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Research Article

Phylogeny of *Euonymus* inferred from molecular and morphological data

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Abstract *Euonymus*, a cosmopolitan genus of the family Celastraceae, has its species diversity centered in East Asia. It exhibits a complex pattern of morphological variation, making its taxonomy difficult. Monophyly of the genus remains uncertain, and the evolutionary implications of the infrageneric division and delimitation of many species are debatable. In this study, we sampled a total of 62 accessions representing 48 species/taxa covering a broad range of diversity of the genus and its allies. We first investigated the phylogeny of *Euonymus* using DNA sequences of multiple nuclear and plastid markers, and then used this phylogeny to discuss the circumscription and classification of the genus in combination with evolution of fruit characters, which has been used as an important criterion for the generic subdivision. The resultant data revealed the monophyly of *Euonymus* sensu lato with inclusion of *Glyptopetalum*, resolved the major lines of phylogeny of the genus, and clarified the echinate and winged capsules each as lineage-specific. Therefore, among the five sections accepted by *Flora of China*, only Sect. *Echinococcus* (with spiny fruits) and Sect. *Kalonymus* (with winged fruits) correspond to the molecular grouping. The globose capsule may represent an ancestral character state, and the other types of fruits, that is, the shallowly to deeply lobed capsules, may belong to a broad range of continuous variation derived from the globose.

Key words DNA sequences, *Euonymus*, fruit shape, *Glyptopetalum*, phylogeny.

Euonymus L. (Celastraceae) is a cosmopolitan genus containing approximately 130 species across the tropical to the temperate zones. Its species diversity is centered in East Asia (ca. 95 species) with extensions into Southeast Asia (12 species), Europe (4 species), Australia (2 species), Africa (4 species), and America (7 species) (Ma, 2001). Plants of *Euonymus* are well known for their horticultural and medical use. The fruits of this genus are pod-like capsules that are conspicuously pink-red in color, which make some species widely cultivated for garden landscapes (Ma, 2001). Some species like *E. tingens* Wall. and *E. alatus* (Thunb.) Siebold are used medically; particularly, the winglike cork of *E. alatus* is commonly used in traditional Chinese medicine (Ma, 2001; Simmons et al., 2012).

The taxonomic classification of this genus remains controversial, especially with regards to generic status, circumscription, infrageneric division, and species delimitation. This may be due to the complex patterns of morphological variation. Previously, the circumscription of the genus was determined by a few or merely a single morphological trait of the flower or fruit. Blume (1825) was the first to use fruit traits (i.e., whether a capsule is angular or whether the dehiscent of fruit is deep) for the taxonomy of *Euonymus*. Although his work only covered five species, he recognized the importance of using fruit features in *Euonymus* classification, which has been crucial for the subsequent taxonomic efforts. Sprague (1908) grouped all species with echinate capsules into Sect. *Echinococcus*, and Nakai (1941) recognized 36 species and divided them into eight sections, of which Sect. *Echinococcus*, Sect. *Melanocarya*, and Sect. *Ilicifolia* were defined by fruit shapes. Not until Loesener (1902) had people started to pay attention to multiple traits on vegetative organs in addition to merely the fruit and flower characters. By 1942, Loesener accomplished a monograph of the genus with much more information on anatomy, embryology, pollination, and fruits. Blakelock (1951) published a synopsis of *Euonymus* using multidisciplinary data including morphology, biogeography, and paleontology. He recognized 177 species under 2 subgenera, 7 sections, and 14 series. Although this work has contributed greatly to the taxonomy of *Euonymus*, some authors, such as Ma (2001) and Simmons et al. (2012), considered Blakelock's "species" over-splitting.

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China, especially its southwest region with extension to the Himalayas, is the center of the species diversity of *Euonymus*. It harbors ca. 89 species, which is 69% of the total worldwide (Ma, 2001). However, earlier work on *Euonymus* rarely paid attention to this species-rich region. The first valuable account of the Chinese species was given by Loesener (1902). This was followed by Wang (1939), who recognized 61 Chinese *Euonymus* species, and Cheng & Huang (1999) provided a relatively complete taxonomy in *Flora Reipublicae Popularis Sinicae*, which recorded 111 species under 5 sections and 10 series. In a later revision of the taxonomy of *Euonymus*, Ma (2001) recognized 129 species worldwide. Concurring with most of the previous researchers that fruit shape was an important character, Ma set up a system of two subgenera (following Blakelock, 1951) and five sections based on fruit types.

Despite the aforementioned taxonomic efforts, the phylogeny of *Euonymus* remains obscure. Particularly, debates on the systematic position of the closely related *Glyptopetalum*, a small genus containing about 20 species, has raised the question on monophyly of *Euonymus*. In morphology, *Euonymus* and *Glyptopetalum* share several synapomorphies. However, a few reproductive traits, such as a single ovule, raphe with branches, and persistent axile placentation, have led to *Glyptopetalum* being treated as a separate genus (Thwaites, 1856; Bentham & Hooker, 1862; Baillon, 1880; Hou, 1963; Simmons et al., 2012). Taxonomic controversies often exist when complex relationships are inferred merely from morphological characters that are often influenced by the environment. To solve such problems, neutral molecular markers have been widely used in phylogenetic studies (Stuessy, 2009).

The internal transcribed spacer (ITS) of the nuclear ribosomal DNA has been a workhorse marker for species-level phylogenetic studies despite the acknowledged difficulty that may arise from its multiple copies (Baldwin et al., 1995; Álvarez & Wendel, 2003; Feliner & Rosselló, 2007). The external transcribed spacer (ETS) is less applied in plant molecular phylogenetic studies, but it has proven to be informative for the genus *Celastrus*, a close relative of *Euonymus* (Mu et al., 2012). From the plastid genome, three non-coding regions, *psbA-trnH*, *rp136-infA-rps8*, and *trnC-ycf6*, contain relatively high phylogenetic information at low taxonomic ranks (Hollingsworth et al., 2009) and have been used for DNA barcoding for some *Euonymus* and related taxa (Millen et al., 2001; Moore et al., 2010; Bruni et al., 2012). We therefore used sequences from these nuclear and plastid DNA non-coding regions to decipher the phylogeny of *Euonymus*, especially of its taxa from China.

We sampled all the representative taxa covering the morphological diversity of *Euonymus*. To clarify the circumscription of the genus, we also sampled its generic allies *Glyptopetalum*, *Celastrus*, and *Tripterygium* according to earlier studies on the family Celastraceae (Simmons & Hedin, 1999; Simmons et al., 2001a, 2001b). The main objectives of this study are, first, to examine the monophyly of the genus *Euonymus*; second, to reevaluate previous classification systems which were based on morphological characters; third, to interpret, in the light of molecular phylogeny, taxonomic implications and evolutionary trends of fruit types that were used as important criteria for generic subdivisions; and finally, to provide insight into systematic positions of a few taxa that remain of taxonomic debate.

