Botanical Journal of the Linnean Society, 2017, **184**, 204–223. With 6 figures.

An integrative study of evolutionary diversification of *Eutrema* (Eutremeae, Brassicaceae)

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Received 18 January 2016; revised 5 October 2016; accepted for publication 2 April 2017

In this comprehensive study of *Eutrema* (Brassicaceae), five DNA markers [nuclear internal transcribed spacer (ITS) and plastid *matK*, *rbcL*, *trnH-psbA* and *trnL-F*] were sequenced for 183 individuals of 32 species of *Eutrema s.l.* and the closely related members of tribe Eutremeae, *Chalcanthus* and *Pegaeophyton*. The genetic-gap analyses showed that five previously described taxa are polyphyletic, and we identified 37 potential species units or independently evolving lineages. Further phylogenetic analyses were based on sequence variations of these five-marker barcodes for the typical representatives of all species units and they showed that the monospecific *Chalcanthus*, *Pegaeophyton nepalense* and *P. scapiflorum* are nested in and should be transferred to *Eutrema*, whereas *P. watsonii* was sister to *Pycnoplinthopsis* of tribe Euclidieae. Three clades were recovered in the expanded *Eutrema* based on the plastid DNA phylogenetic trees. However, phylogenetic relationships of some species in two later diverging clades are incongruent between ITS and plastid DNA trees and within them. These incongruences suggest possible hybridizations, incomplete lineage sorting or parallel evolution during recent species diversification in the genus. Furthermore, molecular dating and biogeographical analyses suggested that the recircumscribed *Eutrema s.l.* probably originated in eastern Asia, probably in central China. The origin and early divergence between three major clades of *Eutrema*, which are distributed mainly in central China, central Asia and the Qinghai–Tibetan Plateau (QTP), occurred in the Late Oligocene and Early Miocene, probably reflecting the extensive plateau uplifts and Asian aridification during that period. However, further diversification events from the Late Miocene to the Pleistocene, especially in response to the later QTP uplifts and climatic oscillations, might have promoted speciation of more current species through allopatric divergence and hybridization in that region. Several important taxonomic traits seem to have arisen multiple times with obvious parallel evolution. The new name ***E. baimashanicum*** and the new combinations ***E. nepalense***, ***E. purii***, ***E. renifolium***, ***E. robustum***, ***E. scapiflorum***, ***E. xingshanensis*** and ***Aphragmus minutus*** are proposed. This case study highlights the importance of using DNA barcode sequences from multiple individuals or populations to solve evolutionary questions in a given genus.

ADDITIONAL KEYWORDS: biogeography – Cruciferae – DNA barcodes – diversification – generic circumscription – phylogeny – species delimitation.

INTRODUCTION

Several authors (e.g. Mayr, 1982; de Queiroz, 1998; de Queiroz & Donoghue, 1988; Sites & Marshall, 2003; Wiens, 2007; Stockman & Bond, 2007; Bickford, 2007; McKay *et al.*, 2013) have indicated that species delimitation, generic circumscription, phylogenetic relationships and biogeographical histories are clearly interrelated in understanding evolutionary diversification. Therefore, DNA barcode sequence variation

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is used to address comprehensively various unsolved questions in a given genus. The sequence variations from commonly used DNA barcodes seem to a good choice for such an aim because they are easier and less costly to amplify and to sequence across different families with distant relationships (Wang, Yu & Lui, 2011). Two plastid DNA fragments, *rbcL* and *trnH-psbA*, were proposed as the core barcode for plants by Kress & Erickson (2007), and *matK* and *trnL-F* fragments were suggested later as barcodes to discriminate between closely related species (Hollingsworth, Graham & Little, 2011). However, the nuclear ribosomal internal transcribed spacer (nrITS) was strongly recommended as an additional core barcode for plants, on the basis of results from a large comparative dataset (Li *et al.*, 2011), although there are problems with ITS in some taxa. Except for identifying known and well-recognized species, few studies based on these barcoding sequence variations have been designed to conduct an integrative study of evolutionary diversification in plants, although they have occasionally been used to establish genetic gaps to delimit discrete and objective species units (e.g. Hu *et al.*, 2015; Su *et al.*, 2015). In these case studies, species are assumed as evolutionarily distinct and monophyletic lineages with distinct genetic gaps, which could reconcile different sources for species delimitations, including morphological distinction, reproductive isolation, terminations of gene flow and geographical isolation. In addition, sequence variation in the DNA fragments used as barcodes has also been widely used for reconstructing interspecific relationships, generic circumscription and biogeographical history of plant groups (e.g. Liu *et al.*, 2002, 2006; Mao *et al.*, 2010; Sun, McLewin & Fay, 2012; Ren, Conti & Salamin, 2015). Here we aim to address several unresolved evolutionary issues in *Eutrema* R.Br. (Brassicaceae) based on DNA barcode sequence variation.

Eutrema is an important genus and includes a model plant for salt-tolerance studies, *E. salsugineum* (Pall.) Al-Shehbaz & Warwick, and the economically important wasabi, *E. japonicum* (Miq.) Koidz. Based on phylogenetic analyses of nrITS sequence variations of this and related genera (Warwick, Al-Shehbaz & Sauder, 2006), *Eutrema s.l.* was expanded to comprise 26 species (Al-Shehbaz & Warwick, 2005), with 16 transferred from four previously independent genera, *Taphrospermum* C.A.Mey., *Thellungiella* O.E.Schulz, *Neomartinella* Pilger and *Platycraspedum* O.E.Schulz (Al-Shehbaz & Warwick 2005; Warwick *et al.*, 2006). Recent molecular phylogenetic studies (Beilstein Al-Shehbaz & Kellogg, 2006; Beilstein *et al.*, 2008; Warwick *et al.*, 2010) have shown that *Eutrema s.l.* is closely related to the south-western Asian *Chalcanthus* Boiss. (one species) and Sino-Himalayan *Pegaeophyton* Hayek & Hand.-Mazz. (seven species). The three genera alone are currently placed in tribe Eutremeae (Al-Shehbaz, 2012). However, generic boundaries between *Eutrema s.l.* and the other genera remain unclear due to the addition of new species based on recently reported molecular and morphological evidence (Ning *et al.*, 2006; Al-Shehbaz, 2007; Gan & Li, 2014; Xiao *et al.*, 2015; Hao *et al.*, 2015). All species of *Eutrema s.l.* show great variation in diagnostic characters, including habit, leaf venation, rhizome formation, growth form and raceme bracts. However, whether these diagnostic characters have acquired adaptive advantages and evolved independently remains untested. Therefore, a complete phylogenetic tree would be desirable to trace the evolution of the morphological traits on which species circumscription can be based. In addition, such a phylogenetic tree would be useful for tracing the biogeographical origin and dispersal of a particular genus in Brassicaceae (e.g. Mummenhoff, Brüggemann & Bowman, 2001; Carlsen *et al.*, 2009). *Eutrema s.l.* occurs from central to eastern Asia and the Qinghai–Tibet Plateau (QTP), with a further extension into northern Asia and north-western America. Most species are mainly distributed in eastern Asia and the QTP. The other temperate genera occurring in these regions were revealed to show contrasting biogeographical patterns. For example, some genera originated in the QTP and then migrated to other regions (e.g. Zhang *et al.*, 2009; Xu *et al.*, 2010; Jia *et al.*, 2012), whereas others probably originated in central Asia, but diversified extensively in the QTP and eastern Asia (e.g. Sun *et al.*, 2001; Liu *et al.*, 2002; Mao *et al.*, 2010; Tu *et al.*, 2010).

In this study, five DNA barcodes (plastid *matK*, *rbcL*, *trnH-psbA* and *trnL-F* and nuclear ITS) were sequenced for 183 individuals of 32 species of *Eutrema s.l.*, *Chalcanthus* and *Pegaeophyton* to address the following questions. (1) How many species units should be recognized based on genetic gaps and distinct lineages among all sampled individuals? (2) Are *Eutrema s.l.*, *Chalcanthus* and *Pegaeophyton* monophyletic or should they be combined into one broadly circumscribed genus? (3) What are the phylogenetic relationships of species in the recircumscribed *Eutrema s.l.* and how did the main diagnostic traits evolve? (4) Where and when did the genus originate and diversify?

MATERIAL AND METHODS

Samples

In the present study, 183 individuals from 73 populations of *Eutrema s.l.* and the closely related *Pegaeophyton* and *Chalcanthus* were sampled. Of these, 164 individuals of 54 populations (26 species) were collected in the field and 19 accessions representing 11 species

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were taken from herbarium specimens (MO, GOET). The samples cover 24 of the 26 recognized species in *Eutrema s.l.* plus four recently described species (Ning *et al.*, 2006; Al-Shehbaz, 2007; Xiao *et al.*, 2015; Hao *et al.*, 2015). One species, *E. platypetalum* (Schrenk) Al-Shehbaz & Warwick, endemic to Kazakhstan and known from a few collections, was not included in this study as we failed to find it in its type locality. Based on molecular phylogenetic studies (German *et al.*, 2009), *Eutrema parvula* (Schrenk) Al-Shehbaz & Warwick has recently been placed in the monospecific *Schrenkiella* D.A.German & Al-Shehbaz (German and Al-Shehbaz, 2010) and was therefore not included in this study. Four of the seven *Pegaeophyton* spp. (Al-Shehbaz, 2000) were unavailable due to their limited representation in herbaria. The sampled species, voucher information and GenBank accession numbers for the six datasets are listed in the Appendix (Supporting Information, Table S1).

DNA EXTRACTION AND SEQUENCING

We followed the protocols described by Hao *et al.* (2015) and Hu *et al.* (2015) for total DNA extraction and sequencing of *matK*, *rbcL*, *trnH-psbA*, *trnL-F* and ITS. Some directly sequenced ITS showed double peaks with numerous additive sites. These samples were further cloned using vector pGEM-T (Promega, Madison, WI, USA). Ten positive clones were chosen for sequencing using primers 'sp6' and 't7'. However, for most individuals with only one or two additive sites, the directly sequenced ITS sequences with degenerate sites were used for all analyses. For ITS, 183 individuals were sequenced. For plastid regions, 182, 181, 181 and 180 individuals were sequenced for *trnH-psbA*, *matK*, *rbcL* and *trnL-F*, respectively. All newly obtained sequences were submitted to GenBank (Supporting Information, Table S1).

