Molecular phylogeny, divergence time estimates and historical biogeography within one of the world’s largest monocot genera

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Abstract. A primary aim of historical biogeography is to identify the causal factors or processes that have shaped the composition and distribution of biotas over time. Another is to infer the evolution of geographic ranges of species and clades in a phylogenetic context. To this end, historical biogeography addresses important questions such as: Where were ancestors distributed? Where did lineages originate? Which processes cause geographic ranges to evolve through time? Allium subgenus Anguinum comprises approximately twelve taxa with a disjunct distribution in the high mountains from south-western Europe to eastern Asia and in northeastern North America. Although both the systematic position and the geographical limits of Anguinum have been identified, to date no molecular systematic study has been performed utilizing a comprehensive sampling of these species. With an emphasis on the Anguinum eastern Asian geographical group, the goals of the present study were: (i) to infer species-level phylogenetic relationships within Anguinum, (ii) to assess molecular divergence and estimated the times of the major splits in Anguinum and (iii) to trace the biogeographic history of the subgenus. Four DNA sequences (ITS, matK, trnH-psbA, rps16) were used to reconstruct the phylogeny of Allium subgen. Anguinum. RbcL sequences were used to estimate divergences time for Allium, and sequences of ITS were used to estimate the divergence times for Anguinum and its main lineages and to provide implications for the evolutionary history of the subgenus. Phylogenetic analyses for all Allium corroborate that Anguinum is monophyletic and indicate that Anguinum is composed of two sister groups: one with a Eurasian–American distribution, and the other restricted to eastern Asia. In the eastern Asian geographical group, incongruence between gene trees and morphology-based taxonomies was recovered as was incongruence between data from plastid and nuclear sequences. This incongruence is likely due to the combined effects of a recent radiation, incomplete lineage sorting, and hybridization/introggression. Divergence time estimates suggest that the crown group of Anguinum originated during the late Miocene (ca. 7.16 Mya) and then diverged and dispersed. Biogeographic analyses using statistical dispersal–vicariance analysis (S-DIVA) and a likelihood method support an eastern Asia origin of Anguinum. It is inferred that in the late Pliocene/Early Pleistocene, with cooling climates and the uplift of the Himalayas and Hengduan Mountains, the ancestor of the eastern Asian alliance clade underwent a very recent radiation.

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Keywords: Allium; anguinum; divergence time; historical biogeography; hybridization/introggression; incomplete lineage sorting; phylogeny; radiation.

Introduction

A primary aim of historical biogeography is to identify the causal factors or processes that have shaped the composition and distribution of biotas over time (Sanmartín 2007). Another major focus is to infer the evolution of geographic ranges of species and clades in a phylogenetic context (Ree and Smith 2008). To this end, many questions about the historical distributions of species may be of interest, such as: Where were ancestors distributed? Where did lineages originate? Which processes (e.g., vicariance or dispersal) cause geographic ranges to evolve through time? (Ree and Smith 2008; Xie et al. 2009). Many studies have addressed these biogeographic questions using phylogenetic analyses, molecular dating, and reconstruction of ancestral geographic ranges (e.g., Sytsma et al. 2004; Nie et al. 2006; Bell 2007; Sanmartín et al. 2008; Xie et al. 2009).

A brief introduction of the paleontological history of the Northern Hemisphere since the late Cretaceous helps to understand the biogeographic history of plant biota. Overall, the Earth’s climate became cooler through the Tertiary (Zachos et al. 2001), and the climate cooled gently from 50 to 35 million years (Myr) ago, then fluctuated until 15 Myr ago, after which the climate cooled progressively, culminating in the Quaternary (2–0 Myr ago) glaciations (Milne and Abbott 2002). Cooling climates in the latter part of the Tertiary forced the boreotropical flora to retreat southwards to large refugial regions that preserved the warm wet climate that they needed. These refugia include eastern Asia, south-eastern Europe, eastern and western North America, and western Asia, and the floras concerned are termed Tertiary relict floras (Tiffney 1985a,b; Wen 1999; Milne and Abbott 2002). Over the 2 million years of the Quaternary, these data indicate considerable and continuous climatic variation (Mitchell 1976; Smiley et al. 1991). Up to 24 glacial events of about 50–100 000 years each have occurred (van Donk 1976). The climatic oscillations of the Quaternary resulted in repeated drastic environmental changes that profoundly shaped the current distributions and genetic structures of many plant species in temperate zones of the Northern Hemisphere (Hewitt 1996, 2000, 2004). When the Tertiary period began, North America was connected via Greenland to north-eastern Europe—a connection known as the North Atlantic land bridge (NALB; Tiffney 1985b). Different perspectives exist for the timing of the break up of the NALB (Tiffney 1985a,b; Tiffney 2000; Wen 2000; Tiffney and Manchester 2001; Milne and Abbott 2002; Milne 2006), but it is thought that the NALB was available for plant exchanges from the early Tertiary to possibly as late as 15 Myr ago (Milne and Abbott 2002). It is known that the BLB connected eastern Asia and western North America at one time or another throughout the late Cretaceous and the Tertiary and was available for floristic exchanges until ca. 3.5 millions of years (Ma; Tiffney 1985a,b; Wen 1999; Gladenkov et al. 2002; Milne 2006).