1 Material and methods

1.1 Taxon sampling

Samples analyzed for this study were mostly collected from natural populations in China where the species diversity of *Euonymus* is centered. Taxa covering the broad range of species diversity across all five sections sensu Ma (Ma, 2001; Ma et al., 2008) were analyzed. In most cases, one specimen was analyzed for one species/taxon, whereas for those that are widely distributed, more than one accession was sampled from different locations. Three species of *Glyptopetalum* and four species of *Celastrus* and *Tripterygium* were also sampled according to earlier studies on the family Celastraceae (Simmons & Hedin, 1999; Simmons et al., 2001a, 2001b, 2012). As *Glyptopetalum* was found paraphyletic to *Euonymus* (Simmons et al., 2012), only *Celastrus* and *Tripterygium* were analyzed as outgroups. Therefore, a total of 62 specimens of 48 taxa were sequenced in this study (Table 1). In addition, nine ITS sequences of *Euonymus* and *Glyptopetalum* downloaded from NCBI GenBank were incorporated into our analysis, making the individual ITS dataset containing 71 sequences of 56 taxa.

Identification of the vouchers mainly followed Ma (2001) and Ma et al. (2008) with a few exceptions where we agreed with Cheng & Huang (1999) for their taxonomic treatments on some species, for example, *E. porphyreus* Loes. All voucher specimens were deposited in the herbarium of Beijing Forestry University (BJFC).

Table 1 Taxa, voucher information of the specimens analyzed in this study, and GenBank accession numbers of the sequences

