an unresolved polytomy that is well supported by bootstrap value (90%). NJ analysis results in a tree with similar topology (Fig. 1b) to that of the parsimony tree. *Hippophae* is also divided into two clades and *H. tibetana* is at the basal position. In the NJ tree, however, the clade including the remaining taxa except *H. tibetana* is supported by a higher bootstrap value (96%). Further-

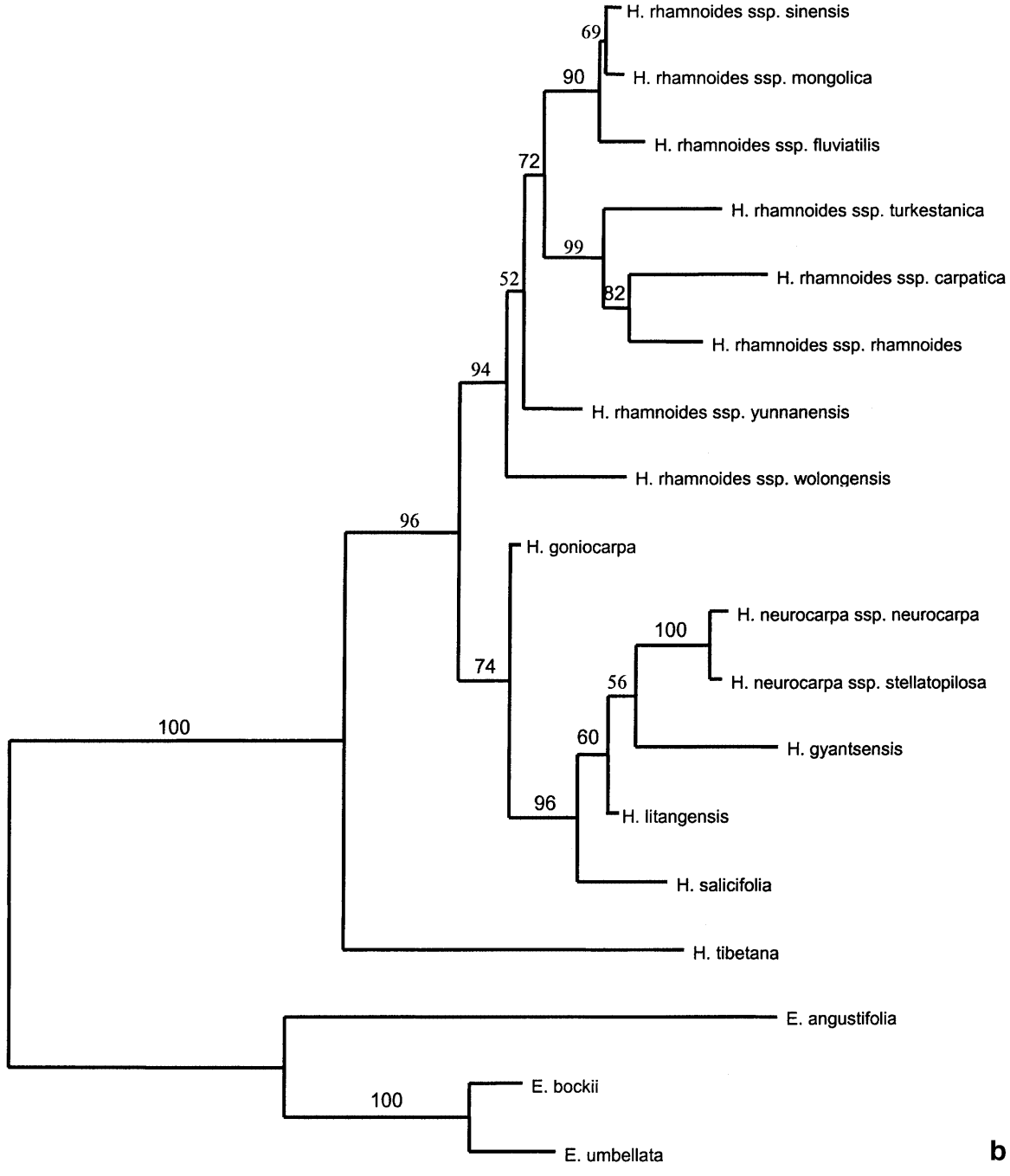

Fig. 1 (continued)

more, the two unresolved polytomies in the maximum parsimony analysis (Fig. 1a) are well resolved on the NJ tree (Fig. 1b).