The genus Allium comprises about 920 species (Seregin et al. 2015), making it one of the largest monocotyledonous genera. Allium is a member of order Asparagales, family Amaryllidaceae, subfamily Allioideae (Fay and Chase 1996; APG III 2009; Chase et al. 2009). After Fay and Chase (1996), Friesen et al. (2000) and Chase et al. (2009), Allium (including Caloscordum Herb., Milula Prain and Nectaroscordum Lindl.) is the only genus in tribe Allieae. Previous molecular data suggested that Allium evolution proceeded in three separate evolutionary lines; subgenus Anguinum is a member of the second evolutionary line (Fritsch 2001; Fritsch and Friesen 2002; Friesen et al. 2006; Li et al. 2010; Choi et al. 2012). Anguinum contains approximately twelve taxa (nine species and three varieties) with a disjunct distribution in the high mountains from south-western Europe to eastern Asia and in northeastern North America (Fritsch and Friesen 2002). It is characterized by specific root anatomical characters (Fritsch 1992a), leaf and bulb structure (Pastor and Valdes 1985), hypogaeal seed germination and A. victorialis-type seedlings (Druselmann 1992), uniovulate locules (Hanelt, 1992), narrow, branched and lengthwise-twisted septal nectarines (Fritsch 1992b) and a short vegetative period with the adaptation of the light regime under deciduous forest conditions (Pistrick 1992; Li et al. 2010). Unlike other Allium lineages, the seed testa sculpturing is very simple among species of Anguinum (Kruse 1984, 1988). Species of Anguinum also share similar metaphase chromosomes and the basic chromosome number x = 8 and all reported karyotypes are 2A type (Jing et al. 1999). Based on the consistency of its morphological, anatomical and cytological characteristics, it is a rather distinct and specialized group (Li et al. 2010). Previous molecular studies indicated that Anguinum is monophyletic and shares a more recent
common ancestor with Vvedenskya, Porphyroprason and Melanocrommyum and is the sister group to Caloscordum (Friesen et al. 2006; Li et al. 2010). According to Friesen et al. (2006) and Li et al. (2010), two sister groups comprise this subgenus: one with a Eurasian–American distribution, and the other restricted to the Hengduan Mountains and adjacent areas.

Although both the systematic position and the geographical limits of Anguinum have been identified, to date no molecular systematic study has been performed utilizing a comprehensive sampling of these species. Thus, in order to better understand the phylogeny and historical biogeography of Anguinum, an extended population sampling of Anguinum species endemic to eastern Asia was incorporated in this study. With an emphasis on the Anguinum eastern Asian geographical group, the goals of the present study were: (i) to infer species-level phylogenetic relationships within Anguinum using four molecular markers ITS, matK, trnH-psbA, and rps16, (ii) to assess molecular divergence and estimated the times of the major splits in Anguinum and related these divergence times with external factors that might have contributed to the diversification of the subgenus and (iii) to trace the biogeographic history of the subgenus by provided a time frame to the biogeographic studies.

**Methods**

**Taxon sampling**

Our sampling strategy was designed to cover those taxonomic and geographic Anguinum groups that were underrepresented in previous analyses, especially from eastern Asia, and to build on previous studies (Friesen et al. 2006; Li et al. 2010). Fifty samples (ITS sequences for four samples downloaded in Genbank) representing twelve taxa of Anguinum with a focus on the eastern Asian geographical group, were sampled as the ingroup for phylogenetic reconstructions. Two species from subgenus Caloscordum (A. neriniflorum, A. tubiflorum) were designated as outgroups according to previous studies (Fritsch and Friesen 2002; Friesen et al. 2006; Nguyen et al. 2008; Li et al. 2010). GenBank accession numbers and voucher details referred to the above-mentioned taxa are given in [see Supporting Information, Appendix S1].

There are no known fossils of Anguinum or even Allium, so we first intend to estimate the divergence time of Allium, based on a relatively broad analysis of rbcL sequences of Allium (i.e., representatives almost in every subgenus of Allium) together with other samples from the order Asparagales and other monocots. According to previous systematic studies (e.g. Fay and Chase 1996; Vinnersten and Bremer 2001; Davis et al. 2004; Tamura et al. 2004; Leebens-Mack et al. 2005; Müller et al. 2006; Meng et al. 2008; Kim et al. 2010) and our preliminary phylogenetic analysis for 255 sequences of the groups of monocots, 152 sequences were chosen covering all orders and as many families as possible referred to the present study, in which 32 sequences (26 sequences were generated in this study) representing Allium spp. [see Supporting Information, Appendix S2]. To infer divergence times within Anguinum, 144 ITS sequences (34 sequences were generated in this study) that represented Anguinum and other subgenera and sections in Allium, plus two sequences of Nothoscordum gracile (family Amaryllidaceae, subfamily Allioideae, tribe Gilliesieae) and Tulbaghia violacea (family Amaryllidaceae, subfamily Allioideae, tribe Tulbaghieae) were used, and the selection of Anguinum taxa is based on haplotype analysis, and sequences belong to the same haplotype form the same taxa are removed (except A. nanodes) [see Supporting Information, Appendix S3].