Species	Locality	Voucher	GenBank accession number				
			ITS	ETS	*psbA-trnH*	*rp136-infA-rps8*	*trnC-ycf6*
Euonymus acanthocarpa Franch.	Wen Mt., Yunnan, China	Y.-C. Zheng, YN47 (BJFC)	KF282154	KF282032	KF282278	KF282093	KF282216
Euonymus alatus (Thunb.) Sieb	Dongling Mt., Beijing, China	Y.-N. Li, XLM6 (BJFC)	KF282155	KF282033	KF282279	KF282094	KF282217
Euonymus alatus (Thunb.) Sieb	Qinling Mt., Shaanxi, China	G.-M. Zhang & X. Liu, XYB014 (BJFC)	KF282156	KF282034	KF282280	KF282095	KF282218
Euonymus balansae Sprague	Wen Mt., Yunnan, China	Y.-C. Zheng, YN59 (BJFC)	KF282157	KF282035	KF282281	KF282096	KF282219
Euonymus bockii Loes. ex Diels	Wen Mt., Yunnan, China	Y.-C. Zheng, YN63 (BJFC)	KF282158	KF282036	KF282282	KF282097	KF282220
Euonymus carnosus Hemsl.	Huang Mt., Anhui, China	Y.-N. Li & H. Wang, HSM7 (BJFC)	KF282159	KF282037	KF282283	KF282098	KF282221
Euonymus carnosus Hemsl.	Wuyi Mt., Fujian, China	Y.-N. Li & Q. Tu, WYM7 (BJFC)	KF282160	KF282038	KF282284	KF282099	KF282222
Euonymus centidens H. Lev.	Jiulian Mt., Jiangxi, China	Y.-N. Li & Q. Tu, JLS4 (BJFC)	KF282161	KF282039	KF282285	KF282100	KF282223
Euonymus chenmoui W. C. Cheng	Tianmu Mt., Zhejiang, China	H. Wang, LA1 (BJFC)	KF282162	KF282040	KF282286	KF282101	KF282224
Euonymus chuii Hand.-Mazz	Emei Mt., Sichuan, China	Y.-C. Zheng, EMM2 (BJFC)	KF282163	KF282041	KF282287	KF282102	KF282225
Euonymus cornutus Hemsl.	Emei Mt., Sichuan, China	Y.-C. Zheng, EMM1 (BJFC)	KF282164	KF282042	KF282288	KF282103	KF282226
Euonymus cornutus Hemsl.	Huanglong, Sichuan, China	X.-Y. Mu, HL057 (BJFC)	KF282165	KF282043	KF282289	KF282104	KF282227
Euonymus dielsiana Loes. ex Diels	Emei Mt., Sichuan, China	Y.-C. Zheng, EMM21 (BJFC)	KF282166	KF282044	KF282290	KF282105	KF282228
Euonymus dielsiana Loes. ex Diels	Houhe, Hubei, China	J.-Y. Li & Y.-N. Li, Hh001 (BJFC)	KF282167	KF282045	KF282291	KF282106	KF282229
Euonymus dolichopa Merr. ex J. S. Ma	Longzhou, Guangxi, China	X.-Y. Mu, GX025 (BJFC)	KF282168	KF282046	KF282292	KF282107	KF282230
Euonymus echinatus Wall.	Shiwan Mt., Guangxi, China	X.-Y. Mu, GX001 (BJFC)	KF282169	KF282047	KF282293	KF282108	KF282231
Euonymus europaeus L.	Bot. Garden, Univ. Oldenbürg, Germany	D. Albach, Euo (BJFC)	KF282170	KF282048	KF282294	KF282109	KF282232
Euonymus frigida Wall.	Changdu, Tibet, China	L. He, XZ2794 (BJFC)	KF282171	KF282049	KF282295	KF282110	KF282233
Euonymus giraldii Loes. ex Diels	Shennongjia, Hubei, China	J.-Y. Li & J. Zhang, SN179 (BJFC)	KF282172	KF282050	KF282296	KF282111	KF282234
Euonymus gracillimus Hemsl.	Shiwan Mt., Guangxi, China	X.-Y. Mu, GX007 (BJFC)	KF282173	KF282051	KF282297	KF282112	KF282235
Euonymus grandiflora Wall.	Wen Mt., Yunnan, China	Y.-C. Zheng, YN44 (BJFC)	KF282174	–	KF282298	KF282113	KF282236
Euonymus hamiltonianus Wall. ex Roxb.	Huang Mt., Anhui, China	Y.-N. Li & H. Wang, HSM21 (BJFC)	KF282175	KF282052	KF282299	KF282114	KF282237
Euonymus hamiltonianus Wall. ex Roxb.	Wuyi Mt., Fujian, China	Y.-N. Li & Q. Tu, WYM4 (BJFC)	KF282176	KF282053	KF282300	KF282115	KF282238
Euonymus hederacea Champ. ex Benth.	Jigong Mt., Henan, China	Y.-N. Li & Y.-C. Zheng, J9 (BJFC)	KF282177	KF282054	KF282301	KF282116	KF282239
Euonymus laxiflora Champ. ex Benth.	Changdu, Tibet, China	L. He, XZ1728 (BJFC)	KF282178	KF282055	KF282302	KF282117	KF282240
Euonymus laxiflora Champ. ex Benth.	Wen Mt., Yunnan, China	Y.-C. Zheng, YN53 (BJFC)	KF282179	KF282056	KF282303	KF282118	KF282241
Euonymus maackii Rupr.	Dongling Mt., Beijing, China	Y.-N. Li, XY2 (BJFC)	KF282180	KF282057	KF282304	KF282119	KF282242
Euonymus macroptera Rupr.	Maoer Mt., Heilongjiang, China	Q. Tu, MEM1 (BJFC)	KF282181	KF282058	KF282305	KF282120	KF282243
Euonymus mengtseanus (Loes.) Sprague	Wen Mt., Yunnan, China	Y.-C. Zheng, YN54 (BJFC)	KF282182	KF282059	KF282306	KF282121	KF282244
Euonymus microcarpa (Oliv. ex Loes) Sprague	Pingwu, Sichuan, China	Z.-X. Zhang, PW (BJFC)	KF282183	KF282060	KF282307	KF282122	KF282245
Euonymus myrianthus Hemsl.	Houhe, Hubei, China	J.-Y. Li & Y.-N. Li, Hh003 (BJFC)	KF282184	KF282061	KF282308	KF282123	KF282246
Euonymus myrianthus Hemsl.	Jiulian Mt., Jiangxi, China	Y.-N. Li & Y.-C. Zheng, JLS10 (BJFC)	KF282185	KF282062	KF282309	KF282124	KF282247
Euonymus myrianthus Hemsl.	Wuyi Mt., Fujian, China	Y.-N. Li & Q. Tu, WYM8 (BJFC)	KF282186	KF282063	KF282310	KF282125	KF282248
Euonymus nanoides Loes. & Rehder	Heng Mt., Shanxi, China	Y.-C. Zheng, HM1 (BJFC)	KF282187	KF282064	KF282311	KF282126	KF282249
Euonymus nitidus Benth.	Shiwan Mt.,Guangxi, China	X.-Y. Mu, GX005 (BJFC)	KF282188	KF282065	KF282312	KF282127	KF282250
Euonymus oblongifolius Loes. & Rehd.	Xingyi, Guizhou, China	S.-Y. Meng, GZ001 (BNU)	KF282189	KF282066	KF282313	KF282128	KF282251
Euonymus oblongifolius Loes. & Rehd.	Xingyi, Guizhou, China	S.-Y. Meng, GZ002 (BNU)	KF282200	KF282077	KF282324	KF282139	KF282262
Euonymus oxyphyllus Miq.	Huang Mt., Anhui, China	Y.-N. Li & H. Wang, HSM16 (BJFC)	KF282190	KF282067	KF282314	KF282129	KF282252
Euonymus oxyphyllus Miq.	Qinling Mt., Shaanxi,China	G.-M. Zhang & X. Liu, XYB020 (BJFC)	KF282191	KF282068	KF282315	KF282130	KF282253
Euonymus phellomana Loes. ex Diels	Laojun Mt., Henan, China	Y.-C. Zheng & M. Xiao, LJM21 (BJFC)	KF282193	KF282070	KF282317	KF282132	KF282255
Euonymus phellomana Loes. ex Diels	Qinling Mt., Shaanxi,China	G.-M. Zhang & X. Liu, XYB025 (BJFC)	KF282194	KF282071	KF282318	KF282133	KF282256
Euonymus porphyreus Loes.	Laojun Mt., Henan, China	Y.-C. Zheng & M. Xiao, LJM20 (BJFC)	KF282195	KF282072	KF282319	KF282134	KF282257
Euonymus porphyreus Loes.	Qinling Mt., Shaanxi,China	G.-M. Zhang & X. Liu, XYB026 (BJFC)	KF282196	KF282073	KF282320	KF282135	KF282258
Euonymus sanguinea Loes. ex Diels	Huanglong, Sichuan, China	X.-Y. Mu, HL058 (BJFC)	KF282198	KF282075	KF282322	KF282137	KF282260
Euonymus sanguinea Loes. ex Diels	Changdu, Tibet, China	L. He, XZ2675 (BJFC)	KF282199	KF282076	KF282323	KF282138	KF282261
Euonymus semenovii Regel & Herder	Hongdougu, Shanxi, China	Z.-X. Zhang, HDG1 (BJFC)	KF282197	KF282074	KF282321	KF282136	KF282259
Euonymus sp.	Shennongjia, Hubei, China	J.-Y. Li & J. Zhang, SN584 (BJFC)	KF282201	KF282078	KF282325	KF282140	KF282263

Continued

Table 1 Continued

Species	Locality	Voucher	GenBank accession number				
			ITS	ETS	*psbA-trnH*	*rp136-infA-rps8*	*trnC-ycf6*
Euonymus subsessilis Sprague	Emei Mt., Sichuan, China	Y.-C. Zheng, EMM5 (BJFC)	KF282202	KF282079	KF282326	KF282141	KF282264
Euonymus subsessilis Sprague	Shiwan Mt., Guangxi, China	X.-Y. Mu, GX017 (BJFC)	KF282203	KF282080	KF282327	KF282142	KF282265
Euonymus theacola C. Y. Cheng ex T. L. Xu & Q. H. Chen	Fangcheng, Guangxi, China	X.-Y. Mu, GX006 (BJFC)	KF282204	KF282081	KF282328	KF282143	KF282266
Euonymus tingens Wall.	Wen Mt., Yunnan, China	Y.-C. Zheng, YN70 (BJFC)	KF282205	KF282082	KF282329	KF282144	KF282267
Euonymus verrucosa Scop.	Laoju Mt., Gansu, China	S.-B. Dong, GLJ1 (BJFC)	KF282206	KF282083	KF282330	–	KF282268
Euonymus verrucosoides Loes.	Qinling Mt., Shaanxi, China	G.-M. Zhang & X. Liu, XYB024 (BJFC)	KF282207	KF282084	KF282331	KF282145	KF282269
Euonymus viburnoides Prain	Wen Mt., Yunnan, China	Y.-C. Zheng, YN49 (BJFC)	KF282208	KF282085	KF282332	KF282146	KF282270
Euonymus wilsonii Sprague	Emei Mt., Sichuan, China	Y.-C. Zheng, EMM3 (BJFC)	KF282209	KF282086	KF282333	KF282147	KF282271
Celastrus angulatus Maxim.	Yunnan, China	X.-Y. Mu, 013 (BJFC)	KF282152	KF282030	KF282276	KF282091	KF282214
Celastrus orbiculatus Thunb.	Yunnan, China	X.-Y. Mu, 001 (BJFC)	KF282153	KF282031	KF282277	KF282092	KF282215
Glyptopetalum continentale (Chun & How) C. Y. Cheng & J. S. Ma	Yunnan, China	Q.-R. Liu, L001 (BNU)	KF282210	KF282087	–	KF282148	KF282272
Glyptopetalum pallidifolium (Hayata) Q. R. Liu & S. Y. Meng	Kenting, Taiwan, China	P.-J. Liao, TW (BJFC)	KF282192	KF282069	KF282316	KF282131	KF282254
Glyptopetalum rhytidophyllum (Chun & F. C. How) C. Y. Cheng	Yunnan, China	X.-Y. Mu, 023 (BJFC)	KF282211	KF282088	KF282334	KF282149	KF282273
Tripterygium regelii Sprague & Takeda	Yunnan, China	B.-J. Zheng, Zheng008 (NEFI)	KF282212	KF282089	KF282335	KF282150	KF282274
Tripterygium wilfordii Hook. f.	Yunnan, China	B. Xu, Xu001 (BJFC)	KF282213	KF282090	KF282336	KF282151	KF282275