Only three most parsimonious trees of 345 steps were generated with CI = 0.8551 and RI = 0.8649 when two putative hybrid species were excluded. The strict consensus tree displays similar topology as that of Fig. 1a but the bootstrap values increase considerably (Fig. 2a). It can be shown that the phylogenetic relationships among *H. salicifolia*, *H. neurocarpa* and *H. gyantsensis* are well resolved after two

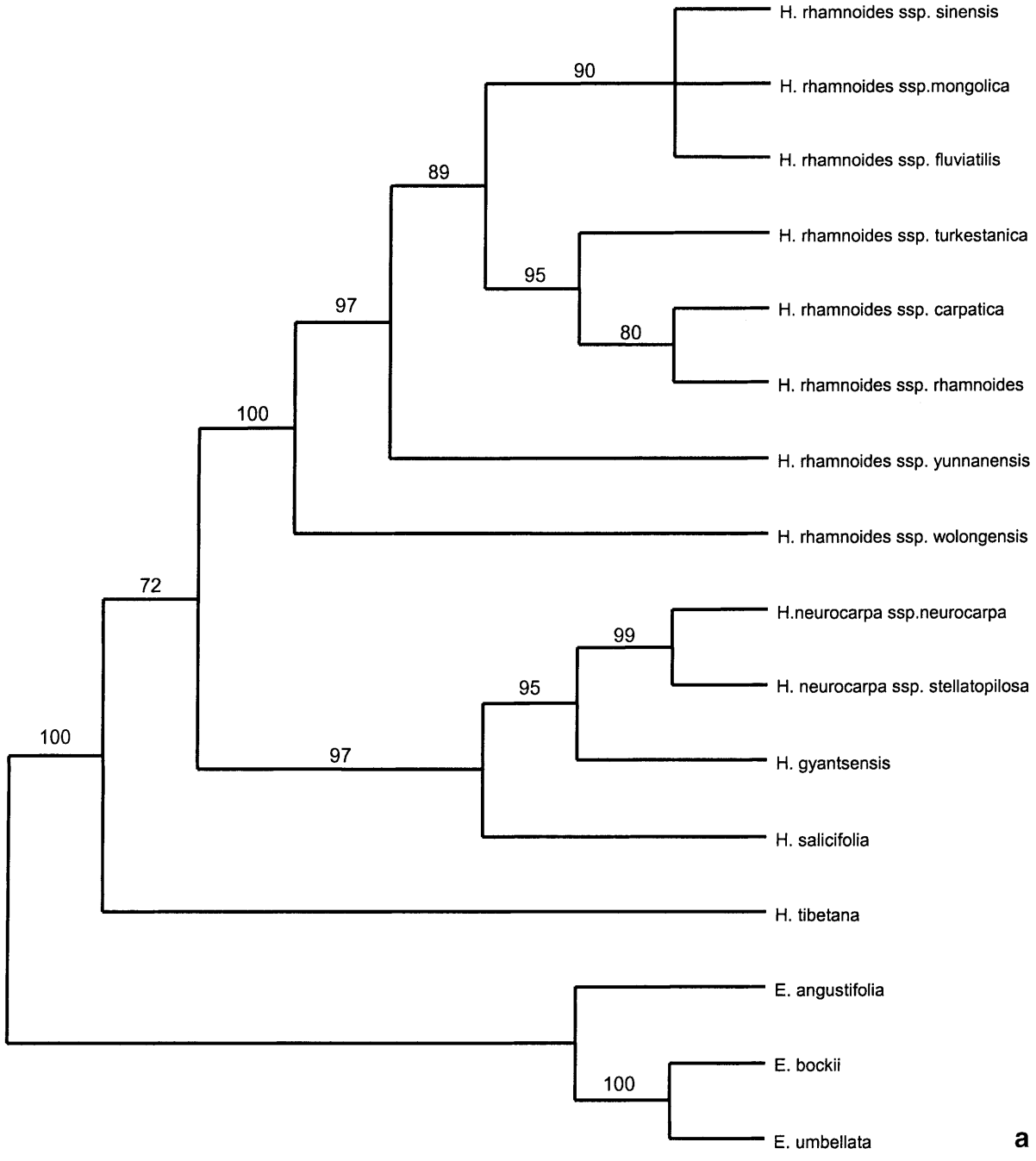

Fig. 2. Phylogenetic relationships among *Hippophae* species based on ITS sequences with two putative hybrid species excluded. **a** Strict consensus tree of three equally most parsimonious trees (tree length = 345, CI = 0.855, RI = 0.865). **b** Neighbor-Joining tree using the Kimura two-parameter distance estimates. Bootstrap percentages more than 50% are shown above each branch

putative hybrids are removed from the analysis. The clade including two subspecies of *H. neurocarpa* is supported by 99% bootstrap value with *H. gyantsensis* as the sister group (bootstrap value 95%). The NJ tree shows almost same topology with slightly different bootstrap values (Fig. 2b), and the exception is that the polytomic clade is resolved.