**DNA extraction, amplification and sequencing**

Genomic DNA was extracted from silica gel-dried or fresh leaves using the method of Doyle and Doyle (1987). Primers ITS4 and ITS5 (White et al. 1990) were used to amplify the ITS region followed the protocol of Li et al. (2010). The rps16 intron was amplified with primers rpsF and rpsR2 (Oxelman 1999) in accordance with the protocol of Marazzi et al. (2006). The intergenic spacer trnH-psbA was amplified using the primers trnH (GUG) F and psbAR (Hamilton 1999). The PCR programme was as follows: 94 °C for 4 min; 30 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min; and 72 °C for 7 min. The rbcL was amplified with primers rbcL N° and DRRBAS2 (Terachi et al. 1987) for 26 sequences. The matK was amplified with primers 3F_KIM and IR_KIM (Kim, unpublished). Their PCR parameters were same as follows: 94 °C for 4 min; 30 cycles of 94 °C for 1 min, 52 °C for 1 min 30 s; and 72 °C for 10 min. PCR products were separated using 1.5 % agarose TAE gel and purified using Wizard PCR preps DNA Purification System (Promega, Madison, WI, USA) following the manufacturer’s instructions. The purified PCR products were sequenced in an ABI 310 Genetic Analyzer (Applied Biosystems Inc.) using the PCR primers.

**Sequence comparisons and phylogenetic analyses**

Forward and reverse sequences were assembled and edited with SeqMan (DNAstar package; DNAStar Inc., Madison, WI, USA). DNA sequences were initially aligned using the default pairwise and multiple alignment parameters in Clustal X (Jeanmougin et al. 1998) and then
rechecked and adjusted manually as necessary using MEGA4 (Tamura et al. 2007). Gaps were positioned to minimize nucleotide mismatches and treated as missing data in phylogenetic analyses. Phylogenetic analyses were conducted by employing maximum-parsimony (MP) criteria and Bayesian inference (BI), using the programs PAUP* version 4.0b10 (Swofford 2003) and MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003), respectively. For MP, heuristic searches were carried with 1000 random addition sequence replicates. One tree was saved at each step during stepwise addition, and tree-bisection-reconnection (TBR) was used to swap branches. All characters were unordered and equally weighted. Gaps were treated as missing data. Bootstrap values were calculated from 6000 000 replicate analyses using ‘fast’ stepwise-addition of taxa and only those values compatible with the majority-rule consensus tree were recorded. Prior to a Bayesian analysis, MrModelfest version 2.2 (Nylander 2004) was used to select a best-fit model of nucleotide substitution under the Akaikes information criterion. The Bayesian Markov chain Monte Carlo (MCMC) settings consisted of four independent runs with four chains each (one cold chain and three incrementally heated chains) for 6 million generations starting from random trees and sampling one of every 100 generations, by using default priors and estimating all parameters during the analysis. The first 25% of the trees were discarded as burn-in. A 50% majority-rule consensus tree of the remaining trees was produced.

The incongruence length difference (ILD) test of ITS vs. the combined chloroplast sequences (rps16, matK, and tmH-psbA) was carried out in PAUP* (Farris et al. 1994) to assess potential conflicts between the phylogenetic signal from different genomes. This test was implemented with 100 partition-homogeneity test replicates, using a heuristic search option with the simple addition of taxa, TBR branch swapping and MaxTrees set to 1000.

Divergence time estimation

Since the strict molecular clock model was rejected for rbcL with \(P<0.05\) in Likelihood ratio tests (LRTs; Felsenstein 1988), divergences time of Allium was estimated using a Bayesian approach with an uncorrelated lognormal relaxed molecular clock model (Drummond et al. 2006), as implemented in the program BEAST v1.5.2 (Drummond and Rambaut 2007). An advantage of BEAST is its ability to estimate the topology and divergence times simultaneously. The BEAST analysis was run for \(5 \times 10^{7}\) generations with parameters sampled every 1000 generations and used the GTR+I+G substitution model selected by MrModeltest and randomly generated Starting Tree and the Yule tree prior. The fossil record of monocots is comparatively poor primarily due to problems of preservation (Herendeen and Crane 1995; Crepet, Nixon and Gandolfo 2004). A few fossils of the Asparagales have been reported from the late Eocene (Couper 1960; Muller 1981; Herendeen and Crane 1995) and they all may be too young to calibrate the crown clade of the order (Wikström et al. 2001; Janssen and Bremer 2004). Based on five molecular markers, Magallón and Castillo (2009) estimated the age of the crown Asparagales as 124.95 million years ago (Mya) for relaxed datings. We therefore used this age as the calibration point for the crown group Asparagales node, with a normally distributed standard deviation of 0.35. Based on eight reference fossils, Bremer (2000) estimated the split between Acorus and all other monocots to be more than 134 Myr old. Using a calibration point outside the monocots (within the eudicot order Fagales), Wikström et al. (2001) produced an age for extant (crown group node) monocots of 127–141 Mya. These two estimates fall into the range of ages estimated by Sanderson and Doyle (2001) and are in fairly close agreement with Good-Avila et al. (2006) who estimated the crown age of monocots to be 132 Myr old. As Chase (2004) wrote, we can draw some generalities about the ages of monocots from these studies. Thus, following Janssen and Bremer (2004) and Meng et al. (2008), we calibrated the crown age of monocots as 134 Mya, with a normally distributed standard deviation of 4.25. We also set the crown age of Amaryllidaceae at 87 Mya based on Janssen and Bremer (2004), with a normally distributed standard deviation of 0.01. The results were evaluated by the Tracer program 1.4 (Rambaut and Drummond 2007) and the first 10% of the generations were discarded as burn-in. Samples from the posterior were summarized on the maximum clade credibility tree using TreeAnnotator version 1.5.2. (Drummond and Rambaut 2007) and the tree was visualized using FigTree ver. 1.3.1 (Rambaut 2009). The divergence times are given as the mean node heights and the 95% highest posterior density (HPD) intervals in millions of years (Ma). According to Magallón and Castillo (2009), we rooted the tree with four species (Ascarina swamyana, Chloranthera nervosus, Hedyosmum arboreascens and Sarcedera chloranthoides) from order Chloranthales.