Sequences were aligned using CLUSTALX version 1.83 (Thompson *et al.*, 1997) followed by manual adjustments in MEGA 5.10 (Tamura *et al.*, 2011). DNASP 5.0 (Librado & Rozas, 2009) was also used to identify insertions/deletions (indels) and single nucleotide polymorphisms between different individuals. Sequences from all four plastid DNA regions were concatenated into a single matrix for all analyses because of their common inheritance without obvious recombination.

DATA ANALYSES

Species delimitation

All sequences of the examined individuals were used for species delimitation. Three sequence datasets (plastid DNA, ITS and plastid DNA+ITS) were separately analysed. Because indels may contain potential phylogenetic information (Simmons *et al.*, 2001), most were also coded using the simple code method applied by GapCoder (Young & Healy, 2003) and added for species delimitation and following phylogenetic analyses. However, when sequence alignments were difficult, the inserted indels were treated as missing data. Because species delimitations were mainly determined based on genetic gaps, neighbor-joining (NJ) analyses were used for that purpose, instead of maximum parsimony (MP) or maximum likelihood (ML), as demonstrated to be effective for species barcoding and delimitations (Li *et al.*, 2011; Su *et al.*, 2015).

Phylogenetic analyses

Only one individual was used to represent each monophyletic cluster, as it probably represents a separate lineage or species unit for phylogenetic analyses. To test whether *Eutrema*, *Chacanthus* and *Pegaeohyton* comprise a monophyletic group in Brassicaceae, eight other genera were selected (Supporting Information, Table S1), including *Cleome spinosa* Jacq. (Cleomaceae) as the outgroup because Cleomaceae and Brassicaceae are closely related sister families (Hall, Sytsma & Iltis, 2002). Plastid DNA and ITS sequence datasets were analysed separately and combined for the final phylogenetic analyses using MP, ML and Bayesian inference (BI). The MP analyses were carried out using heuristic searches of 1000 replicates with random stepwise taxon addition, tree bisection–reconnection (TBR) branch swapping, MulTrees on and the Collapse option selected in PAUP* v.4.0b10 (Swofford, 2003). All characters were treated equally and support of each clade was evaluated based on bootstrap values (BS) with 100 replicates using a heuristic search with simple taxon addition, TBR and MULPARS options implemented (Felsenstein, 1985). The ML analyses were performed through RAxML 7.2.6 (Stamatakis, 2006) with the order: raxmlHPC -f a -s sequence. phy -n boot2 -m GTRGAMMA -x 1234 -# 1000 -n outname. The GTRGAMMA model was chosen and ML bootstrap analyses were estimated with 1000 replicates. MrBayes v.3.12 (Ronquist & Huelsenbeck, 2003) was used for the BI analysis (Rannala & Yang, 1996) and the Akaike information criterion (AIC; Akaike, 1974) was applied to select the best model by MrModeltest v.2.0 (Nylander, 2004). The Markov chain Monte Carlo (MCMC) algorithm was run for 20 million generations with one cold and three heated chains, starting from random trees. The resulting log likelihood and number of generations were plotted to determine the point after which the log likelihood had stabilized through the 'sum parameters' command. The posterior probability (PP) of each clade node was estimated from the 50% consensus trees of the last 18 001 trees, with the first 2000 trees discarded as burn-in.

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Lineage-divergence estimation and diversification through time

The simplified plastid DNA ML tree was used to estimate lineage divergence. A likelihood ratio test provided no support for a molecular clock hypothesis ($P < 0.05$). Thus, the divergence times were estimated by using a Bayesian approach implemented in BEAST v.1.6.1 (Drummond & Rambaut, 2007) under a log-normal relaxed molecular clock (Drummond *et al.*, 2006) with birth–death prior, estimated base frequencies, gamma shape distribution (with four categories) and a proportion of invariant sites. MrModeltest v.2.0 was used to select the best model. The posterior distributions of all estimates were approximated by using two independent MCMC analyses of 20 million generations with a 10% burn-in. Samples from the two runs, which yielded similar results, were combined and convergence of the chains was checked using the program Tracer v.1.4 (Drummond & Rambaut, 2007). The samples from the posterior distribution were summarized on the maximum clade credibility (MCC) tree, which has the maximum sum of posterior probabilities on its internal nodes, using TreeAnnotator v.1.5.4 (Drummond & Rambaut, 2007) with the PP limit set at 0.5 and mean node heights summarized. The MCC tree was visualized using FigTree v.1.3.1, from which we obtained the means and 95% higher posterior.

Three calibration points were set based on previous studies to estimate lineage divergences. First, the crown age of Brassicaceae (the split between *Aethionema* W.T.Aiton and core Brassicaceae) was set to 37.6 Mya (Koch, Haubold & Mitchell-Olds, 2000, 2001; Couvreur *et al.*, 2010). Second, the age of the core Brassicaceae (the split between three main lineages of Brassicaceae) was set to 32.3 Mya (Ermolaeva *et al.*, 2003; Henry, Bedhomme & Blanc, 2006; Schranz & Mitchell-Olds, 2006). Finally, the split between tribes Brassiceae and Sisymbrieae in Lineage II was assumed to be 17.3 Mya (Lysák, 2005; Couvreur *et al.*, 2010). A lineage diversification through time (LTT) plot by R 2.9.0 (R Development Core Team, 2009) was estimated with 'laser' (Rabosky, 2006), 'geiger' (Harmon *et al.*, 2008) and 'ape' (Paradis, Claude & Strimmer, 2004) packages loaded. The beast chronogram was used to produce an LTT plot for major clades and the total genus. Relative cladogenesis was estimated to detect possible rapid shifts in species diversification rates by GEIGER 1.3-1.

Ancestral state reconstruction and parallel evolution of key taxonomic traits

Ancestral state reconstruction was conducted to examine possible parallel evolution of six diagnostic morphological characters, including perennial/annual–biennial duration, rosette/non-rosette growth form, pinnate/palmate leaf venation, silicle/silique fruit type, absence/presence of inflorescence bracts and presence/absence of distinct rhizomes (Appendix, Supporting Information, Table S2). Bayesian Binary MCMC (BBM) in RASP, which is not limited to historical biogeographical applications, was used to perform the reconstruction of ancestral morphological states on the reduced plastid DNA tree obtained from the BEAST analyses. All default settings were adopted, the analyses were run for one million MCMC generations and the F81 + G model was used for changes between states. For each character pair, two models, based on the 'homology' and 'independent origin' hypotheses, were compared to test parallel evolution of these characters with the likelihood sensitivity analyses (Oakley & Cunningham, 2002). One thousand bootstrap trees were constructed using RAxML 7.2.6 (see the settings above) to estimate the likelihood values under the character states by BayesMultiState in the BayesTraits package (Pagel, Meade & Barker, 2004). The *t*-test was used to examine the significance of the likelihood differences between two contrasted hypotheses.

Biogeographical reconstructions

BBM was used to investigate the biogeographical history of *Eutrema* in RASP v.3.2 (Yu *et al.*, 2015). The plastid DNA phylogenetic tree was used for the biogeographical analyses and four areas were defined based on the distribution of the sampled species: (A) the QTP and adjacent high-elevation (> 1500 m) regions; (B) eastern Asia; (C) central Asia and adjacent parts of Siberia; and (D) northern Asia and north-western America. BBM analyses in RASP were carried out as described above.

RESULTS

Sequence characteristics

Sequences of four plastid DNAs were obtained from 180 individuals excluding those from herbarium specimens of *E. pseudocordifolium* Turcz. ex Ledeb. and *P. watsonii* Al-Shehbaz. The aligned sequence matrix of the combined plastid sequences was 2984 characters long and contained 206 polymorphic sites, of which 159 were potentially parsimony informative. ITS sequences were obtained for all of 183 individuals representing 32 putative species. The ITS data set comprised 645 characters, of which 141 were polymorphic and 116 were potentially parsimony informative. The matrix of the combined plastid and ITS datasets, which was constructed for only 180 individuals, was 3629 characters long and comprised 329 polymorphic sites, of which 280 were potentially parsimony informative.

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Species delimitation

The NJ analyses of the plastid DNA sequence data comprised 180 individuals (representing 30 species) and identified 32 clusters (Supporting Information, Fig. S2) of which five species [*E. heterophyllum* (W.W.Sm.) H.Hara, *E. deltoideum* (Hook.f. & Thomson) O.E.Schulz, *E. yunnanense* Franch., *E. tenue* (Miq.) Makino and *P. scapiflorum* (Hook.f. & Thomson) C.Marq. & Airy-Shaw] contained non-sister clusters. However, *E. halophilum* (C.A.Mey.) Al-Shehbaz & Warwick shared the same sequence with one population of *E. salsugineum* (Pall.) Al-Shehbaz & Warwick, and *E. bulbiferum* Y.Xiao & D.K.Tian and *E. japonicum* (Miq.) Koidz. were nested in different groups of *E. tenue*.

The ITS sequence dataset of 183 individuals (32 species) recovered 36 clusters (Supporting Information, Fig. S3), of which five species contained non-sister clusters as in the plastid analyses. However, *E. bulbiferum* formed a single cluster distinct from that of *E. tenue* and *E. japonicum* and from the other group of *E. tenue*. In addition, *E. salsugineum* and *E. halophilum* shared identical ITS sequences and were nested in the same cluster. The final analysis of the combined plastid DNA and ITS sequence data for 180 individuals (31 putative species) recovered 34 clusters (Fig. 1). Five species were paraphyletic, whereas *E. bulbiferum* and *E. tenue*, *E. tenue*, and *E. japonicum* were distinguished from each other and *E. halophilum* shared the same sequences with one population of *E. salsugineum*.