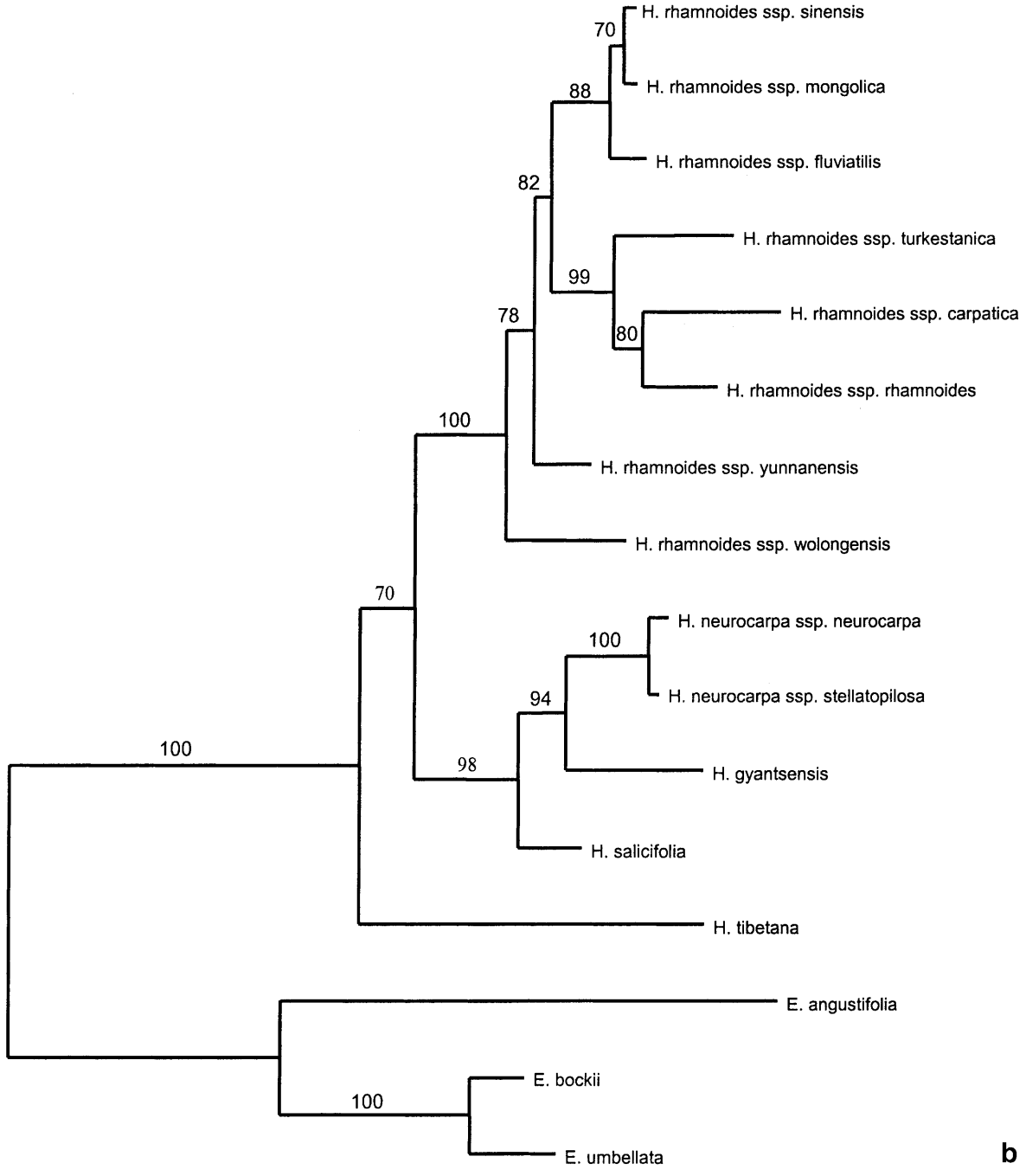

Fig. 2 (continued)

Discussion

Circumscription of *H. rhamnoides* and relationships among subspecies within the species. In the genus *Hippophae*, *H. rhamnoides* is the only one with wide distribution in Eurasia. This species was found to be extremely heterogeneous and was divided into several subspecies with different distributions (Rousi 1971, Lian 1988, Lian et al. 2000). These treatments agreed very well with isozyme studies of

Hippophae (Yao and Tigerstedt 1993). As pointed out by Rousi (1971), however, these subspecies were not always easily distinguished. For some subspecies of *H. rhamnoides*, e.g. *H. rhamnoides* subsp. *sinensis*, high diversity in morphology had been detected, especially for fruit, shape of hairs and chemical components (Rousi 1971, Zhao et al. 1991, Yao and Tigerstedt 1992, Lian et al. 2000). Phylogeny of *Hippophae* based on morphological characters indicated that the monophyly and circumscription of *H. rhamnoides* recognized by Rousi (1971) and Lian (1988) were problematic (Hyvonen 1996). According to Hyvonen (1996), *H. rhamnoides* subsp. *yunnanensis* and subsp. *sinensis* were excluded from this species, while *H. tibetana* and *H. neurocarpa* were included. The systematic position of subsp. *yunnanensis* and subsp. *sinensis* within *H. rhamnoides* was