Because the likelihood ratio tests rejected the molecular clock for the data (\(P<0.05\)), divergence times within Anguiniun were estimated using the same methods stated above and the GTR+I+G substitution model was selected by MrModeltest. The crown age of Allium was set to 34.26 Mya based on previous analysis, with a normally distributed standard deviation of 0.1. According to previous phylogenetic analyses (Fay and Chase 1996; Mes et al. 1997; Fay et al. 2000; Friesen et al. 2000, 2006;
Nguyen et al. 2008; Li et al. 2010), Nothoscordum gracile and Tulbaghia violacea were used to root the tree.

Biogeographical analysis
To perform our analysis, we used the maximum clade credibility (MCC) phylogeny from the ITS data set by BEAST. Distribution areas of Anguinum and its close allies (Caloscordum, Vvedenskya, Porphyroprasion, and Melanocrommyum) were defined according to the World Checklist of Selected Plant Families maintained by the Royal Botanic Gardens, Kew, UK (http://apps.kew.org/wcsp/home.do) and taxonomic and geographical studies of these Allium species (e.g. Xu and Kamelin 2000; Dale et al. 2002; Choi and Oh 2011). Potential biogeographical scenarios of Anguinum were investigated using statistical dispersal–vicariance analysis (S-DIVA; Yu et al. 2010) and a maximum likelihood-based method (LAGRANGE; Ree et al. 2005; Ree and Smith 2008) implemented in the computer software Reconstruct Ancestral States in Phylogenies (RASP; Yu et al. 2015). In the S-DIVA analysis, allowing reconstruction, the number of ancestral areas was restricted to two. The rationale for such a constraint is that vicariance is a proximate consequence of dispersal. Moreover, extant taxa used in the analyses rarely occur in more than two individual areas. The ML inferences of geographic range evolution using LAGRANGE were conducted for the same distribution matrix under the constraints of maximum areas of two. The connectivity between areas was not constrained.

Results
Molecular datasets
The ILD test conducted on the combined data matrix of common ITS and chloroplast sequences (matK + trnH-psbA + rps16) accessions was significant (ILD probability value = 0.01), indicating that the two datasets are heterogeneous (Cunningham 1997). Finally, four different datasets were generated: dataset 1 for Anguinum ITS phylogenetic analyses, dataset 2 for Anguinum combined chloroplast phylogenetic analyses, dataset 3 for Allium divergence time estimation, and dataset 4 for Anguinum divergence time estimation. After introducing the necessary gaps, the ITS alignment for dataset 1 was 649 bp in length and resulted in 498 constant characters and 150 variable characters, 110 of which were potentially parsimony-informative; the mean G + C content was 47.5%. For dataset 2, the combined chloroplast sequences (matK + trnH-psbA + rps16) produced a matrix 2296 bp in length, and for which 2193 characters were constant, 39 autapomorphic and 56 potentially parsimony-informative; the mean G + C content was 31.3%. The statistics of chloroplast sequences and nuclear ITS are shown in Table 1. For dataset 3, the aligned rbcL sequences produced a matrix 1328 bp in length, and for which 741 characters were constant, and 418 potentially parsimony-informative; the mean G + C content was 43.6%. For dataset 4, the aligned ITS sequences produced a matrix 754 bp in length, and for which 99 characters were constant, and 547 potentially parsimony-informative; the mean G + C content was 46.6%.

Phylogeny
ITS phylogeny. For dataset 1, trees inferred from BI and MP showed no significant difference in their topologies, and therefore only the Bayesian tree with posterior probabilities (PP) and bootstrap support values (BS) was shown in Fig. 1. In all analyses, the subgen. Anguinum proved to be monophyletic and robustly separated from the outgroup species (PP = 1.00, BS = 100%). The
Anguinum contains two sister groups: one with a Eurasian–American distribution (including *A. victorialis*, *A. listera*, *A. microdictyon*, *A. ochotense*, *A. tricoccum* var. *tricoccum*, *A. tricoccum* var. *burdickii*), and the other restricted to eastern Asia (i.e., the Hengduan Mountains and adjacent areas, including *A. ovalifolium* var. *ovalifolium*, *A. ovalifolium* var. *leuconeurn*, *A. funckiifolium*, *A. nanodes*, *A. prattii*). Within the Eurasian–American distribution clade, *A. ochotense*, *A. tricoccum*, and three species from Eurasia (*A. listera*, *A. microdictyon*, and *A. victorialis*) form a trichotomy (PP = 0.85, BS = 80%); three accessions of *A. listera* join together (PP = 0.99, BS = 63%). Within the eastern Asia distribution clade, species from eastern Asia...
(*A. ovalifolium* var. *ovalifolium*, A. *ovalifolium* var. *cordifolium*, A. *ovalifolium* var. *leuconeurn*, A. *nanodes*, A. *funckiifolium*, A. *prattii*) form a large basal-most polytomy (PP = 1.00, BS = 100%), and inside some small polytomies; two accessions of *A. nanodes* grouped together (PP = 0.95, BS = 63%). In all cases, although there are small clades of alleles from the same taxa, *A. ovalifolium* var. *ovalifolium*, *A. ovalifolium* var. *leuconeurn* and *A. prattii* are non-monophyletic. Considering only one accession involved in the present study, the monophyly/non-monophyly of *A. ovalifolium* var. *cordifolium* and A. *funckiifolium* requires further investigation. Overall, ITS phylogeny is populated by both short internal and terminal branches; therefore, it is not surprising that there are unresolved polytomies.