–, No sequence available for that accession. ETS, external transcribed spacer; ITS, internal transcribed spacer; Mt., mountains.

1.2 DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from 30 mg silica gel-dried leaf materials or from fresh leaf fragments using the Plant Kit (Tiangen Biotech, Beijing, China) following the manufacturer's protocol. Some difficult samples were extracted using the 2× CTAB method (Doyle & Doyle, 1987).

Two nuclear DNA regions, the ITS and the ETS, and three chloroplast DNA intergenic spacers, *psbA-trnH*, *rp136-infA-rps8*, and *trnC-ycf6*, were sequenced. The nuclear ribosomal DNA regions were amplified and sequenced using primer pairs ITS4/ITS5 (White et al., 1990) and ETS-1F/18S-IGS (Baldwin & Markos, 1998; Weeks et al., 2005), respectively. For ITS, internal primer pairs, N-nc18S10/C26A (Wen & Zimmer, 1996) and the specific ITS-1f, ITS-1r, and ITS-2f, ITS-2r (designed in this study) were applied when amplification repeatedly failed with universal primers. We followed Demesure et al. (1995), Hamilton (1999), Lee & Wen (2004), and Kress et al. (2005) for primers to amplify three chloroplast DNA regions. All the primer information is provided in Table 2. The amplified products were purified with the Plant Kit (Tiangen Biotech) and then used for direct sequencing (carried out by SinoGenoMax, Beijing, China). All the sequences were deposited in NCBI GenBank under accession numbers KF282030–KF282336 (Table 1).

1.3 Phylogenetic analyses

Sequences were assembled and aligned with Sequencher 4.1.4 (Gene Codes Corp., Ann Arbor, MI, USA). The alignments were adjusted manually in Se-Al 2.0 (Rambaut, 2002). Areas with ambiguous alignment or containing poly-N stretches were excluded from the phylogenetic analyses.

Our datasets were examined with the incongruence length difference (ILD) test (Farris et al., 1994) as implemented in the partition homogeneity test in PAUP* (Swofford, 2003). Heuristic searches were run for 100 replicates with simple sequence addition and the tree bisection–reconnection branch swapping option. For each replicate, a maximum of 100 trees were saved.

Phylogenetic analyses were carried out using maximum parsimony (MP) analysis implemented in PAUP*4.0b10 (Swofford, 2003) and Bayesian inference (BI) in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). For the MP analysis, heuristic searches were carried out with tree bisection–reconnection branch swapping, random addition sequence with 1000 replicates, and multiple trees saved for each replicate (no more than 10 trees with scores higher than 10 were saved per replicate). Internal node supports were

Table 2 Primers used for amplification and sequencing of two nuclear DNA regions (internal transcribed spacer (ITS) and external transcribed spacer (ETS)) and three chloroplast DNA intergenic spacers (*psbA-trnH*, *rp136-infA-rps8*, and *trnC-ycf6*) of *Euonymus* species

Primer name	Primer sequence (5′–3′)	Refs.
ITS-1f	TTAAACTCAGCGGGTGTTCC	This study
ITS-1r	AAGGTTTCCGTAGGTGAACC	
ITS-2f	GTGTTCCCGCCTGACCTGG	
ITS-2r	TGAACCTGCGGAAGGATCATTGTCG	
ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
ITS5	GGAAGTAAAAGTCGTAACAAGG	
ITS-N-nc18S10	AGGAGAAGTCGTAACAAG	Wen & Zimmer (1996)
ITS-C26A	GTTTCTTTTCCTCCGCT	
ETS-1F	TTCGGTATCCTGTGTTGCTTAC	Weeks et al. (2005)
18S-IGS	GAGACAAGCATATGACTACTGGCAGGATCAACCAG	Baldwin & Markos (1998)
psbA	GTTATGCATGAACGTAATGCTC	Hamilton (1999)
trnH	CGCGCATGGTGGATTCACAAATC	
rp136f	CACAAATTTTACGAACGAAG	Kress et al. (2005)
rps8r	TAATGACAGAYCGAGARGCTCGAC	
trnC	CCAGTTCAAATCTGGGTGTC	Demesure et al. (1995)
petN 1R	CCCAAGCAAGACTTACTATATCC	Lee & Wen (2004)

estimated with 100 bootstrap (Felsenstein, 1985) replicates each with 100 random addition sequences.

Prior to the Bayesian analysis, each dataset was tested for the molecular evolution model using Modeltest 3.7 (Posada & Crandall, 1998) as implemented in MrMTgui (Nuin, 2005) based on the Akaike information criterion (Posada & Buckley, 2004). The best-fit models suggested for each of the datasets are listed in Table 3. Markov chain Monte Carlo searches were run for 3 000 000 generations. All Bayesian analyses produced split frequencies of less than 0.01, showing convergence between the paired runs. Of the 30 000 trees produced, the first 2000 before stationary were discarded as burn-in, and the remaining trees were used to construct the majority-rule consensus trees.