not well supported according to evidences from RAPD analyses of this genus (Bartish et al. 2000). However, the analysis based on combined data sets of cpDNA and morphological characters revealed that currently recognized subspecies of this species formed a monophyletic group (Bartish et al. 2002). The ITS data are not in agreement with the results of morphological and RAPD analyses, and provide strong support for the monophyly of *H. rhamnoides* proposed by Lian et al. (2000) and Rousi (1971) when *H. rhamnoides* subsp. *gyantsensis* is excluded. In ITS trees, all subspecies of *H. rhamnoides* recognized by Rousi (1971) except subsp. *gyantsensis* Rousi, form a monophyletic clade with 97% and 94% (with the putative hybrid included) or 100% (without the putative hybrid) bootstrap support in strict consensus trees or NJ trees respectively (Figs. 1 and 2). Thus, the present results are in good agreement with those of Bartish et al. (2002). *Hippophae rhamnoides* subsp. *gyantsensis*, which was treated as a separate species by Lian (1988), was here clustered with *H. neurocarpa*, *H. goniocarpa*, *H. litangensis* and *H. salicifolia*, in agreement with Bartish et al. (2002). Obviously, ITS phylogeny gives strong support for the treatment of *H. rhamnoides* by Lian (1988) and do not agree with those by Avdeyev (1983) and Hyvonen (1996).