**Chloroplast sequence phylogeny**

In the combined cpDNA analyses, the topology of the Bayesian tree was similar to that of the MP consensus tree. The 50% majority-rule consensus tree from BI is presented in Fig. 2, with PP and BS support values. The monophyly of subgen. *Anguinum* was also recovered (PP = 1.00, BS = 100%). Within *Anguinum*, two sister groups are evident. Within the Eurasian–American distribution clade (PP = 1.00, BS = 100%), accessions of *A. listera* and *A. victorialis* form a polytomy; within the eastern Asia distribution clade (PP = 0.89, BS = 95%), accessions of *A. ovalifolium* var. *ovalifolium*, *A. ovalifolium* var. *cordifolium*, *A. ovalifolium* var. *leuconeurn*, *A. nanodes*, *A. funckiifolium*, and *A. prattii* form a large basal-most polytomy, and also some small polytomies inside the polytomy. The topological patterns of the combined cpDNA phylogeny are more complex than that of ITS. Accessions from following four taxa did not form monophyletic groups: *A. ovalifolium* var. *ovalifolium*, *A. ovalifolium* var. *leuconeurn*, *A. nanodes*, *A. prattii*, although part of the sequences for the same taxa grouped together. Overall, the cpDNA phylogenetic hypothesis is also rich in polytomies.

**Biogeographical analysis**

Eight areas were considered for biogeographical analysis of *Anguinum* and its allies: eastern Asia (a), eastern North America (b), western North America (c), Europe and adjacent areas (d), Siberia (e), West Asia and adjacent areas (f), Central Asia (g) and the Mediterranean (h). The inferred historical biogeographic scenarios from analyses using S-DIVA and LAGRANGE are shown in Fig. 4. Both the analyses strongly supported East Asia as the ancestral area of *Anguinum*.

**Discussion**

**Sequence divergence and polytomies**

For ITS sequences, the average pairwise K2P (Kimura’s two-parameter; Kimura 1980) distance between ingroup *Anguinum* and outgroup *Caloscordum* ranged from 15.73 to 19.58%. The average pairwise K2P distance within *Anguinum* was very low (range 0.00–6.37%), and the average distance was 1.60%. In the Eurasian–American alliance clade, the average distance was 1.40% (range 0.00–3.69%), and in the eastern Asia alliance clade, the average distance was 0.27% (range 0.00–1.09%). For chloroplast sequences, the average pairwise K2P distance between ingroup *Anguinum* and outgroup *Caloscordum* ranged from 1.40 to 2.02%. The average pairwise K2P distance within *Anguinum* was very low (range 0.00–1.17%), and the average distance was 0.32%. In the sister group formed by *A. victorialis* and *A. listera*, the average distance was 0.15% (range 0.00–0.27%), and in the eastern Asia alliance clade, the average distance was 0.23% (range 0.00–0.74%). Overall, ITS data show remarkably low levels of variability within *Anguinum*, and the variation is low in comparison to subgenus *Melanocrommyum* in the same evolutionary line (Gurushidze et al. 2008). Chloroplast sequence data also show remarkably low levels of variability within *Anguinum*. Another conspicuous result is the different level of differentiation at the ITS and the chloroplast sequences within and among species of subgen. *Anguinum*. In several species intraspecies diversity is quite high, and several species show almost no interspecies diversity. Low sequence divergence results in an unresolved ITS and chloroplast phylogeny of *Anguinum*, with both short internal and terminal branches; thus, it is not surprising that there are rich in polytomies. Further studies (utilizing low-copy nuclear gene phylogenies and/or employing marker technology that has broad genomic coverage) are needed to obtain more information on species-level relationships of *Anguinum*.
Phylogenetic implications, incomplete lineage sorting, hybridization/introggression

The results presented here support the earlier finding that subgen. Anguinum is monophyletic (Friesen et al. 2006; Li et al. 2010). In agreement with Friesen et al. (2006) and Li et al. (2010), two sister groups comprise this subgenus: one with a Eurasian–American distribution and the other restricted to eastern Asia. In the Eurasian–American geographical group, A. listera, A. microdictyon, and A. victoriensis are closely related species and form a polytomy, which may indicate that this group diverged rapidly. In the ITS tree, accessions of three A. listera populations are clustered together, implying that A. listera is monophyletic. In the eastern Asian geographical