2 Results

2.1 Sequence data and ILD tests

The ILD test yielded non-significant incongruence between all pairs of the plastid ($P > 0.05$) as well as between the nuclear ITS and ETS datasets ($P = 0.09$). Therefore, sequences of the plastid loci were concatenated into one matrix containing 2369 aligned positions, and those of ITS and ETS into a matrix of 885 bp in length. In contrast, neither the individual nuclear ETS/nuclear ITS nor their combined dataset presented confident congruence with the plastid ($P = 0.01$). However, considering the problems of the ILD test in revealing the true positions and level of data conflict (for details, see Section 3) (De Queiroz, 1993; Bergh et al., 2011), we still combined sequences from all five loci.

The sequence variation and other molecular evolutionary characters of each individual and combined datasets are provided in Table 3. Phylogenetic analyses were first carried out separately on the combined nuclear (Fig. 1: A), the combined plastid (Fig. 1: B), and the ITS datasets which contained nine additional sequences downloaded from GenBank (Fig. S1), and then on the all-combined data (Fig. 2). As the nuclear and plastid trees agreed with each other in resolving major clades and displayed only conflicts

Table 3 DNA loci of *Euonymus* species analyzed, their sequence characteristics, and evolutionary model

Gene region	No. of samples	Aligned length (bp) (with/without gaps)	Variable sites	Parsimony informative sites	Tree length	CI	RI	AIC model
ITS	62	771/706	65	207	830	0.4855	0.5145	GTR + I + G
ITS (ext.)[†]	71	789/706	99	221	1067	0.4602	0.5398	GTR + I + G
ETS	61	335/320	41	147	513	0.5107	0.4893	TVM + G
Combined nuclear DNA	62	885/841	80	311	1193	0.4728	0.5272	GTR + G
Combined plastid DNA	62	2369/1867	182	229	653	0.7213	0.2787	TVM + I + G
All combined	62	3254/2703	262	540	1905	0.5433	0.4567	GTR + G
psbA-trnH	61	723/545	83	107	323	0.7214	0.2786	TVM + G
rp136-infA-rps8	61	552/496	31	36	96	0.7292	0.2708	TVM + I + G
trnC-ycf6	62	1096/874	73	86	200	0.875	0.125	TVM + G

[†]The extended (ext.) internal transcribed spacer (ITS) dataset with nine additional sequences was downloaded from NCBI GenBank. AIC, Akaike information criterion; CI, consistency index; ETS, external transcribed spacer; RI, retention index.

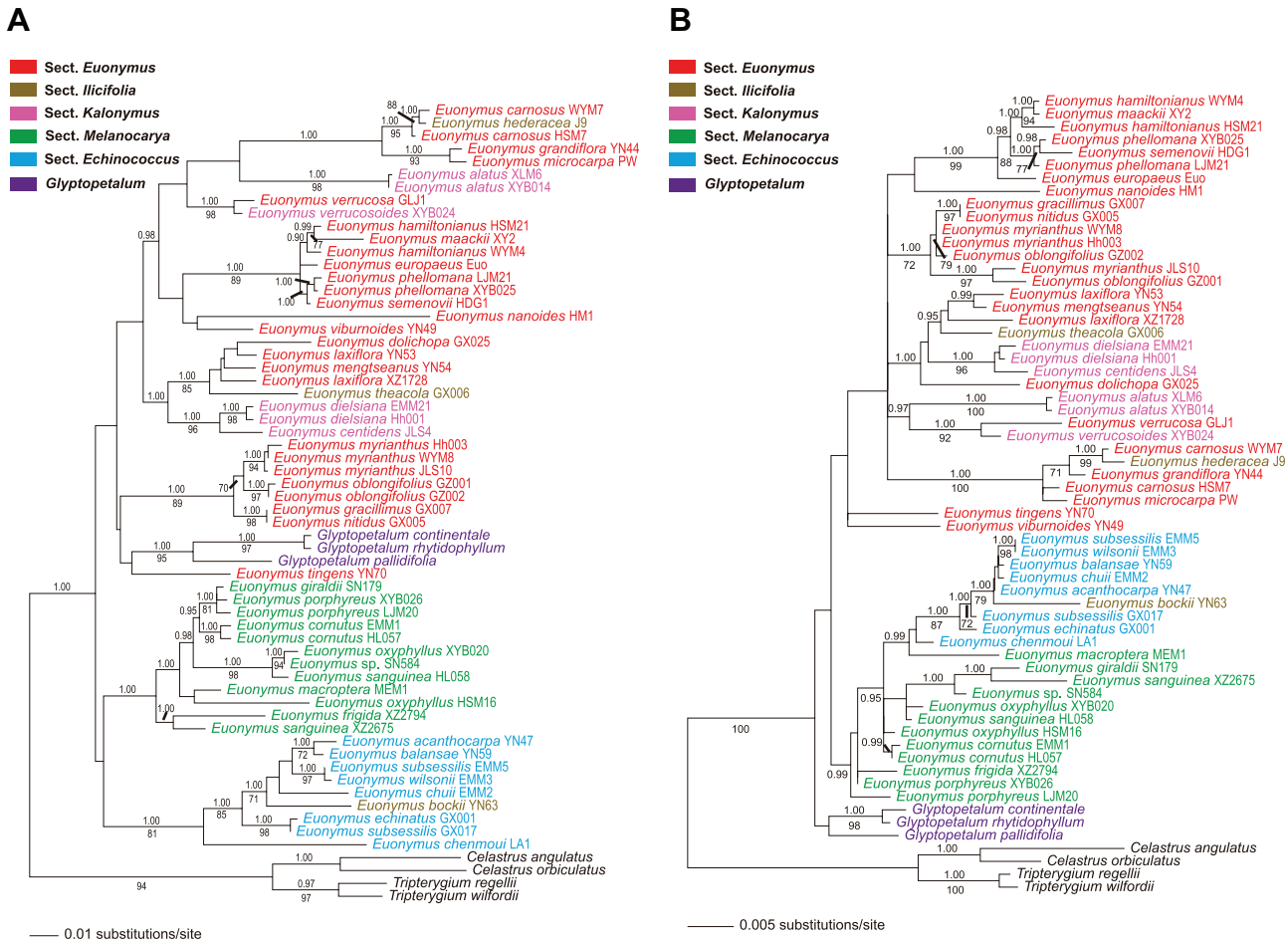

Fig. 1. Bayesian consensus tree of the *Euonymus* species based on the combined nuclear dataset (**A**) and combined plastid dataset (**B**). Bayesian posterior probabilities (>0.9) and bootstrap values (>70%) of the maximum parsimony analysis are shown above and below the branches, respectively. Terminal nodes are in six different colors corresponding to the five sections of *Euonymus* defined in *Flora of China* (Ma et al., 2008) and the genus *Glyptopetalum*.

on a few poorly supported branches (e.g., the first branching) and as the all-combined data gave the best power of resolution, we refer to the all-combined tree (Fig. 2) for phylogenetic inference with consideration of conflicting information from different individual datasets.