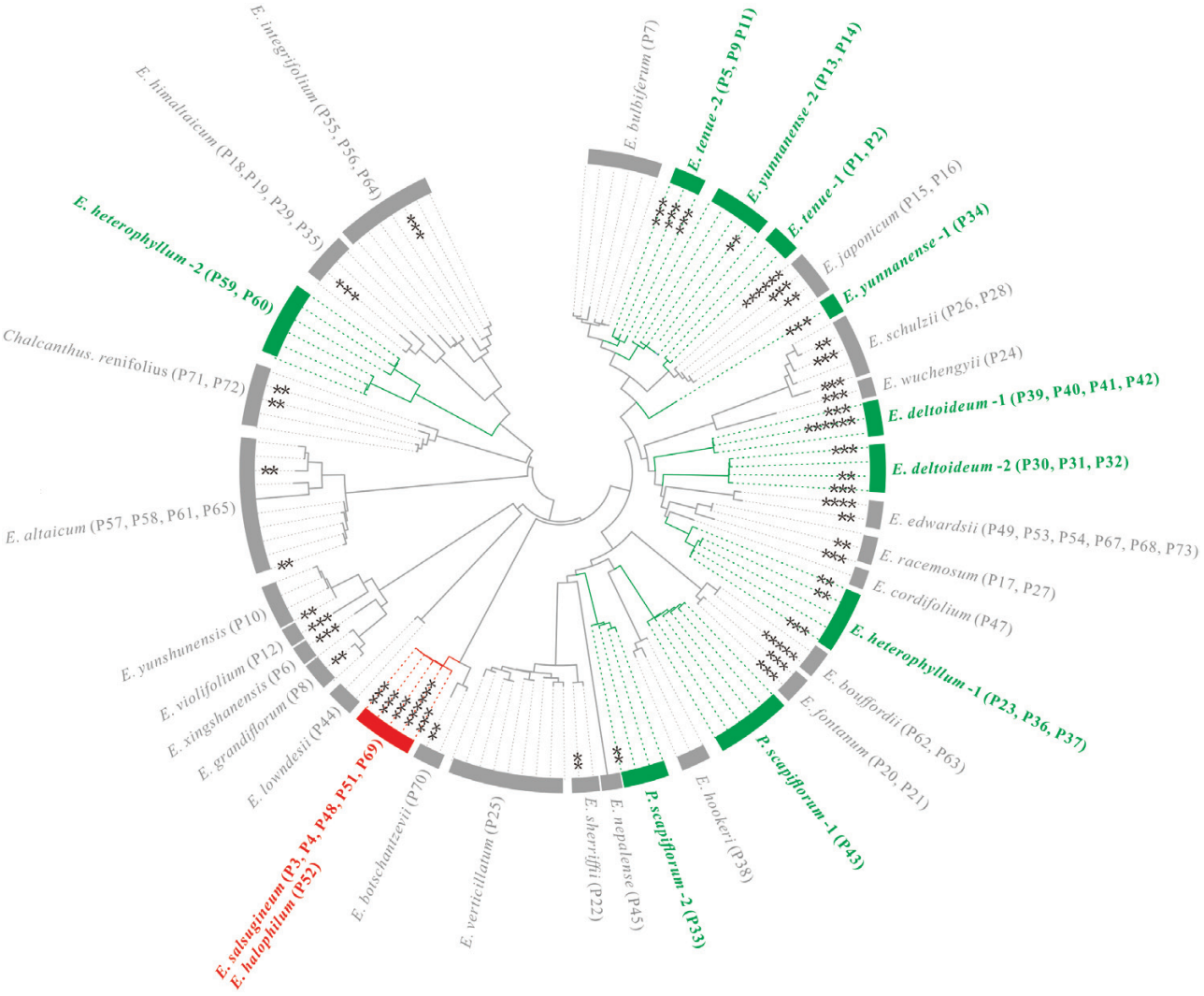

Figure 1. Neighbor-joining tree of circle-based analyses of all unique sequences through a combination of all plastid DNA and nrITS fragments for all sampled individuals of *Eutrema s.l.* Green blocks represent paraphyletic taxa and red blocks represent two species that share identical sequence. When two to five samples shared the sequence, '*' on the branches represents the numbers of samples

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When all these analyses were combined, 36 clusters were recovered and they may represent tentative species units as independently evolving lineages based on the present genetic gaps, sister relationships of the recovered clusters and morphological distinctions used by taxonomists. However, although *E. halophilum* shared plastid and ITS sequences with some populations of *E. salsugineum*, these two species are considered to be distinct from each other in both morphology and breeding systems (Koch & German 2013). Therefore, they were treated as two distinct species and 37 tentative species units were used for the following analyses.

Phylogenetic analyses

One individual was selected from each of the 37 clusters for phylogenetic analyses of ITS data. Previous studies (e.g. Beilstein *et al.*, 2006, 2008; Warwick *et al.*, 2010) were followed to determine the systematic position of *Pegaeophyton*, *Chalcanthus* and *Eutrema*. Initial phylogenetic analyses suggested that *P. watsonii* fell outside the expanded *Eutrema s.l.*, and *Eutrema s.l.* included three putative *Pegaeophyton* species units and the monospecific *Chalcanthus* (Supporting Information, Fig. S4). To maintain maximum informative sites during the sequence alignments, *Brassica rapa* L. and *Pycnoplinthopsis bhutanica* Jafri were used as outgroups for further analyses of the phylogenetic relationships in *Eutrema s.l.* All MP, ML and Bayesian analyses produced similar tree topologies although with different support values for each node (Fig. 2A). Only two well-supported clades were obtained. The first (clade A) comprised four species occurring in central China and clade B included the remaining 32 putative species. In clade B, *C. renifolius* (Boiss. & Hohen.) Boiss. was sister to *E. pseudocordifolium*. One species unit classified as *P. scapiflorum* and *P. nepalense* Al-Shehbaz comprised a monophyletic group. In addition, five monophyletic groups (all with support values > 70%) comprised two or three *Eutrema* spp. However, the inter-relationships between these groups and between another *P. scapiflorum* species unit and other *Eutrema* spp. remained unsolved.

Because plastid DNA sequences were not obtained for *E. pseudocordifolium* or *P. watsonii*, only 35 putative species units of the expanded *Eutrema s.l.* were used for the phylogenetic analyses of plastid data and the combined plastid DNA+ITS. Due to fewer indels, it was easier to align all plastid DNA sequences with those of other Brassicaceae. Therefore, ten species of Brassicaceae (representing different major lineages or clades) were included as outgroups in the phylogenetic analyses of the plastid DNA sequence data. Similarly, all phylogenetic analyses produced similar tree topologies (Fig. 2B) and the phylogenetic relationships between the major lineages of Brassicaceae were basically consistent with those inferred before (Warwick *et al.*, 2006). However, in *Eutrema s.l.*, three clades with moderate to relatively high support were recovered. Similar to the ITS tree, clade A comprised four species from central China, but the remaining species clustered into two clades (B1 and B2). Clade B1 comprised species predominantly occurring in central Asia and the western Himalayas, whereas B2 included the remaining ones that mainly occur in the QTP and adjacent high-elevation regions. In each clade, the relationships among species and subclades were relatively resolved, although the support values changed greatly.

Compared to the recovered monophyletic groups with relatively high ITS support, the sister relationships were still maintained between *Eutrema schulzii* Al-Shehbaz & Warwick and *E. wuchengyii* (Al-Shehbaz, T.Y.Cheo, L.L.Lu & G.Yang) Al-Shehbaz & Warwick, between *E. fontanum* (Maxim.) Al-Shehbaz & Warwick and *E. bouffordii* Al-Shehbaz, between *E. verticillatum* (Jeffrey & W.W.Sm.) Al-Shehbaz & Warwick and *E. sherriffii* Al-Shehbaz & Warwick, and between *E. botschantzevii* (D.A.German) Al-Shehbaz & Warwick and *E. salsugineum*/*E. halophilum*. Three groups, *E. himalaicum* Hook.f. & Thomson, one *E. heterophyllum* species unit and *E. integrifolium* (DC.) Bunge, were similarly recovered to comprise a clade with high support values, although the relationships among them differed from those in the ITS tree. However, two *P. scapiflorum* units clustered as a monophyletic group in the plastid DNA phylogenetic tree. Similarly, six species units morphologically identified as *E. yunnanense*, *E. japonicum*, *E. tenue* and *E. bulbiferum*, clustered as a monophyletic group with moderate support values. However, the inter-relationships between these differed from those recovered from the ITS tree. Such inconsistent phylogenetic relationships between plastid DNA and ITS trees were also found for a group comprising *E. deltoideum*, *E. racemosum* Al-Shehbaz, G.Q.Hao & J.Quan Liu, *E. cordifolium* Turcz. ex Ledeb., *E. edwardsii* R.Br. and *E. heterophyllum*. Phylogenetic relationships between these groups in clades B1 and B2 were largely discerned despite the medium support values (Fig. 2B).

Significant incongruence was detected between the ITS and plastid DNA datasets by the incongruence length difference test ($P < 0.001$). All phylogenetic analyses of the combined datasets produced similar tree topologies (Supporting Information, Fig. S5). The identified major clades and phylogenetic relationships between species or species groups were largely consistent with those recovered from analyses of the plastid DNA dataset due to there being more informative sites for plastid DNA than ITS, although inconsistencies were found between ITS and plastid DNA phylogenetic trees.