On the basis of the ITS sequences, the mean divergence values between subspecies of *H. rhamnoides* vary from 0.15% (between *H. rhamnoides* subsp. *sinensis* and subsp. *mongolica* Rousi) to 6.22% (between the published subspecies and subsp. *carpatica* Rousi), suggesting that *H. rhamnoides* subsp. *wolongensis* was strongly differentiated in *H. rhamnoides*, and thus deserve taxonomic treatment at subspecies or even at species level. On the other hand, three subspecies, *H. rhamnoides* subsp. *sinensis*, subsp. *mongolica* and subsp. *fluviatilis* van Soest form a closely related group supported by high bootstrap values in both parsimonious and NJ trees, which is in agreement with Bartish et al. (2002) where *H. rhamnoides* subsp. *sinensis* is basal to other subspecies of *H. rhamnoides*. The close relationship between subsp. *yunnanensis* and subsp. *sinensis* suggested by Rousi (1971) and Hyvonen (1996) is not evidenced by our ITS data. The present ITS data also indicate that three subspecies, subsp. *turkestanica* Rousi, subsp. *carpatica* and subsp. *rhamnoides*, are most closely related.

Phylogenetic relationships among species of *Hippophae*. The number of species in *Hippophae* recognized so far by different authors varied greatly (Rousi 1971, Liu and He 1978, Avdeyev 1983, Lian 1988, Hyvonen 1996, Lian et al. 2000, Bartish et al. 2002) though Rousi (1971) pointed out that species boundaries of this genus were quite distinct. Lian (1988) recognized five species and eight subspecies mainly following Rousi (1971), and further divided the genus into two sections based on fruit morphology. However, by claiming that the genus *Hippophae* included only two species, *H. salicifolia* and *H. rhamnoides*, Hyvonen (1996) treated *H. tibetana*, *H. neurocarpa* and *H. gyantsensis* as subspecies of *H. rhamnoides*, and transferred *H. rhamnoides* subsp. *sinensis* and *H. rhamnoides* subsp. *yunnanensis* to *H. salicifolia* as subspecies, respectively. In their recent phylogenetic study on this genus, Bartish et al. (2002) recognized eight species

and seven subspecies, including two species (*Hippophae goniocarpa* and *H. litangensis*) and one subspecies (*H. neurocarpa* subsp. *stellato-pilosa*) that were previously published as nomina nuda and were validated by them. It is evident from the ITS data that Hyvonen's (1996) treatment is not justified, but rather the treatments of Rousi (1971), Lian (1988), Lian et al. (2000) and Bartish et al. (2002) are supported to large degree. Results from ITS data are concordant with those by isozyme (Yao and Tigerstedt 1993), RAPD analyses (Bartish et al. 2000) and the combined cpDNA and morphological data (Bartish et al. 2002).

As can be seen in Table 3, the ITS region within *Hippophae* is remarkably variable. The sequence divergence between species is relatively high except the divergence between subspecies of *H. rhamnoides*, and of *H. neurocarpa* as well as that between the hybrids and their putative parents. The greatest sequence divergence within the genus is measured between *H. tibetana* and all other groups, with the mean distances from 8.07% to 11.88%. Morphological analysis also indicate that *H. tibetana* stood away from the rest of this genus (Rousi 1971). Although *H. tibetana* clustered with *H. gyantsensis*, *H. salicifolia* and *H. neurocarpa* in the combined cpDNA and morphological tree (Bartish et al. 2002), it is basal to all other taxa of *Hippophae* in the ITS trees (Figs. 1 and 2). Apparently, the sister relationship between *H. tibetana* and *H. neurocarpa* suggested by morphological cladistic analysis (Hyvonen 1996) and isozyme studies (Yao and Tigerstedt 1993) is not verified by our ITS data as well as RAPD and cpDNA analyses (Bartish et al. 2000, 2002).