Figure 2. Phylogenetic tree resulting from a Bayesian analysis of the concatenated plastid sequences (rps16 + matK + trnH-psbA) from species of subgenus Anguinum plus two outgroups. Branch lengths correspond to the genetic distances (substitutions per site). Values along branches represent Bayesian posterior probabilities (PP) and parsimony bootstrap (BS), respectively. Numbers following taxon names refer to populations identified in the Appendix S1. Letters (a–d) indicate relevant nodes discussed in the text.
Figure 3. Chronogram of Allium from the order Asparagales and other monocots based on rbcL data. Divergence times are shown using the computer program BEAST. The tree was rooted using species from order Chloranthales and calibrated using an estimated age of 134 Myr for the age of the crown group of monocots (node 1). The crown group of Asparagales (node 2) and Amaryllidaceae (node 3) was set to be 124.95 Mya and 87 Mya, respectively. The division of the geologic time according to the ‘Geologic Time Scale’ compiled by Walker and Geissman (2009).
Figure 4. Dated phylogeny for Anguinum based on a maximum clade credibility tree obtained from a BEAST analysis of 146 ITS sequences under an uncorrelated lognormal molecular clock. Branch lengths represent millions of years (Ma). Crown age of Allium was set to be 34.26 Mya (node 1) based on the estimated date of Allium from the order Asparagales and other monocots. The division of the geologic time according to the ‘Geologic Time Scale’ compiled by Walker and Geissman (2009). Biogeographic analysis of Anguinum was based on the S-DIVA (shown in front of the slashes) and the ML (shown behind the slashes) analyses. The optimal ancestral areas with an asterisk at some node presented under LAGRANGE are the ones with the highest probabilities among the alternatives. Letters (a–d) indicate relevant nodes discussed in the text.
group, for both the ITS and the cpDNA tree, accessions of *A. ovalifolium* var. *ovalifolium*, *A. ovalifolium* var. *cordifolium*, *A. ovalifolium* var. *leucuneurum*, *A. nanodes*, *A. funckiifolium*, and *A. prattii* form a large basal-most polytomy, and also some small polytomies inside the polytomy. Accessions of two *A. nanodes* populations form a monophyletic group in the ITS tree, and are not clustered together in chloroplast tree. Both ITS tree and chloroplast tree sufficiently indicate that *A. ovalifolium* var. *ovalifolium*, *A. ovalifolium* var. *leucuneurum* and *A. prattii* are non-monophyletic, although there are small clades of alleles from the same taxa. In total, in the eastern Asian geographical group, incongruence between gene trees and morphology-based taxonomies, and incongruence between trees from plastid sequences and nuclear sequences are recovered. This leaves the phylogenetic relationships among the species ambiguous.

Incongruence among molecular data and morphology-based taxonomies (e. g. Gurushidze et al. 2008, 2010; Aduse-Poku et al. 2009; Waters et al. 2010), and incongruence between nuclear and plastid data (e. g. Fehrer et al. 2007; Kim and Donoghue 2008; Pelser et al. 2010) have been repeatedly reported. Experimental and theoretical studies (e. g. reviewed in Wendel and Doyle 1998; Whitfield and Lockhart 2007; Waterman et al. 2009; Gurushidze et al. 2010) have shown that some factors can lead to gene trees incongruence and gene tree—species tree incongruence, including stochastic error, systematic error, convergence, evolutionary rate heterogeneity, lineage sorting, and reticulation, namely hybridization, introgression, homoploid and polyplody. When incongruence is recovered among gene trees, the first consideration is whether it is due to inadequate sampling. The second consideration is stochastic error and systematic error. Once these factors are ruled out, biological factors in the evolutionary process may be considered, including convergence and rapid radiation, horizontal gene transfer, hybridization/introgression, and incomplete lineage sorting. Our sampling scheme was designed to cover all taxa of the Anguinum eastern Asia geographical group; with the exception of *A. ovalifolium* var. *cordifolium* and *A. funckiifolium*, all remaining taxa were represented by accessions from multiple populations. ITS sequences and three chloroplast sequences (*matK*, *trnH-psbA*, *rps16*) were selected as molecular markers in order to have markers representing different genomic compartments. Phylogenetic analyses of the ITS sequences and the combined chloroplast sequences were performed separately, and in BI, a best-fit model of nucleotide substitution was selected for each chloroplast sequence. Consistency index (CI; Kluge and Farris 1969) and the retention index (RI; Farris 1989) obtained from the parsimony analysis are as follows: CI and RI obtained from the ITS sequences were separately 0.94 and 0.98, and CI and RI obtained from the combined chloroplast sequences were separately 0.89 and 0.94, which illustrate that the levels of homoplasy found in our data set are similar to those of other angiosperm groups (Alvarez and Wendel 2003). Given these tests, it is likely that inadequate sampling as well as stochastic error and systematic error can be ruled out, indicating a biological factor for the observed incongruence.

As a single genetic change in a regulatory region can cause dramatic morphological transformations, which typically are unaccompanied by similar levels of molecular divergence, and the processes that drive molecular divergence can lag behind the phenotypic divergence, the result will be incongruence between molecular phylogenies and morphology (reviewed in Wendel and Doyle 1998). While, data from ITS sequences and three chloroplast sequences provide neither resolved topologies nor congruent hypotheses about species-level relationships in the *Anguinum* eastern Asia geographical group, it is possible that other loci or genomic elements that are more rapidly evolving may provide phylogenetic resolution for this group.