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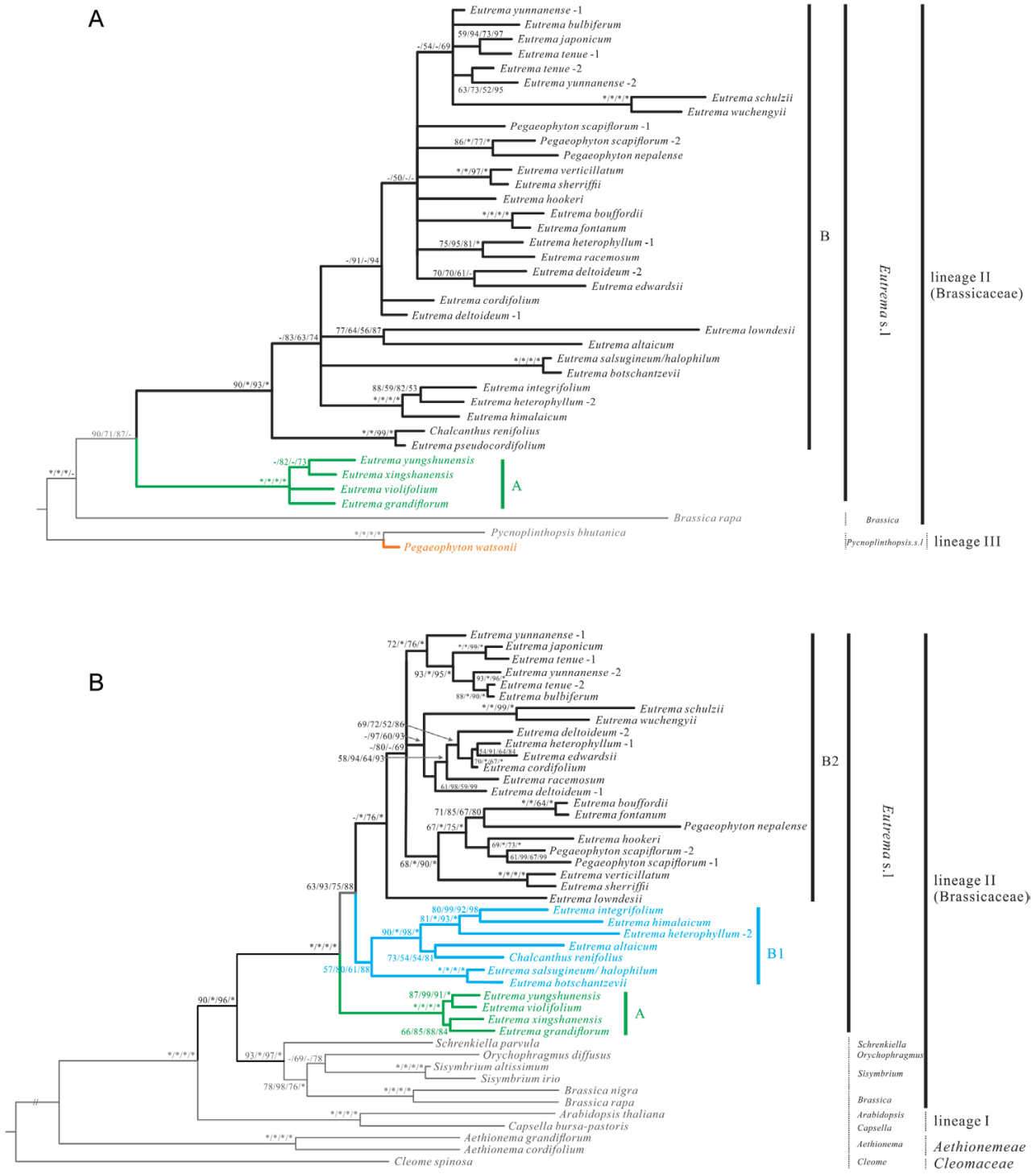

Figure 2. A, Bayesian majority-rule consensus tree inferred from ITS data. B, Bayesian majority-rule consensus tree inferred from the data of combined five plastid DNA markers. Numbers above branches are maximum parsimony bootstrap support values, Bayesian posterior possibilities, maximum likelihood bootstrap support values, and BEAST posterior possibilities. An '*' represents values of 100%, while '-' represents <50%.

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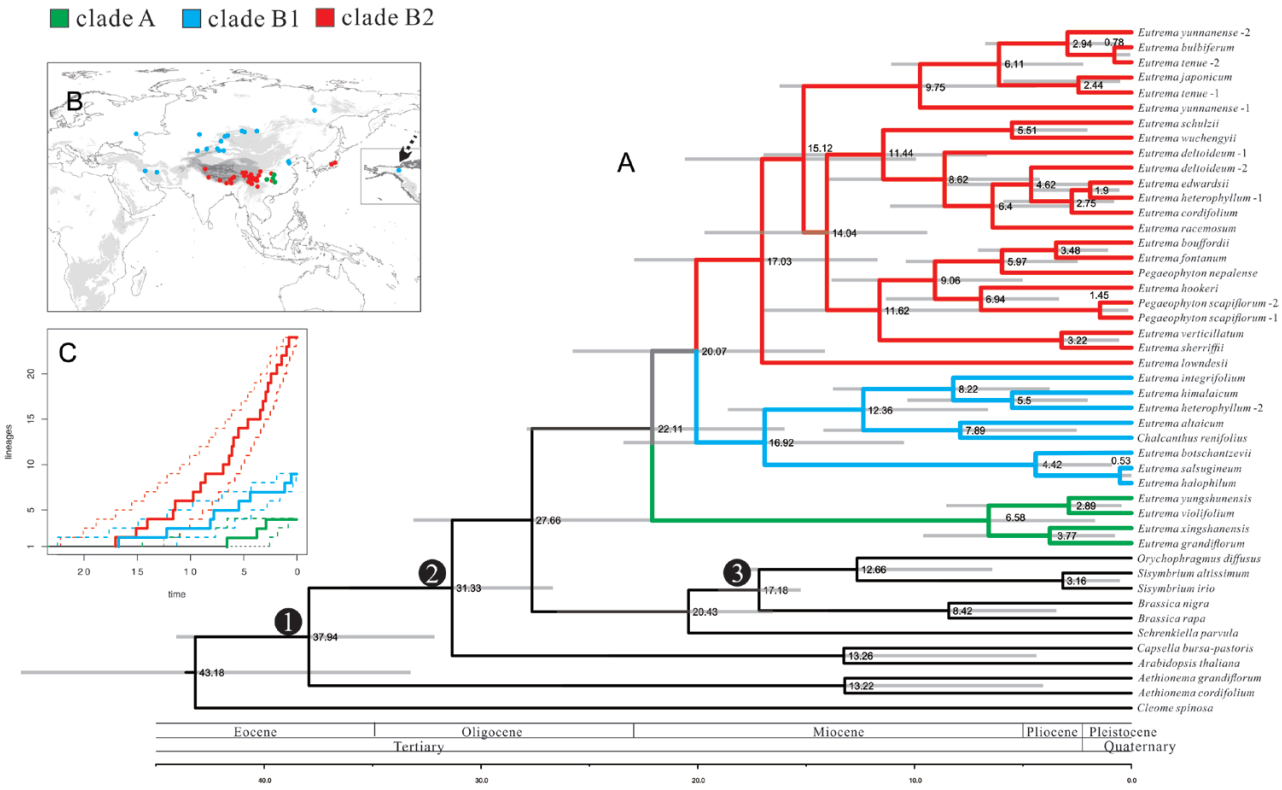

Figure 3. A, divergence-time scale of *Eutrema s.l.* determined by BEAST. Letters in black circles represent time calibration points. Each point represents one population used in this study. B, distribution of the three clades of *Eutrema s.l.* C, lineage-through-time (LTT) plot for three clades of *Eutrema s.l.* The dotted lines represent the upper and lower bounds of all (LTT plots. The solid lines correspond to the maximum credibility tree from the BEAST dating analyses.

Molecular dating and diversification analyses

The stem age of the expanded *Eutrema s.l.* was dated at 27.7 Mya [95% highest posterior density (HPD): 22.1–33.1] based on the plastid DNA tree. Clade A diverged from clades B1 and B2 in the Early Miocene (22.1 Mya, 95% HPD: 16–27.9) (Fig. 3B). The divergence between the remaining clades occurred around 20.1 Mya (95% HPD: 14.1–25.8). No accelerated speciation events were detected for the genus or any of its clades (Fig. 3C). However, most current species evolved between the Mid-Miocene and the Quaternary (8–2 Mya) through a series of diversification events.

Reconstruction of character-state evolution

The plastid DNA phylogenetic tree of *Eutrema s.l.* was used to trace the evolutionary history of six main diagnostic traits (perennial vs. annual–biennial habit, silique vs. silicle, pinnate vs. palmate leaf venation, distinct vs. indistinct rhizomes, rosette vs. non-rosette growth form and bracteate vs. ebracteate racemes). The analyses revealed that the annual or biennial duration, formation of leaf rosettes, lack of a distinct rhizome, palmate venation, absence of bracts and production of siliques appear to be plesiomorphic, whereas the perennial duration, non-rosette growth habit, presence of distinct rhizomes, pinnate venation, presence of bracts and formation of silicles were each derived more than once (Figs 4, 5). All likelihood tests rejected the 'homology' hypothesis for each of six derived traits ($P < 0.05$), whereas the 'independent origin' hypotheses for these traits were supported with high significance ($P < 0.05$).

Ancestral-area reconstruction

The biogeographical reconstruction comprised only species of *Eutrema s.l.* because the sister group of the genus remains unclear. The results from the BBM analysis suggested that the ancestral area of

Figure 4. Ancestral-state reconstruction in *Eutrema s.l.* for (A) habit. In some floras *Eutrema altaicum* is reported as perennial, but following a detailed examination, this may be a mistake. B, growth form; C, rhizome; D, leaf venation; E, inflorescence bracts; F, fruit shape. Scale bars = 5 cm.