The species *H. neurocarpa* is very unique in the genus *Hippophae* mainly because its fruit is brown and almost without juice. To some degree, this species is more or less similar to *H. gyantsensis* in fruit characters. Because all subspecies of *H. rhamnoides* forms a highly supported clade while *H. neurocarpa* formed a highly supported clade with other four species (Figs. 1 and 2), the treatment of *H. neurocarpa* as a subspecies of *H. rhamnoides* by Hyvonen (1996) is not supported. Instead, *H. neurocarpa* is closely related to *H. gyantsensis*, *H. litangensis*, *H. goniocarpa* and to some degree to *H. salicifolia* (Figs. 1 and 2). The two subspecies of *H. neurocarpa* which were recognized by Lian et al. (1997) mainly based on shape of hairs on leaf surface and validly published by Bartish et al. (2002), are sister groups as shown in Figs. 1 and 2. The clear differentiation between two subspecies of *H. neurocarpa* revealed by morphological characters (Lian et al. 2000) as well as cpDNA and RAPD studies (Bartish et al. 2000, 2002) is further supported here. *H. gyantsensis*, which was ever treated as a subspecies of *H. rhamnoides* by Rousi (1971) and Hyvonen (1996), is sister to *H. neurocarpa* (Figs. 1 and 2). Therefore, its species status proposed by Lian (1988) was justified, and has also been suggested by RAPD data (Bartish et al. 2000). In addition, Rousi (1971) concluded that *H. salicifolia* represented an extreme in the genus in many respects and came closest to *H. rhamnoides* subsp. *yunanensis*. Lian (1988) further treated *H. salicifolia* and *H. rhamnoides* together in Sect. *Rhamnoides*. However, no evidence of a close relationship between *H. salicifolia* and *H. rhamnoides* was found in RAPD and cpDNA analyses (Bartish et al. 2000, 2002) and our ITS result.

In conclusion, ITS phylogeny gives support to the taxonomic treatments proposed by Rousi (1971), Lian (1988), Lian et al. (1997, 1998, 2000) and Bartish et al. (2002). However, phylogenetic relationships among species of *Hippophae* revealed by ITS data are not consistent with most conclusions proposed previously, but in agreement with that of Bartish et al. (2002) to a large extent.

Origin of the deduced hybrid *H. goniocarpa* and *H. litangensis*. Plants of hybrid origin typically exhibit additivity of parental genomes, and molecular markers thus can be used to identify the origin of hybrids. DNA sequencing data, especially the nuclear rDNA sequence, is a useful method in detection of hybrid and reticulate evolution and has been successfully used to reveal the history of several hybrid species (Kim and Jansen 1994,

Table 3. Kimura 2-parameter distances of taxa in the genus *Hippophae* and the outgroups