2.2 Phylogenetic inference

According to fruit shapes, Ma (2001) and Ma et al. (2008) divided *Euonymus* into five sections, Sect. *Echinococcus*, Sect. *Kalonymus*, Sect. *Melanocarya*, Sect. *Euonymus*, and Sect. *Ilicifolia*. We herein map these sections on the phylogenetic tree (Fig. 2) for clarification and for the discussion below.

The MP analysis and BI were congruent in resolving some of the deep clades and most of the shallow branches. Nevertheless, the MP tree, showing lower resolution than the BI tree, generated a large polytomy including all the major clades. Therefore, only the BI tree is presented and bootstrap supports (BS) higher than 70% from the MP analysis are shown on the BI tree (Fig. 2).

Rooted with species of two outgroup genera, *Celastrus* and *Triterygium*, both the BI and MP trees on the all-combined data resolved a strongly supported *Euonymus* + *Glyptopetalum* group (posterior probability (*PP*) = 1.00; *BS* = 100). Within this group we can recognize four clades with strong to moderate supports (Fig. 2). Clade I (*PP* = 0.97; *BS* < 70) comprised two well-supported subclades, Ia (*PP* = 1.00; *BS* = 98) and Ib (*PP* = 1.00; *BS* = 82), corresponding to Sect. *Echinococcus* and Sect. *Kalonymus*, respectively. Clade II (*PP* = 0.92; *BS* < 70) contained three *Glyptopetalum* and one *Euonymus* species. The clade formed by the three *Glyptopetalum* species was strongly supported (*PP* = 1.00; *BS* = 99). In the ITS tree (Fig. S1), with an additional sequence of *Glyptopetalum palawanense* Merr. from the Philippines (sequence

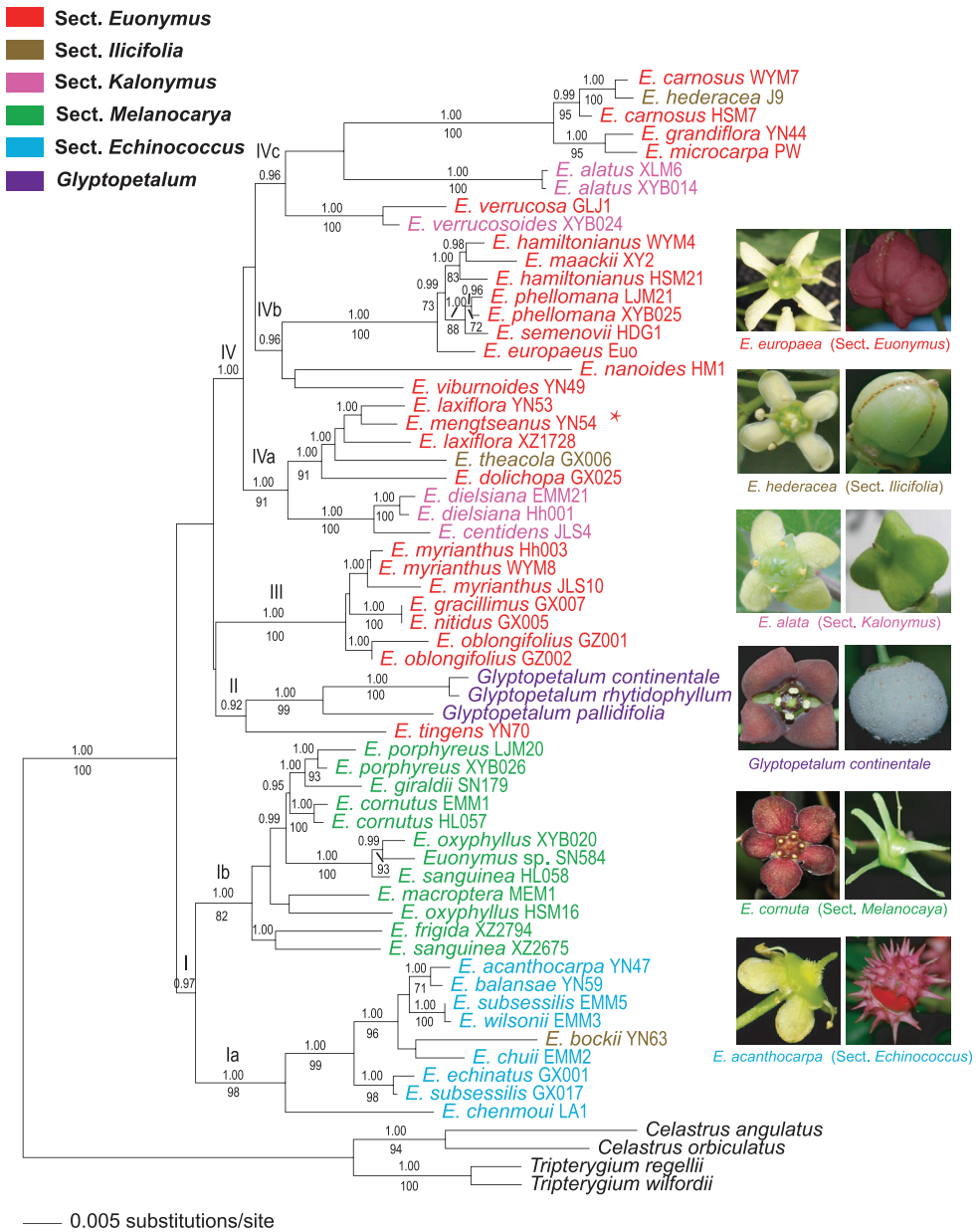

Fig. 2. Bayesian consensus tree of the *Euonymus* species based on a combined dataset of nuclear internal transcribed spacer and external transcribed spacer and plastid *psbA-trnH*, *rp136-infA-rps8*, and *trnC-ycf6*. Bayesian posterior probabilities (>0.9) and bootstrap values (>70%) of the maximum parsimony analysis are shown above and below the branches, respectively. Terminal nodes are in six different colors corresponding to the five sections of *Euonymus* defined in *Flora of China* (Ma et al., 2008) and the genus *Glyptopetalum*. Flower and fruit morphology of a representative species of each of the five sections and *Glyptopetalum* are mapped beside the corresponding clades or branches. I–IVc represents each clade. **Euonymus mengtseanus*, the systematic position of which is clarified in this study.

obtained from GenBank, Simmons et al., 2012), the *Glyptopetalum* species still formed a strongly supported clade ($PP = 1.00$; $BS = 99$). Clade III ($PP = 1.00$; $BS = 100$) was exclusively of species of Sect. *Euonymus*. Clade IV ($PP = 1.00$; $BS < 70$) contained all members of Sect. *Melanocarya*, and most of Sect. *Euonymus* and Sect. *Ilicifolia*. Within this clade, only IVb was purely of species from Sect. *Euonymus*, whereas IVa and IVc were composed of species from the three sections.