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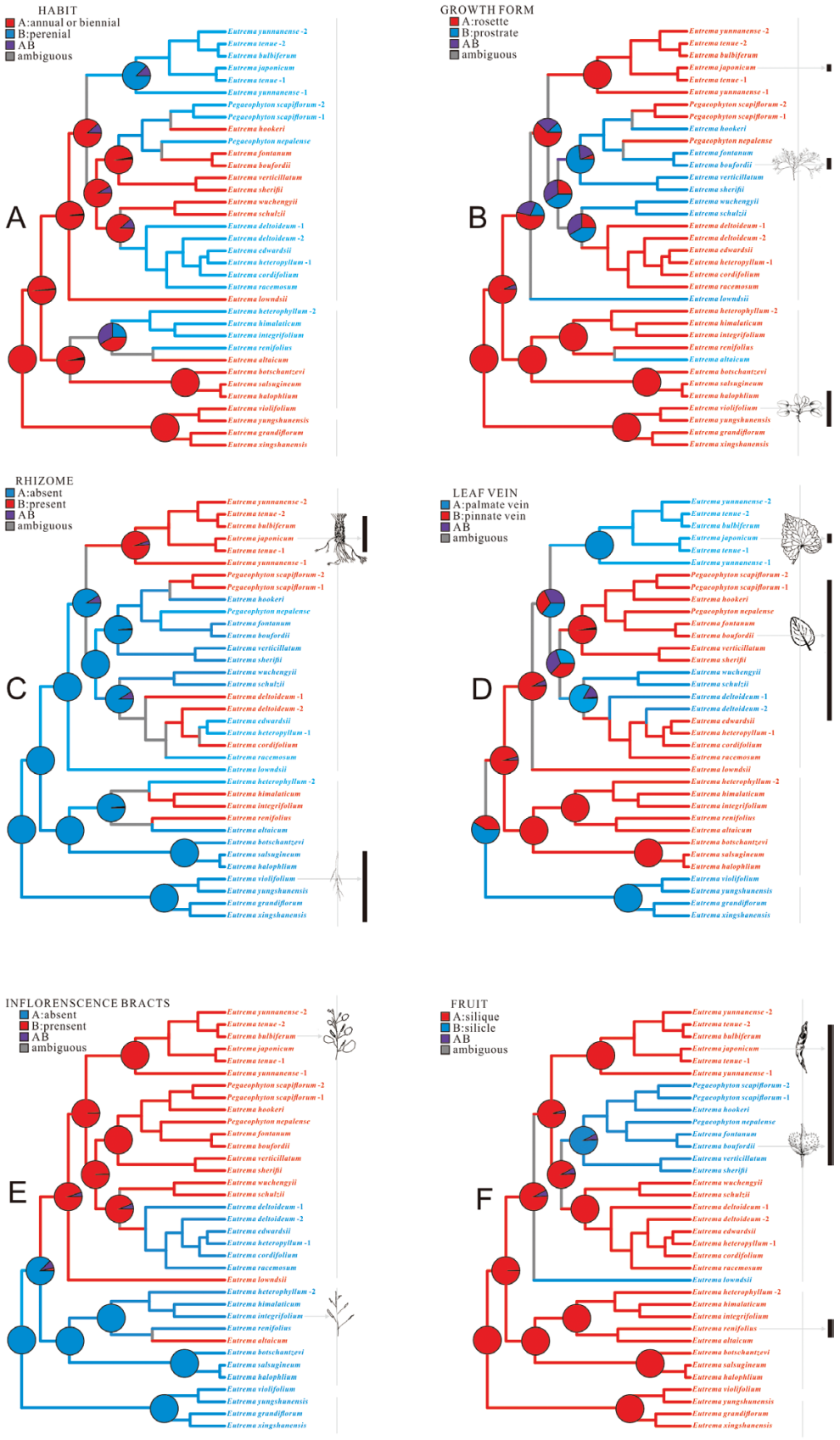

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Figure 5. Habit of *Eutrema s.l.* A, *E. verticillatum*, *Ljq-hao14-094*, non-rosette. B, *E. fontanum*, *Ljq-hao13-144*, non-rosette. C, *E. violifolium*, *Ljq-hao14-007*, short-lived rosette. D, *E. yunnanense*, *Ljq-hao14-036*, rosette, perennation. E, *E. integrifolium*, *Ljq-hao13-051*, rosette, perennation. The grey bars indicate the plant height; scale bars = 10 cm.

recircumscribed *Eutrema* should be eastern Asia (Fig. 6), most likely in central China, because the earliest branching clade is exclusively distributed there. Following that, geographical vicariance seems to have occurred between the other two clades because the common ancestor of clade B1 was in the QTP and adjacent regions and that of clade B2 in central Asia. Geographical exchanges may have occurred between the latter two clades and the reverse dispersals occurred from the QTP to eastern Asia.

DISCUSSION

Based on genetic gaps from sequence variations of four plastid regions and ITS for multiple individuals of each species, the clustering analyses of 32 species of *Eutrema s.l.* and related genera suggested that five species are polyphyletic. Further phylogenetic analyses indicated that the monospecific *Chalcanthus* and *P. scapiflorum*, the generitype, and *P. nepalense* should be transferred to *Eutrema s.l.* Further phylogenetic analyses of all recircumscribed species identified three tentative clades in Eutrema *s.l.* and their species are mainly distributed in central China, central Asia and the QTP and adjacent regions. Biogeographical analyses and molecular dating suggested that *Eutrema* probably originated in eastern Asia and underwent vicariant divergences between three major geographical clades in the Early Miocene. Diversification events since the Mid-Miocene to the Quaternary produced most of the current species and many taxonomic traits evolved independently. We will discuss these findings in detail and provide a taxonomic treatment.

SPECIES DELIMITATION AND INTRASPECIFIC DIVERGENCE

Although the definition of a species remains debated, it is widely accepted that a species should be delimited as an evolutionarily distinct lineage (de Queiroz, 1998, 2007; Stockman & Bond, 2007; Fujita *et al.*, 2012; Hendrixson *et al.*, 2013; McKay *et al.*, 2013). The concatenated plastid DNA, ITS and combined plastid DNA+ITS sequence datasets for multiple individuals of each species were used to identify tentative species units of *Eutrema s.l.*, *Pegaeophyton* and *Chalcanthus*. As expected, multiple individuals of 26 out of 32 species comprised monophyletic clusters based on analyses of the three datasets. Although some individuals of *E. halophilum* and *E. salsugineum* shared the same sequence for plastid DNA and ITS, they are treated as independent species (see above and Koch & German, 2013).

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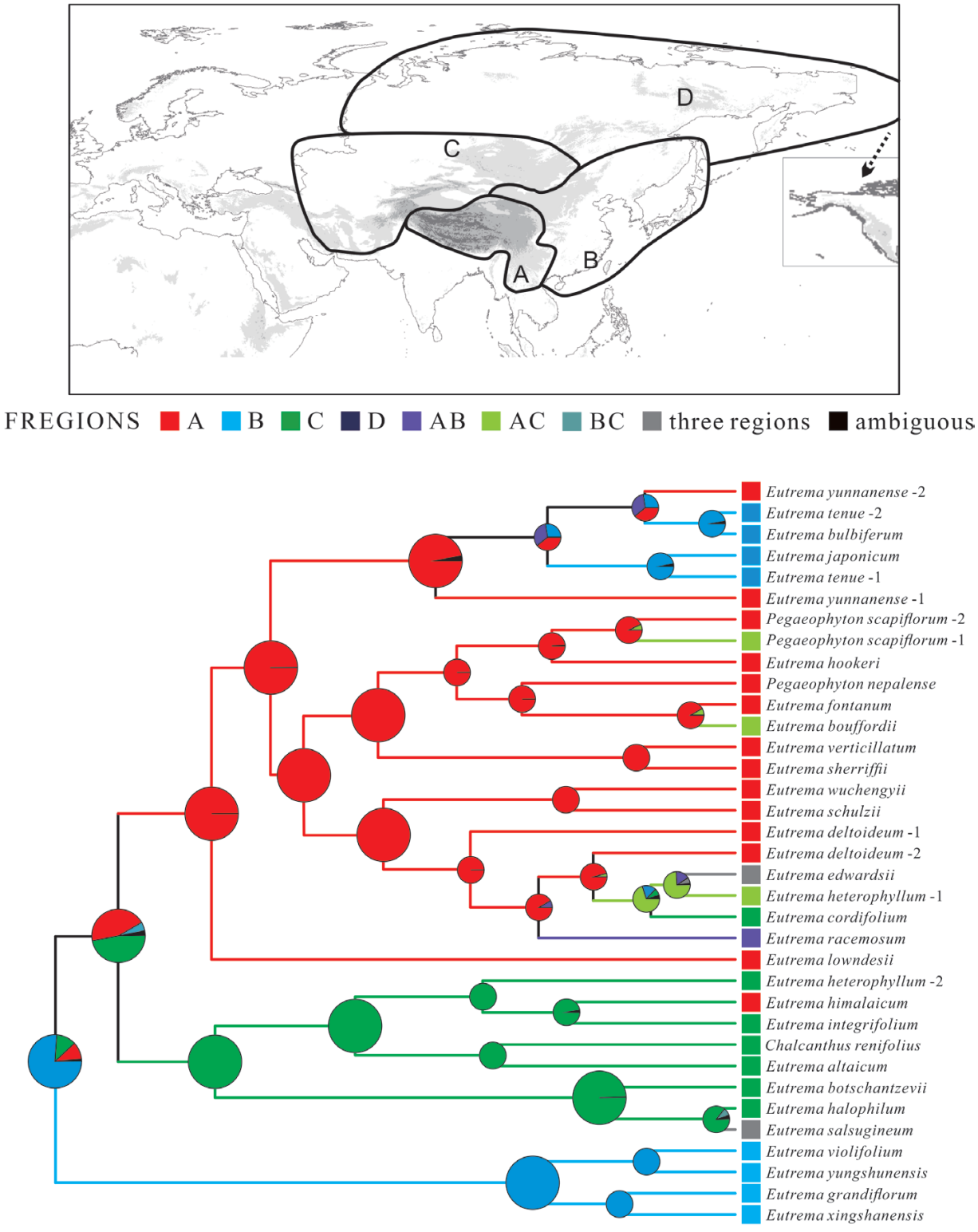

Figure 6. Ancestral area reconstruction (AAR) in *Eutrema s.l.* based on a reduced BEAST combined plastid DNA chronogram. The AARs are shown as coloured circles at each node. Partitioning of the distribution area, based on centres of endemism of *Eutrema*, is shown in the map: A, QTP (Qinghai–Tibetan Plateau, including adjacent regions); B, central China and adjacent parts of Siberia; C, central Asia; D, North Asia and north-western America.

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However, individuals of five species (*P. scapiflorum*, *E. heterophyllum*, *E. yunnanense*, *E. deltoideum* and *E. tenue*) clustered into two non-sister clades (Figs 1, S2, S3). Further MP, ML and Bayesian analyses, using one individual for each cluster and possibly representing separate species units, similarly suggested that they are polyphyletic (Figs 2A, B, S4, S5). Two subspecies were previously recognized for *P. scapiflorum* (Marquand, 1929; Al-Shehbaz, 2000). Phylogenetic analyses based on plastid DNA and plastid DNA+ITS datasets clustered two *P. scapiflorum* units into a monophyletic group, but they remained polyphyletic in the phylogenetic analyses of the ITS dataset (Fig. 2A). These two units are distributed in the western and eastern QTP (Supporting Information, Fig. S1) and they suggest possible divergences due to geographical isolation. Furthermore, the directly sequenced ITS of *P. scapiflorum*-1 from the western QTP had one additive site. Therefore, it is likely that this species unit may have experienced historical hybridization with sympatrically distributed congeners, which eventually led to the divergence of this western unit from the eastern one. In addition, ten more individuals from two populations of the western unit (unpublished) were examined and found to be fixed for the mutations found there, but additive sites disappeared in some individuals. The type specimen of this species was collected from the western QTP. Due to the well-differentiated sequences and geographical isolation, the eastern unit should be separated as a new species (see below). Similarly, two species units of *E. heterophyllum* were found not to be sister groups and have disjunct distributions. Of these, *E. heterophyllum*-2 is distributed in the Tianshan Mountains of central Asia and *E. heterophyllum*-1 occurs in the eastern QTP. More material from the Tianshan Mountains is needed before a meaningful conclusion can be reached.