Given the nature of the data, however, it is most probable that incomplete lineage sorting and hybridization/introgression are the main contributing factors to the conflicts found among sequences for the *Anguinum* eastern Asia geographical group. The incongruence occurs between gene trees and morphology-based taxonomies, and between trees from plastid sequences and nuclear ribosomal sequences. These findings are consistent with other studies in *Allium* subgen. *Melanocrommyum* (Gurushidze et al. 2010) and other plant genera (e. g., Dobes et al. 2004; Jakob and Blattner 2006; Vargas et al. 2009) indicating that the combined effects of incomplete lineage sorting and hybridization/introgression can obscure organismal-level relationships in a phylogenetic framework. Incomplete lineage sorting (ILS) is the persistence of ancestral polymorphisms through speciation events; with time and subsequent extinction of gene lineages, descendant populations will have randomly sorted separate nuclear and organellar sequences (Wendel and Doyle 1998; Avise 2000). ILS is especially likely when species rapidly radiate and population sizes are large (Maddison 1997; Sang 2002; Funk and Omland 2003). A consequence of incomplete lineage sorting is that species will appear to be non-monophyletic. The ITS tree indicates that, *A. nanodes* is monophyletic, while *A. ovalifolium* var. *ovalifolium*, *A. ovalifolium* var. *leucuneurum*, and *A. prattii* are resolved as non-monophyletic. Evidence suggests that nuclear genes move across hybrid boundaries less freely than organellar genes (Rieseberg and Wendel 1993;...
Soltis and Soltis 1995; Hardig et al. 2000), so we conclude that incomplete lineage sorting could most likely account for the difference in the plastid tree vs. the nuclear ITS tree for this group. It cannot be ruled out from these data, however, that ancient reticulation has occurred in the past. During glacial periods in the Quaternary, previously isolated populations of the Anguinum eastern Asian geographical group may have come into contact, allowing hybridisation and introgression, resulting in species non-monophyly as evidenced by the DNA data presented here. The combined plastid DNA tree indicated that A. nanodes, A. ovalifolium var. ovalifolium, A. ovalifolium var. leuconeurum, and A. pratii are non-monophyletic, while accessions of partial populations of A. ovalifolium var. ovalifolium, A. ovalifolium var. cordifolium, and A. pratii are clustered together (Fig. 2, node c), and accessions of partial populations of A. ovalifolium var. ovalifolium, A. ovalifolium var. leuconeurum, and A. pratii are clustered together (Fig. 2, node d). Above evidence suggests that, the lineage sorting process is either not finished, or that some hybridization is still going on. The coalescence of organelle DNA is four times faster than nuclear genes (Moore 1995), and therefore it is unlikely that the lineage sorting for nuclear genes had been completed if lineage sorting for chloroplast genes is not yet complete. Overall, we propose that the process of lineage sorting is ongoing for both nuclear ribosomal and chloroplast genes in the Anguinum eastern Asian geographical group, and any lineage sorting for A. nanodes may have been completed, while this process for the other taxa is not yet complete.

Incongruence between phylogenies inferred from nuclear and chloroplast regions, is partially interpreted as being a result of incomplete lineage sorting. However, the incomplete lineage sorting is not the only process that could produce such incongruence. Incongruence between nuclear and chloroplast data, and the sharing of chloroplast haplotypes between species, are also usually interpreted as being a result of hybridization/introgression which results in the chloroplast genome of one species being replaced by that of another species, and such reticulation is easily reflected in the form of cpDNA/nuclear gene tree incongruencies (Anderson 1949; Soltis and Soltis 1995; Rautenberg et al. 2010). By means of molecular markers, ‘footprint’ of hybridization/introgression in the Anguinum eastern Asian geographical group can be found. A prominent example is phenomenon in A. nanodes. Lineage sorting for ITS in this species has been completed (Fig. 1), but in chloroplast tree (Fig. 2), A. nanodes 1 and A. nanodes 2 are not clustered together. A. nanodes 1 is clustered together with multiple populations of A. pratii (Fig. 2, node a) and is the sister group with A. pratii 11 from close localities (Fig. 2, node b), which fully demonstrate that after complete lineage sorting, subsequent hybridization or introgression occurred, and the chloroplast genome of A. nanodes 1 was replaced by that of A. pratii from close localities. It is thus possible that currently delimited morphospecies had insufficient time to develop reproductive barriers, thus promoting hybridisation/introgression. Previous studies (Jing et al. 1999; Xu and Kamelin 2000) suggested that polyploids existed in Allium ovalifolium var. ovalifolium (2n = 16, 24) and A. pratii (2n = 16, 32), which perhaps also imply hybridization/introgression exists in the Anguinum eastern Asian geographical group. While it may be possible to detect hybridization/introgression by means of multiple base calls in ITS sequence electropherograms (Nayes 2006; Carine et al. 2007), the homogenous nature of the ITS data we obtain means that detecting hybridization/introgression using this approach is not possible. Young species groups in particular should be affected by incomplete lineage sorting while its influence should decrease with increasing time due to the gradual loss of polymorphisms and fixation of lineage-specific alleles (Maddison 1997; Wendel and Doyle 1998). As hybridization/introgression also occurs mostly between young and reproductively not completely isolated species (Comes and Abbott 2001; Ramdhani et al. 2009), incomplete lineage sorting and hybridization/introgression may not be mutually exclusive. It is much more difficult to distinguish between incomplete lineage sorting and hybridization/introgression because these processes can produce almost identical outputs at the level of tree discordances (Wendel and Doyle 1998; Ramdhani et al. 2009; Lu et al. 2010). With the existing information it is impossible to distinguish the relative impact of incomplete lineage sorting vs. hybridization/introgression in the Anguinum eastern Asian geographical group.