Despite some discordance, the ITS + ETS tree (Fig. 1: A), the individual ITS and ETS trees (Figs. S1, S2) and the combined plastid tree (Fig. 1: B) were congruent to some extent with each other and with the all-

combined tree. The congruence appeared on three points: (i) *Euonymus* and *Glyptopetalum* together formed a monophyletic group; (ii) species of *Glyptopetalum* always grouped together; and (iii) *E. mengtseanus* (Loes.) Sprague, which was recognized as a member of Sect. *Echinococcus* (Ma, 2001; Ma et al., 2008), consistently appeared close to species of Sect. *Euonymus*. With a broader taxon sampling, the ITS tree (Fig. S1) does not alter the topology but identified a set of similar relationships yielded by the all-combined data.

However, incongruence existed in the relationships between Sect. *Echinococcus* and Sect. *Kalonymus*. In the plastid tree, these two sections were intermingled though they fell in one clade ($PP = 0.99$) (Fig. 1: B), whereas in the other trees, they each formed a well-supported clade (Fig. 1: A; Figs. 2, S1, S2). These two clades further grouped together in the all-combined tree with a BI support of 0.97 (Fig. 2), but without sufficient support in the ITS + ETS tree (Fig. 1: A) or the individual ITS tree (Fig. S1). In the ETS tree, they did not group, but were separated in two poorly supported clades (Fig. S2).

3 Discussion

3.1 Evaluation of data congruence

The use of nuclear ITS, nuclear ETS, and plastid DNA data provided substantial information for our understanding of the phylogeny of *Euonymus* and its generic allies. Our analyses of different datasets identified several similar major clades. However, there were some incongruences, which were reflected by the ILD test. Incongruence between data is common due to the different evolutionary histories of different genomes or different regions of the same genome. In addition, relationships of taxa within *Euonymus* resolved by the present molecular data, either the combined or the individual dataset, were partially in disagreement with that expected from morphological characters. The best fit of our molecular data and morphological traits was on the delimitation of Sect. *Echinococcus* and Sect. *Kalonymus*. In all but the plastid tree, Sect. *Echinococcus* and Sect. *Kalonymus* each appeared as monophyletic (Fig. 1: A; Figs. 2, S1, S2). Even the plastid data supported a broader monophyletic group encompassing both Sect. *Echinococcus* and Sect. *Kalonymus* (Fig. 1: B; for detailed discussion, see below).

In spite of some discordance, the molecular markers used here were shown to be informative for resolving the main lines of phylogeny of *Euonymus*. When more than one DNA loci was sampled, usually the combined data provide the best power of resolution even if "combinability" tests indicate incongruence (Siddall, 1997). Given the analysis using all available data maximized the explanatory power for the phylogeny of *Euonymus*, we refer mainly to the all-combined tree for the following discussion.

3.2 Monophyly of *Euonymus*

Both the Bayesian and the MP analyses revealed that only with the inclusion of *Glyptopetalum*, *Euonymus* became monophyletic (Figs. 1, 2, S1, S2). The two genera are closely related (Thwaites, 1856; Bentham & Hooker, 1862; Baillon, 1880; Hou, 1963; Savinov, 2007; Meng et al., 2011). Simmons et al. (2012) also found the paraphyletic relationship of these two genera; however, due to the limited sampling of both genera and considering a few specific traits of *Glyptopetalum*, he suggested keeping them apart provisionally.

In morphology, the two genera share synapomorphies including: (i) seeds covered by brightly colored aril; (ii) mostly four-merous flowers; and (iii) opposite leaves. They differ by mainly three diagnostic characters of *Glyptopetalum*, single ovule, raphe with branches, and persistent axile placentation. However, none of them are exclusive to *Glyptopetalum*. For example, *E. venosa* Hemsl. has only one ovule, *E. chloranthoides* Yang has branched raphe, and *E. bockii* Loes. ex Diels has persistent axile placentation. *Glyptopetalum* is a tropical to subtropical taxon belonging to the Indo-Malayan flora. The separation of *Glyptopetalum* from *Euonymus* was first proposed by Thwaites (1856) and was supported later by some researchers including Ding Hou who provided an overview of *Glyptopetalum* (Hou, 1963). However, Kurz (1877) suggested that the difference between the two genera could be attributable to the amplitude of variation of *Euonymus*, and should not be over-evaluated for the generic-level classification. Baillon (1880) treated *Glyptopetalum* as a section of *Euonymus*. A recent numerical taxonomic study of *Glyptopetalum* based on 34 morphological characters resolved a group of *Glyptopetalum* nested in the large polytomic clade of *Euonymus* (Meng, 2010). Micromorphological observation also showed that the two genera were of no difference in terms of leaf epidermis, pollen extine, or seed coat sculpture (Meng, 2010; Wang, 2013). Wu (2003) pointed out that *Glyptopetalum* probably was a group of *Euonymus*, which had evolved through adaptation to the special environment of the new India–West Malaysia rainforest. Considering all the data available from previous and present studies, we concur with those authors who believed the difference between the two genera are within the broad variance of *Euonymus*, and regard that *Glyptopetalum* should be

incorporated into *Euonymus* to make a monophyletic *Euonymus* sensu lato.

3.3 Circumscriptions of subgeneric sections and their relationships

Fruit morphology has been commonly used for infrageneric divisions of *Euonymus*. Ma (2001) and Ma et al. (2008) recognized five sections based on capsule morphology: Sect. *Echinococcus* with echinate or spiny, Sect. *Kalonymus* with winged, Sect. *Ilicifolia* with smooth globose, Sect. *Melanocarya* with deeply lobed, and Sect. *Euonymus* with rugose and angulate capsules. Molecular data presented here identified two major groups, one of which was strongly supported (Fig. 2: Clade I). Within Clade I, two subclades corresponded to Sect. *Echinococcus* and Sect. *Kalonymus*, respectively. Taxa of the other three sections all appeared in a poorly supported group, especially Sect. *Ilicifolia*; characterized by global fruits, Sect. *Ilicifolia* turned out to be intricately connected with other sections in clades Ia, IVa, and IVc (Fig. 2). Therefore, only Sect. *Kalonymus* was indeed monophyletic among the five sections sensu Ma (Ma, 2001; Ma et al., 2008); Sect. *Echinococcus* would otherwise correspond perfectly to the molecular grouping if *E. bockii* was excluded from this clade (*E. bockii* has ball-like fruits and was thus classified into Sect. *Ilicifolia*).