Two non-sister units of *E. yunnanense* are distributed in Yunnan and western Sichuan of the eastern QTP. Material from Yunnan is a tetraploid with $2n = 28$ (Du & Gu, 2004) and the material from western Sichuan has the same chromosome number. The units were revealed to be sister groups of different species in the plastid DNA and ITS phylogenetic analyses and therefore qualify as distinct species. However, *E. yunnanense* is extremely variable in plant size and robustness, size, shape and indumentum of basal leaves, absence or presence of bracts, length of the bracteate part, flower size, fruit length and orientation, and style length. It is the most widespread and variable Sino-Himalayan species and its range extends from Yunnan Province in the south into Sichuan, Gansu, Hunan, Shanxi and Anhui provinces. Without thorough molecular and morphological studies, it is premature to divide the species even though our results indicate that it could be divided into two species units, each as sister to a different species.

Two varieties of *E. deltoideum* previously recognized by Schulz (1924, 1926) were found to represent independent lineages closely related to different species. These two units (*E. deltoideum*-1 and *E. deltoideum*-2) are distributed in the western and eastern QTP, respectively, and they differ from each other in flower size and fruit. Therefore, *E. deltoideum*-2 from Baimashan (eastern QTP), which was treated by Schulz (1926) as var. *grandiflorum* O.E.Schulz, should be recognized as a distinct species and it is here given the name *E. baimashanicum*.

Eutrema tenue also forms two species units, of which one occurs in Japan and Korea (*E. tenue*-1) and the other in the Qinling–Daba Mountains (*E. tenue*-2). The Japanese plants have $2n = 28$ (Yoshida, 1974), whereas plants of the Qinling–Daba mountains have $2n = 16$. The two units differ in indumentum, fruit morphology and style length (see below). They should be recognized as distinct species due to their geographical isolation, recognizable morphological differences and reproductive isolation due to having different chromosome numbers. The Chinese plants are here recognized as *E. thibeticum* Franch.

GENERIC CIRCUMSCRIPTION AND PARALLEL EVOLUTION OF DIAGNOSTIC TRAITS

Pegaeophyton differs from other genera of Eutremeae in having solitary flowers originating from a basal rosette and silicles with a flattened replum (a thin false septum) (Al-Shehbaz, 2000). Although no plastid DNA sequence was available for *P. watsonii*, our initial phylogenetic analyses showed that it clearly falls outside *Pegaeophytom* and ought to be placed in another genus (Supporting Information, Fig. S4). Furthermore, the present phylogenetic analyses of ITS and plastid DNA sequence variation suggested that *Pegaeophyton* is polyphyletic and *P. nepalense* and *P. scapiflorum* (three species units recognized here) are deeply nested in *Eutrema* and are closely related to *E. fontanum*, *E. bouffordii*, *E. hookeri*, *E. sherriffii* and *E. verticillatum*, all of which are distributed in the QTP and Himalayan region (Figs 2A, B, S1). The group comprising them and other *Eutrema* spp. has fleshy roots, white flowers, pinnately veined leaves, flattened replums and latiseptate silicles. The three *Pegaeophyton* spp. differ from the above five *Eutrema* spp. in having solitary flowers from the rosette instead of a distinct raceme. However, some solitary flowers are also found in *E. violifolium* (Lév.) Al-Shehbaz & Warwick and the transfer of two *P. scapifolorum* units and *P. nepalense* to *Eutrema* would not alter the generic limits of *Eutrema*.

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The monospecific *Chalcanthus* differs from the other genera of Eutremeae in having tuberous rootstock, linear fruits 7–35 cm long and winged seeds, although it resembles them in having palmately veined, reniform to cordate basal leaves and amplexicaul cauline leaves. The present phylogenetic analyses of *C. renifolius* showed that it is deeply nested in the expanded *Eutrema s.l.* The ITS sequence data suggested that it is closely related to *E. pseudocordifolium* with strong support (Fig. 2A) and together they form a clade in an early branching position in the clade including most *Eutrema* spp. Both *C. renifolius* and *E. pseudocordifolium* have reniform leaves and grow in arid habitats. The plastid DNA phylogenetic tree shows *C. renifolius* clustered with *E. altaicum* in clade B1, comprising the central Asian–Himalayan species. In the ITS tree, *E. altaicum* was closely related to the Himalayan *E. lowndesii* (H.Hara) Al-Shehbaz & Warwick. The inconsistent position of these species on the ITS and plastid DNA trees may suggest hybridization and/or reticulate evolution. However, in all analyses, *C. renifolius* was deeply nested in *Eutrema* and should therefore be transferred to it.

The present study showed that rosette- or non-rosette-forming species with palmate or pinnate venation, bracteate or ebracteate racemes and silique or silicle fruits did not cluster into monophyletic clades (Fig. 2), therefore suggesting that these traits may have evolved independently (Wake, Wake & Specht, 2011). The parallel evolution of these traits was also illustrated by tracing them on the phylogenetic trees (Fig. 4). Our further analyses based on the likelihood sensibility tests similarly supported parallel evolution of these traits. As shown for other plants (Sun *et al.*, 2012), the traits may have had multiple origins in their adaptive response to the arid habitats in central Asia, QTP and adjacent high-altitude regions. Therefore, it is highly likely that reticulate evolution of these traits renders the establishment of some non-monophyletic taxa.

Biogeography and diversification

Only two clades with moderate support (Fig. 2A) were identified based on the phylogenetic analyses of the ITS dataset for *Eutrema s.l.* However, three clades with stronger support values were recovered when plastid DNA sequence data (Fig. 2B) or the combined two datasets (Supporting Information, Fig. S5) were used. As discussed above, the biogeographical history and diversification of *Eutrema* as recircumscribed here were mainly based on the analyses of plastid DNA dataset. Three clades seem to be vicariously distributed mainly in eastern Asia (clade A), central Asia (clade B1), and QTP and adjacent regions (clade B2) (Figs 3, S1), although one to 11 species in each clade co-occur in two regions or in a region dominated by species of the other clade. Ancestral-area reconstruction analyses suggested that the genus probably originated in eastern Asia (Fig. 5), probably in central China, because four species of the earliest diverging clade (A) are exclusively distributed there. After the genus originated *c.* 27 Mya (Fig. 3), the first clade (A) diverged from the other two (B, C) *c*. 22 Mya and the last two diverged from each other *c*. 20 Mya. All of these major divergences occurred in the Late Oligocene and Early Miocene. Due to the lack of fossils for *Eutrema*, all age divergences should be taken as estimates. However, the origin and early divergence of *Eutrema s.l.* corresponded largely to the geological and environmental changes in the regions currently occupied by the diverged clades. The QTP started its first stage of extensive uplift during the Late Oligocene and Early Miocene (Mulch & Chamberlain, 2006), which further trigged Asian desertification due to the formation of the Asian monsoon (Guo *et al.*, 2002, 2004). Therefore, it is likely that at this stage the ancestral species preferring arid habitats in central Asia and high-elevation regions of the QTP diverged respectively from the clade occurring in the wet and warm environment of central China. In fact, similar divergences for major clades were also dated for other genera distributed in these regions, although their ancestral regions were inferred to be different (e.g. Liu *et al.*, 2002; Zhang *et al.*, 2009; Mao *et al.*, 2010).

Four species of clade A [*Eutrema grandiflorum* (Al-Shehbaz) Al-Shehbaz & Warwick, *E. xingshanensis* (Zhao & Ning) G.Q.Hao, Al-Shehbaz & J.Quan Liu, *E. violifolium* and *E. yunshunensis* (Wang) Al-Shehbaz & Warwick] are exclusively distributed in central China, whereas six of eight species of clade B1 [*E. halophilum*, *E. botschantzevii*, *Chalcanthus renifolius*, *E. altaicum* (C.A.Mey.) Al-Shehbaz & Warwick, *E. heterophyllum*-2, *E. integrifolium*] occur predominantly in central Asia. *Eutrema salsugineum*, a model species for studies on salt stress, occurs from central to eastern Asia and into northern North America. Phylogeographical analyses (Wang *et al.*, 2015) and ancestral-area reconstruction suggested that this species diverged from its sister species in central Asia (Fig. 6). Another species of clade B1, *E. himalaicum*, which grows in the Himalayas and QTP, probably diverged from its sister species, *E. integrifolium*, in central Asia. Most of the current species in clade B2 occur in the QTP and on adjacent mountains, but six are distributed there and in central, eastern or northern Asia (*E. racemosum*, *E. cordifolium*, *E. heterophyllum*-1, *E. edwardsii*, *E. bouffordii*, *P. scapiflorum*-1). The reconstruction of ancestral areas suggested that these species probably originated in the QTP and adjacent regions and then dispersed into central and eastern Asia. Four species of this clade occur exclusively in

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eastern Asia, including Japan and eastern and central China (*E. tenue*-1, *E. japonicum*, *E. bulbiferum*, *E. tenue*-2; Fig. 6), and these species or their common ancestor probably had a second dispersal from the QTP into eastern Asia where the current species or species units were formed. Our LTT analyses further suggested that diversification events continued and were more common in clade B2 than in clades A and B1 (Fig. 3C). In clade B2, most current species date from the Mid-Miocene to Quaternary (Fig. 3). It is likely that the QTP uplifts and climatic changes since the Mid-Miocene (Shi. Li & Li, 1998) may have continued to promote diversification events in this region through allopatric divergence and possible hybridization, as indicated by the inconsistent ITS and plastid DNA tree topologies. Such scenarios have also been found in other species-rich genera occurring in the QTP and adjacent regions (e.g. Liu *et al.*, 2006; Koch *et al.*, 2012; Sun *et al.*, 2012). All these findings together suggest that QTP uplifts and climatic changes played an important role in shaping plant species diversity there and adjacent regions. In addition, our biogeographical analyses of *Eutrema* suggest that it originated from the wet and warm habitat and then migrated to the arid regions, which is different from the arid origin and further dispersals for some genera of Brassicaceae (e.g. Mummenhoff *et al.*, 2001; Carlsen *et al.*, 2009).