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. *H. rhamnoides* ssp. *sinensis*	–																
2. *H. goniocarpa*	0.00917	–															
3. *H. neurocarpa* ssp. *neurocarpa*	0.06670	0.00310	–														
4. *H. litangensis*	0.03952	0.00782	0.01111	–													
5. *H. neurocarpa* ssp. *stellatopilosa*	0.06615	0.00463	0.00464	0.00925	–												
6. *H. rhamnoides* ssp. *mongolica*	0.00150	0.01073	0.07127	0.04423	0.07073	–											
7. *H. rhamnoides* ssp. *wolongensis*	0.03217	0.02648	0.07312	0.05085	0.07247	0.03824	–										
8. *H. rhamnoides* ssp. *turkestanica*	0.03854	0.03316	0.08127	0.05758	0.07893	0.04304	0.05090	–									
9. *H. rhamnoides* ssp. *carpatica*	0.04801	0.04417	0.09506	0.06914	0.09582	0.05100	0.06224	0.04139	–								
10. *H. rhamnoides* ssp. *rhamnoides*	0.03529	0.03128	0.09129	0.05730	0.08731	0.03824	0.05246	0.03509	0.03202	–							
11. *H. rhamnoides* ssp. *fluviatilis*	0.00757	0.01700	0.07618	0.04913	0.07567	0.00900	0.04452	0.04140	0.04461	0.03198	–						
12. *H. salicifolia*	0.05301	0.01387	0.03079	0.02329	0.03043	0.05915	0.05606	0.07227	0.08383	0.07540	0.06397	–					
13. *H. rhamnoides* ssp. *yunnanensis*	0.01863	0.01576	0.06744	0.03541	0.06744	0.02323	0.02797	0.03913	0.04575	0.03748	0.02796	0.05238	–				
14. *H. tibetana*	0.09122	0.08073	0.11880	0.09722	0.11364	0.09583	0.09956	0.11338	0.11825	0.10588	0.10248	0.09767	0.09501	–			
15. *H. gyantsensis*	0.07741	0.02349	0.03526	0.03208	0.03482	0.08281	0.07884	0.08852	0.10001	0.09976	0.08852	0.03816	0.07569	0.10809	–		
16. *E. angustifolia*	0.23707	0.22112	0.24454	0.23975	0.24162	0.24209	0.24287	0.26614	0.26731	0.26409	0.24444	0.23045	0.24462	0.25210	0.22726	–	
17. *E. bockii*	0.18138	0.16612	0.19306	0.17979	0.18879	0.18638	0.18696	0.21541	0.21779	0.21100	0.19252	0.17602	0.18737	0.20805	0.19675	0.12164	–
18. *E. umbellata*	0.18716	0.17196	0.20074	0.19197	0.19636	0.19215	0.18853	0.22326	0.22376	0.21695	0.19832	0.18167	0.19329	0.21790	0.20514	0.12338	0.02132

Baldwin et al. 1995, Sang et al. 1995, Wendel et al. 1995, Ainouche and Bayer 1997). In the genus *Hippophae*, hybrid origins of two species, *H. goniocarpa* and *H. litangensis* have been suggested recently (Lian et al. 1997; Bartish et al. 2000, 2002). In the present study, the ITS sequences of two putative hybrids show high rates of polymorphism. Comparative analysis indicates that the polymorphism site in the putative hybrids resulted from the nucleotide additivity between putative parents. As implied by morphology, geographical distribution as well as RAPD and cpDNA studies, ITS sequence data strongly indicate that *H. goniocarpa* and *H. litangensis* are diploid hybrids derived through hybridization between *H. neurocarpa* subsp. *neurocarpa* and *H. rhamnoides* subsp. *sinensis*, between *H. neurocarpa* subsp. *stellatopilosa* and *H. rhamnoides* subsp. *yunnanensis*, respectively. In order to testify this hypothesis, the ITS region of additional individuals from both putative hybrids and their potential parents were collected from different localities, and were then sequenced. The results indicated that many variable sites occur in the ITS region of the hybrids. This explains why the clades comprising *H. gyantsensis, H. salicifolia*, *H. neurocarpa* subsp. *neurocarpa* and *H. neurocarpa* subsp. *stellatopilosa* were collapsed on the ITS trees when *H. goniocarpa* and *H. litangensis* were included in the analysis (Fig. 1a and 1b). The nucleotide additivity of the putative parents and its implications in phylogenetic reconstruction will be discussed elsewere (Sun et al. unpublished).

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