Blakelock (1951) considered the ball-like capsule representative of the primitive state of fruit shape in *Euonymus*. In contrast, Ma (2001) regarded the winged capsule as an ancestral character state. The present molecular data revealed that the global capsule was not lineage-specific. When mapped on the tree, it intermingled with the slight to deep-lobed capsules. We postulate that the slight to deep-lobed capsules may have been derived from the global capsule and then gradually developed into a continuous pattern of variation. Plant species carrying these varying types of fruits, that is, those circumscribed under Sect. *Ilicifolia*, Sect. *Melanocarya*, and Sect. *Euonymus*, may have followed evolutionary trajectories of consecutively rapid lineage splitting, which commonly underlies discordance of gene trees with the species tree (Rosenberg & Tao, 2008; Rosenberg, 2013). Parallel evolution of similar fruit shapes could have also caused the non-monophyly of these sections. The possibility of secondary contacts of split lineages (species) in making conflicting gene trees versus species tree (Doyle, 1992; Hedenas, 2011; Guo et al., 2012) should not be completely ruled out, although no solid evidence of interspecific hybridization was found in *Euonymus*.

In contrast, both the echinate (spined) and winged capsules were lineage-specific. They may have been related to adaptation to special fruit dispersal agents. Plants of *Euonymus* live in wet habitats of woodlands and mixed forests. Their mature capsules show colorful arillate and often attract animals. The spined or winged capsules could be dispersed by animals, wind, and/or water. Mutations leading to such specific types of fruits may have been fixed by selection when special seed agents were available. It is also possible that the fruit types of Sect. *Echinococcus* and Sect. *Kalonymus*, mainly occurring in isolated mountainous environments in the Hengduan Mountains, SW China, evolved by genetic drift in small populations, which fixed the morphological changes as well as substitutions in neutral markers leading to monophyly of the sections.

In summary, at the subgeneric level, we suggest maintaining sections *Echinococcus* and *Kalonymus*, and incorporating the three other sections sensu Ma (Ma, 2001; Ma et al., 2008) into Sect. *Euonymus*.

3.4 Remarks on delimitation and systematic positions of some species

Due to the complex patterns and the broad morphological variation of *Euonymus*, much controversy has remained not only for the circumscription of infrageneric division, but also for the species delimitation and/or their phylogenetic positions. For instance, a long-standing problem concerns the positions of *E. mengtseanus* due to insufficient information provided on its specimen type. This species was first regarded as a variety of *E. theifolia* Wall. (Loesener, 1902), and then by Sprague (1908) as a separate species similar to *E. griffithii* Kurz. Later, Blakelock (1951) treated it under Ser. *Myrianthi* rather than under Ser. *Japonici*, to which *E. griffithii* belonged. Cheng & Huang (1999) and Ma (2001) pointed out that *E. mengtseanus* belonged to Sect. *Echinococcus* because its type specimen (Henry 10684) looked very similar to the species of Sect. *Echinococcus* in terms of flowers, branches, and leaves even though it lacks fruits. However, the systematic position of this species was clarified by Zheng et al. (2012). In 2011, Zheng collected a plant with fruits at the type locality of *E. mengtseanus* (Wenshan, Yunnan, China) (Fig. S3). All of its characters of vegetative organs and flower fully match the type specimen Henry 10684 of *E. mengtseanus*. Thus, it is the only specimen of *E. mengtseanus* containing fruit information. However, its fruits were rugose with five lobes (Fig. S3: E), which is not the feature of Sect. *Echinococcus*. Therefore, Zheng suggested that *E. mengtseanus* should not be a member of Sect. *Echinococcus* (Zheng et al., 2012). This treatment was supported by the present molecular

data: *E. mengtseanus* had the closest relationship with *E. laxiflora* Champ. ex Benth. of Sect. *Euonymus*.

Within Sect. *Kalonymus*, *E. porphyreus* was incorporated into *E. frigida* Wall. by Ma (2001) according to several morphological traits. But with the molecular sequence data, they were clearly separated in two branches (Fig. 2). We, therefore, suggest keeping them as independent species.

The phylogenetic position of *E. bockii* defined under the polyphyletic Sect. *Ilicifolia* is particularly deviated from the morphological classification based on fruit shape (Fig. 2). This is probably due to secondary contacts of *E. bockii* with species of Sect. *Echinococcus*. The geographic distribution of *E. bockii* overlaps that of Sect. *Echinococcus*, both in SW China and adjacent areas like Yunnan, Sichuan, Guizhou, and Guangxi. Although *E. bockii* has ball-like capsules similar to other members of Sect. *Ilicifolia*, it does show a slight difference; unlike the smooth fruits of the other species, capsules of *E. bockii* are densely white-spotted, sometimes with white scales, whereas those of the others are smooth. However, no solid evidence of hybridization has been found, and this needs further investigation.

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Supporting Information

The following supplementary material is available for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.12068/suppinfo:

Fig. S1. Bayesian consensus tree of the *Euonymus* species based on the nuclear internal transcribed spacer

dataset. Bayesian posterior probabilities (>0.9) are shown above and bootstrap values (>70%) of the maximum parsimony analysis below the branches. Names of terminal nodes are written in six different colors corresponding to the five sections of *Euonymus* defined in *Flora of China* (Ma et al., 2008) and to the genus *Glyptopetalum*. *Sequences from previous studies (Simmons, 2012; NCBI accession numbers: HQ393697, HQ393702, HQ393705, HQ393709, HQ393710, HQ393715, HQ393718, HQ393720, HQ393722).

Fig. S2. Bayesian consensus tree of the *Euonymus* species based on the nuclear external transcribed spacer dataset. Bayesian posterior probabilities (>0.9) and bootstrap values (>70%) of the maximum parsimony analysis are shown above and below the branches, respectively. Names of terminal nodes are written in six different colors corresponding to the five sections of *Euonymus* defined in *Flora of China* (Ma et al., 2008) and to the genus *Glyptopetalum*, respectively.

Fig. S3. *Euonymus mengtseanus*. **A,** Habitat. **B,** Type specimen (Henry, 10684). **C**, branches. **D**, Flower. **E**, Growing lobed fruit, characteristic of *Euonymus* sect. *Euonymus*.