TAXONOMIC CONSIDERATION

As discussed above, the monospecific *Chalcanthus* and two species (three species units) of *Pegaeophyton*, including the type, are nested in the larger and earlier-published *Eutrema* and therefore the three genera ought to be united. However, since only two of the seven *Pegaeophyton* spp. qualify for being transferred to *Eutrema*, the generic placement of the remaining five species should also be addressed. In addition to the expansion of the boundaries of *Eutrema*, *P. minutum* is transferred here to *Aphragmus*, but the generic assignment of *P. watsonii*, *P. angustiseptatum* and *P. sulphureum* will be addressed in a forthcoming paper.

EUTREMA

In uniting *Chalcanthus* and *Pegaeophyton* with *Eutrema*, the generic boundaries of *Eutrema* are expanded to accommodate the transfer of four species and one subspecies. A detailed description of the combined genus is given below, along with several new combinations.

Eutrema R.Br., Chlor. Melvill., 193. 1823. Type species: *E. edwardsii* R.Br.

Chalcanthus Boiss., Fl. Orient. 1: 211. 1867. Type: *C. renifolius* (Boiss. & Hohen.) Boiss.

Esquiroliella H.Lév., Mondes Plantes, ser. 2, 18: 31. 1916. Type species: *E. violifolia* (H.Lév.) H.Lév. (based on *Martinella violifolia* H.Lév.).

Glaribraya H.Hara, J. Jap. Bot. 53: 135. 1978. Type species: *G. lowndesii* H.Hara.

Martinella H.Lév. Bull. Soc. Bot. France 60: 290. 1904; not *Martinella* Baill., Hist. Pl. 10: 30. 1891; not *Martinella* (Cooke & Massee ex Cooke) Sacc., Syll. Fung. 10: 409. 1892. Type species: *M. violifolia* H.Lév.

Neomartinella Pilger in Engler & Prantl, Nat. Pflanzenfam. Nachtr. 3: 134. 1906. Type species: *N. violifolia* (H.Lév.) Pilger (based on *Martinella violifolia* H.Lév.).

Pegaeophyton Hayek & Hand.-Mazz., Anz. Akad. Wiss. Wien, Math.-Naturwiss. Kl. 59: 246. 1922. Type: *P. sinense* (Hemsl.) Hayek & Hand.-Mazz. (= *P. scapiflorum* (Hook.f. & Thomson) C.Marq. & Airy Shaw).

Platycraspedum O.E.Schulz, Repert. Sp. Nov. Regni Veg. Beih. 12: 386. 1922. Type species: *P. tibeticum* O.E.Schulz.

Taphrospermum C.A.Mey. in Ledebour, Fl. Altaic. 3: 172. 1831. Type species: *T. altaicum* C.A.Mey.

Thellungiella O.E.Schulz in Engler, Pflanzenreich IV. 105 (Heft 86): 251. 1924. Lectotype species designated by Kotov (1979: 144) in Florae partis europaeae URSS [Fl. Evrop. Chasti SSSR] 5: *T. salsuginea* (Pall.) O.E.Schulz (based on *Sisymbrium salsugineum* Pall.).

Wasabia Matsum., Bot. Mag. (Tokyo) 13: 71. 1899. Lectotype species is designated by Hara in Farr *et al*. (1979: 1861) in Index Nominum Genericorum: *W. pungens* Matsum., nom. illeg. [≡ *Lunaria japonica* Miq.].

Herbs, annual, biennial or perennial, with rhizomes, caudex or fleshy and fusiform or slender and non-fleshy roots, rarely with a tuberous rootstock. Trichomes absent or simple. Multicellular glands absent. Stems erect or ascending to decumbent or prostrate, simple or branched at base and/or apically, leafy or rarely leafless, unarmed, sometimes reduced to tiny portions added annually to apices of caudex and its branches. Basal leaves petiolate, rosulate or not, simple, entire, dentate or pinnately or palmately lobed; cauline leaves petiolate or sessile and cuneate or auriculate to amplexicaul at base, entire, dentate or crenate, lowermost alternate or rarely verticillate, sometimes absent; ultimate veins ending or not with apiculate callosities. Racemes bracteate throughout or basally or ebracteate, elongated considerably or not in fruit, sometimes flowers solitary on long pedicels originating from axils of rosette leaves; fruiting pedicels erect and subappressed to

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stem, ascending, divaricate or rarely reflexed, persistent. Sepals ovate or oblong, free, deciduous or rarely persistent, erect or ascending to spreading, equal, base of inner pair not saccate; petals white, rarely pink, purple or blue, sometimes veins darker than blade, longer or shorter than sepals; blade spatulate, obovate, oblong or obcordate, rarely suborbicular, apex obtuse to rounded or emarginate; claw usually undifferentiated from blade, shorter than sepals, glabrous, unappendaged, entire; stamens six, exserted or included, tetradynamous to subequal in length, erect to spreading; filaments wingless or rarely flattened and laterally toothed, unappendaged, glabrous, free; anthers ovate or oblong or rarely linear, obtuse or apiculate at apex; nectar glands lateral or confluent and subtending bases of all stamens, median glands present or absent; ovules two to 96 per ovary; placentation parietal. Fruit dehiscent, capsular siliques or silicles, linear, oblong, ovoid, obcordate, conical, ovate or lanceolate, terete, slightly four-angled, latiseptate or angustiseptate, not inflated, unsegmented; valves papery or rarely thick leathery, with an obscure or prominent midvein, glabrous or rarely papillate, smooth or torulose, keeled or not, wingless; gynophore obsolete or to 5 mm; replum terete or flattened throughout or only basally, visible; septum complete or rarely perforated and reduced to rim or absent, veinless; style obsolete or distinct and up to 3 mm, slender or clavate; stigma capitate, entire, rarely two-lobed and lobes opposite replum, not decurrent, unappendaged. Seeds uniseriate or biseriate, wingless or rarely narrowly winged, oblong to ovate, plump or flattened; seed coat obscurely reticulate to foveolate or papillate, slightly mucilaginous or not when wetted; cotyledons incumbent, oblique or rarely accumbent.

In addition to the 25 *Eutrema* spp. recognized by Al-Shehbaz & Warwick (2005) and the five recently described *E. xingshanensis* (as *Neomartinella xingshanensis* Z.E.Chao, Z.L.Ning & X.W.Hu; Ning *et al.*, 2005), *E. bouffordii* (Al-Shehbaz, 2007), *E. zhuxiense* (Gan & Li, 2014), *E. bulbiferum* (Xiao *et al.*, 2015) and *E. racemosum* (Hao *et al.*, 2015), the addition of the following seven species below expands the genus to include 37 species distributed primarily in Asia, especially China, and only two species, *E. salsugineum* and *E. edwardsii*, are widespread in northern North America and northern and central Asia.

Eutrema baimashanicum Al-Shehbaz, G.Q.Hao & J.Quan Liu, nom. nov., *Eutrema deltoideum* var. *grandiflorum* O.E.Schulz, Notizbl. Bot. Gart. Berlin-Dahlem 9: 476. 1926, non *Eutrema grandiflorum* (Al-Shehbaz) Al-Shehbaz & Warwick, Harvard Pap. Bot. 10: 132. 2005. Type: China. NW Yunnan [S Hengduan], Peimeishan, Mekong-Yangtze divide between Atuntze (Dêqên) and Pungtzera, Jul. 1923, *J. F. Rock 9944* [lectotype designated by Al-Shehbaz (2015a): US 00099944; isolectotypes: E, GH 00112016, P].

Distribution: China (Sichuan, Tibet, Yunnan)

Notes: Eutrema baimashanicum, which was not recognized by Zhou *et al.* (2001) or Al-Shehbaz (2015b) and treated as part of highly variable *E. deltoideum*, is undoubtedly most closely related to the latter, from which it is distinguished by having larger flowers with petals 7–8 × 3–4 mm, straight, lanceolate fruit with basally carinate valves and two-lobed stigmas with lobes opposite the valves. By contrast, *E. deltoideum*, which is distributed in Bhutan, India (Sikkim) and neighbouring Tibet, has smaller flowers with petals 5–6 × 2–3 mm, usually curved, oblong to ovate fruit not basally carinate on the valve, and entire or subentire stigmas.

Eutrema nepalense (Al-Shehbaz, Kats.Arai & H.Ohba) Al-Shehbaz, G.Q.Hao & J.Quan Liu, comb. nov. Basionym: *Pegaeophyton nepalense* Al-Shehbaz, Kats.Arai & H.Ohba, Novon 8: 327. 1998. Type: Nepal. Around Lamni Nama, 4200–4900 m, 15 Aug. 1977, *H. Ohashi, H. Kani, H. Ohba & Y. Tateishi 775117* (holotype: TI; isotype: MO 05083454).

Distribution: Bhutan, China (Tibet), India (Sikkim), central and eastern Nepal.

Eutrema purii (D.S.Rawat, L.R.Dangwal & R.D.Gaur) Al-Shehbaz, G.Q.Hao & J.Quan Liu, comb. nov. Basionym: *Dilophia purii* D.S.Rawat, L.R.Dangwal & R.D.Gaur, J. Bombay Nat. Hist. Soc. 93: 262. 1996. Type: India, NW Himalaya, Roopkund, 4850 m, 11 Aug. 1993, *D. S. Rawat s.n.* (holotype: GUH 22498, n.v.; isotype: MO 5658792).

Synonym: Pegaeophyton purii (D.S.Rawat, L.R.Dangwal & R.D.Gaur) Al-Shehbaz, Novon 14: 157. 2004.

Distribution: Known only from the type locality.

Notes: Although the species was not sampled for the present molecular phylogenetic study, it is most closely related morphologically to *Eutrema nepalense*. From the latter, *E. purii* is readily distinguished by having glabrous, slightly flattend fruit, glabrous stems and leaves, and about eight ovules per ovary. By contrast, *E. nepalense* has apically puberulent, terete fruit, puberulent stems and leaves, and two to four ovules per ovary.

Eutrema renifolium (Boiss. & Hohen.) Al-Shehbaz, G.Q.Hao & J.Quan Liu, comb. nov. Basionym: *Hesperis renifolia* Boiss. & Hohen., Diagn. Pl. Or. Nov. Ser 1, 8: 22. 1849. Type: Iran, near Tehran, in valle Latkan prope Shah Neshin in Mt. Tuchal, 7000–8000 ft [2133.6–2438.4 m], *C. G. T. Kotschy*

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228 (holotype, G-BOIS; isotypes, BM 000552465, E 00386046, G 00389773, GOET 002734, K 000653966).

Synonym: Chalcanthus renifolius (Boiss. & Hohen.) Boiss., Fl. Orient. 1: 212. 1867.

Distribution: Afghanistan, Iran, Kazakhstan, Tajikistan, Turkemenistan, Uzbekistan.

Notes: Except for its enormously large rootstock, long fruit and winged seeds, *Eutrema renifolium* is quite similar to several *Eutrema* spp. in foliage and flowers. However, it differs drastically from the other species of *Eutrema s.l.* in having a tuberous rootstock 7–15 × 1.5–3.5 cm, linear fruit (7–)14–26(–35) cm × 5–6 mm, and winged seeds 4–6 × 2.5–4.0 mm with a continuous wing 0.5–1.5 mm wide apically.

Eutrema robustum (O.E.Schulz) Al-Shehbaz, G.Q.Hao & J.Quan Liu, comb. & stat. nov. Basionym: *Pegaeophyton sinense* (Hemsl.) Hayek & Hand.-Mazz. var. *robustum* O.E.Schulz, Notizbl. Bot. Gart. Berlin-Dahlem 9: 477. 1926. Type: China, Yunnan, Mount Lauchünshan, SW of the Yangtze bend at Shiku, swampy meadow, Jun. 1923, *J. F. Rock 9577* [lectotype designated by Al-Shehbaz *et al*. in Al-Shehbaz (2000: 164): B 100272069; isolectotypes: E 00107445, GH 00112004, P 00747206, US, W 1926-0015876].

Synonyms: Pegaeophyton scapiflorum subsp. *robustum* (O.E.Schulz) Al-Shehbaz, T.Y.Cheo, L.L.Li & G.Yang in Al-Shehbaz, Edinburgh Journal of Botany 57: 164. 2000; *P. scapiflorum* var. *robustum* (O.E.Schulz) R.L.Guo & T.Y.Cheo, Bull. Bot. Lab. North-East. Forest. Inst. 6(6): 28. 1980.

Distribution: Bhutan, China (Sichuan, Tibet, Yunnan), northern Myanmar.

Notes: Eutrema robustum, which corresponds to *Pegaeophyton scapiflorum*-2 of the present study, differs substantially from *E. scapiflorum* (*P. scapiflorum*-1). It is distinguished from the latter by having mostly stout caudex (5–)8–20(–30) mm in diameter and simple or rarely branched at apex; petals (6–)8–12(–15) mm long and (5–)6–9(–10) mm wide with the length (1.0–)1.2–1.5 times the width, and seeds (2.0–)2.5–3.5(–4.0) mm long and (1.5–)2.0–2.5(–3.0) mm wide. In contrast, *E. scapiflorum* has slender caudex 1–8(–12) mm in diameter and few to many branched or rarely simple at apex, petals (3.5–)5.0–7.0 mm long and (1.5–)2.0–3.0(–3.5) mm wide with the length (1.8–)2.0–2.5(–3.0) times the width, and seeds 1.5–2.0(–2.5) mm long and 1.0–1.6(–1.8) mm wide.

Eutrema scapiflorum (Hook.f. & Thomson) Al-Shehbaz, G.Q.Hao & J.Quan Liu, comb. nov. Basionym: *Cochlearia scapiflora* Hook.f. & Thomson, J. Proc. Linn. Soc., Bot. 5: 154. 1861. Type: India. Sikkim, 15000–17000 m, [4572–5181 m], *J. D. Hooker s.n.* (Lectotype designated by Al-Shehbaz (2015a: 10): K 000247223; isolectotypes: GH, GOET, K000247222, M).

Synonym: Pegaeophyton scapiflorum (Hook.f. & Thomson) C.Marq. & Airy Shaw, J. Linn. Soc. Bot. 48: 229. 1929.

Distribution: China (Gansu, Qinghai, Sichuan, Xinjiang, Xizang), India, Myanmar, Nepal, Pakistan.

Notes: Despite the small overlap in flower and seed size between *E. scapiflorum* and *E. robustum*, the species are readily distinguishable. The latter has been recently treated as a subspecies or variety of the former (see Al-Shehbaz, 2000).

Eutrema thibeticum Franch., Nouv. Arch. Mus. Paris, ser. 2, 8: 201. 1886. Type: China. Tibet [S Hengduan], Moupin, Fl. Mar. & Apr. 1869, *Abbe David* s.n. [holotype: P (P02272673)].

Distribution: China (Guizhou, Sichuan, Tibet, Yunnan).

Notes: As discussed above, *E. tenue sensu* Zhou *et al.* (2001), Al-Shehbaz & Warwick (2005) and Al-Shehbaz (2015b) is polyphyletic and consists of two taxa each sister to a different species of *Eutrema*. The plants from Japan and Korea (*E. tenue*-1 in this study) have $2n = 28$ and they are retained here in *E. tenue* becasue the type collection of the species is from Japan. Furthermore, the above authors correctly listed *E. hederifolium* Franch. & Sav. and *E. bracteata* (S.Moore) Kodzumi in the synonymy of *E. tenue*, and their types are also collected from Japan.

The Chinese collections cited in the above three publications, which belong to our *E. tenue*-2, have $2n = 16$ and the earliest available name for these plants is *E. thibeticum*, a species that was later described by He & Lan (1997) as *Neomartinella guizhouensis* S.Z.He & Y.C.Lan.

Eutrema tenue and *E. thibeticum* are remarkably similar in habit, overall shape, base and margin of basal and cauline leaves, fruit orientation, bract morphology, and flower colour and size. However, *E. tenue* differs by having glabrous or rarely glabrescent distal parts of the stem and by strongly tortuose fruit, with slender style (1.0–)1.5–2.0 mm long. In contrast, *E. thibeticum* has densely or rarely sparsely retrorsely pilose distal stems, with crisped trichomes and smooth or slightly torulose but not tortuose fruit, with stout style 0.5–0.8(–1.0) mm long. More morphological studies at the population level are needed to establish whether there are other characters that separate these two most closely related species.

Eutrema xingshanensis (Zhao & Ning) G.Q.Hao, Al-Shehbaz & J.Quan Liu, comb. nov. Basionym:

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Neomartinella xingshanensis Zhao & Ning J. Wuhan. Bot. Res. 24: 47–48. 2006. Type: China. Hubei, Xingshan Xian, Nanyang town, 26 feb. 2005, Z.N. Zhao 9273 (holotype: HIB).

Distribution: China (Hubei, Hunan).

Notes: This species was described in *Neomartinella* and was mentioned as '*Eutrema xingshanensis*' by Warwick *et al.* (2006). However, its taxonomic tranfer has never been published formally.

APHRAGMUS

As recently delimited (Al-Shehbaz, 2015b), *Aphragmus* Andrz. ex DC. includes 12 species distributed primarily in the Himalayas, with one species each in Russia (Far East, Siberia) and North America (Alaska, Yukon). The generic position of *Pegaeophyton minutum* H.Hara has not been considered since the description of the species > 40 years ago. However, a close examination of the species clearly shows that it is perfectly at home in *Aphragmus* and all of its characters are found in one or more species of the latter genus. For example, the presence of minute trichomes along one side is characteristic of most *Aphragmus* spp., its solitary flowers originating from axils of the basal rosette is found in *A. pygmaeus* Al-Shehbaz, its linear fruits are found in at least three *Aphragmus* spp. and its many-branched, rhizome-like caudex covered with distinct internodes separating whorls of petiolar remains of successive growing seasons is found in *A. nepalense* (H.Hara) Al-Shehbaz. Therefore, the transfer of *P. minutum* to *Aphragmus* makes sense and would not alter the generic limits of the latter.

Aphragmus minutus (H.Hara) Al-Shehbaz, G.Q.Hao & J.Q.Liu, comb. nov. Basionym: *Pegaeophyton minutum* H.Hara, J. Jap. Bot. 47: 270. 1972. Type: India. Sikkim, Oma La-Migothang, ca. 4200 m, 30 Myay 1960, *H. Hara, H. Kanai, G. Murata, M. Togashi & T. Tuyama 6344* [holotype: TI; isotypes: MO (MO05083453), TI].

Distribution: Bhutan, China (Xizang), India, Myanmar, Nepal.

ACKNOWLEDGEMENTS

This research was supported by grants from the National Natural Science Foundation of China (31590821, 91331102) and Sichuan Province Youth Science and Technology innovation team (2014TD003).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Geographical distribution of the populations sampled in this study.

Figure S2. Neighbor-joining tree of circle-based analyses of all unique sequences through ITS for all sampled individuals. Green blocks represent paraphyletic taxa and red blocks represent two species that share identical sequence. When more than one individual shared the same sequence, an asterisk '*' on the branches indicates the number of samples.

Figure S3. Neighbor-joining tree of circle-based analyses of all unique sequences through a combination of all cpDNA fragments for all sampled individuals. Green blocks represent paraphyletic taxa and red blocks represent two species that share identical sequence. When more than one sample shared the same sequence, an asterisk '*' on the branches represents the number of samples.

Figure S4. Bayesian phylogenetic analyses of the representative species of *Eutrema s.l.* and the related genera in the Brassicaceae.

Figure S5. Bayesian phylogenetic tree inferred from the combined five cpDNA fragments and ITS. Numbers above branches are maximum parsimony bootstrap support values, Bayesian posterior possibilities, maximum likelihood bootstrap support values and BEAST posterior possibilities. An asterisk '*' represents values of 100% while '-' indicates values < 50%.

Figure S6. Neighbor-joining (NJ) trees inferred from ITS (a), five cpDNA fragments (b), the combined five cpDNA fragments and ITS (c). Numbers above branches are NJ bootstrap support values. Green represents paraphyletic taxa and red represents two species that share identical sequence. When more than one sample shared the same sequence, an asterisk '*' on the branches represents the number of samples.

Table S1. The sources of materials and GenBank accession numbers.

Table S2. Morphological characters and data matrix used in the ancestral state reconstruction analyses.

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