A hypothesis of the evolutionary history of anguinum

Our analyses revealed that the crown group of Anguinum originated during the late Miocene (ca. 7.16 Mya), and eastern Asia was the ancestral area for Anguinum (node a), where it underwent duplication, and gave rise to two different lineages. during the Mid-Pliocene (ca. 3.64 Mya), one of them, the ancestor of the Eurasian-American alliance clade began to diverge (node b), one descendant probably dispersed to the northeastern North America by the BLB or by the long distance dispersal and diverged into two varieties (A. tri-coccum var. tricoccum and A. tricoccum var. burdickii); the other descendant stayed in eastern Asia (node c) and began to diverge at ca. 3.09 Mya, in which A.
ochotense is the earliest-branching species and gradually dispersed to western North America (Attu Island) and Siberia—while ancestor of the remaining species (A. listera, A. microdictyon, and A. victorialis) originated at the Mid-Pleistocene (ca. 0.98 Mya), in which A. victorialis is the early-branching species and gradually dispersed to Europe and A. microdictyon gradually dispersed to Siberia and Central Asia. Compared to the origin of the Eurasian-American alliance clade, the origin of the eastern Asian alliance (eastern Himalaya and areas south of the Qinlin Mountains of China) clade (node d) is a relatively recent event, which began to diverge at the early Pleistocene (ca. 1.56 Mya), and A. nanodes originated at the late Pleistocene (ca. 0.14 Mya). Geological estimates date the start of the uplift of the Himalayas and Hengduan Mountains unequivocally within the Late Tertiary/mid-Miocene (ca. 15–10 Ma; Royden et al. 2008), and episodes of uplift probably continued throughout the Late Pliocene (ca. 3 Ma) and well into the Quaternary (Li et al. 1979; Zhou et al. 2006). Severe climatic oscillations had dramatic effects on the evolution and distribution of plants in this region (Sun 2002; Qiu et al. 2011). It is inferred that during the late Pliocene/Early Pleistocene, with cooling climates and the uplift of the Himalayas and Hengduan Mountains, the ancestor of the Anguinum eastern Asian lineage underwent a very recent radiation, and gave rise to several closely related species constituting A. ovalifolium var. ovalifolium, A. ovalifolium var. cordifolium, A. ovalifolium var. leuconeurum, A. funckiifolium, A. nanodes, and A. prattii. Recent rapid radiations could result in morphospecies appearing non-monophyletic in DNA-based studies (reviewed by Seehausen 2004; Ramdhan et al. 2009, Lu et al. 2010), as we have found in the Anguinum eastern Asian lineage. These taxa may have had insufficient time to develop reproductive barriers following recent radiation, thus promoting hybridization (Ramdhan et al. 2009), and also insufficient time to finish the lineage sorting process. Due to rapid radiation, phylogenetically inferred internodes on gene trees may be short and difficult to resolve with confidence. This short interior branch phenomenon may be also a common cause of phylogenetic incongruence (Wendel and Doyle 1998). Hybridization appears to facilitate the ability of some plant species to adapt to or evolve in response to changes in climate (Bradshaw and McNeilly 1991). It is inferred that, hybridization/introgression in the Anguinum eastern Asian lineage could be promoted by the ecological heterogeneity and rapidly changing environment resulting from the intense uplift of the Himalaya, resulting in rampant species non-monophyly, and also partially explaining the incongruence between ITS tree and cpDNA tree.

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Contributions by the Authors
Qin-Qin Li conceived the idea and performed the experiment, and then wrote the manuscript. Xian-Qin Wei conducted 1 years of fieldwork. De-Qing Huang made valuable comments on the manuscript. Xing-Jin He and Song-Dong Zhou made valuable comments on the manuscript and provided technical support in the experiments.

Conflicts of Interest Statement
None declared.

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Supporting Information
The following additional information is available in the online version of this article —

Appendix S1. Anguinum material of 50 samples plus two species from Caloscordum (A. neriniflorum, A. tubiflorum), with corresponding locality, voucher information, GenBank reference numbers.

Appendix S2. Taxa, and GenBank accession numbers for all rbcL sequences used in the present study.

Appendix S3. Taxa, references, and GenBank accession numbers for all ITS sequences used in the Anguinum divergence time estimation.

Literature Cited
Aduse-Poku K, Vingerhoedt E, Wahlberg N. 2009. Out-of-Africa again: A phylogenetic hypothesis of the genus Charaxes (Lepidoptera: Nymphalidae) based on five gene regions. Molecular Phylogenetics and Evolution 53:463–478.

Álvarez I, Wendel JF. 2003. Ribosomal ITS sequences and plant phylogenetic inference. Molecular Phylogenetics and Evolution 29:417–430.
Andersen E. 1949. Introggressive hybridization. New York: John Wiley.

APG III 2009. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. Botanical Journal of the Linnean Society 161:105–121.

Bradshaw AD, McNeilll T. 1991. Evolutionary response to global climate change. Annals of Botany 67:5–14.

Bremer K. 2000. Early Cretaceous lineages of monocot flowering plants. Proceedings of the National Academy of Sciences, USA 97:4707–4711.

Carine MA, Robba L, Little R, Russel S, Guerra AS. 2007. Molecular and morphological evidence for hybridization between endemic Canary Island Convulvus. Botanical Journal of the Linnean Society 154:187–204.

Chase MW. 2004. Monocot relationships: an overview. American Journal of Botany 91:1645–1655.

Chase MV, Reveal JL, Fay MF. 2009. A subfamilial classification for the expanded asparagalean families Amaryllidaceae, Asparagaceae and Xanthorrhoeaceae. Botanical Journal of the Linnean Society 161:132–136.

Choi HJ, Oh BU. 2011. A partial revision of Allium (Amaryllidaceae) in Korea and north-eastern China. Botanical Journal of the Linnean Society 167:153–211, with 27 figures.

Choi HJ, Giussani LM, Jang CG, Oh BU, Cota-Sánchez JH. 2012. Systematics of disjunct northeastern Asian and northern North American Allium (Amaryllidaceae). Botany 90:491–508.

Comes HP, Abbott RJ. 2001. Molecular phylogeography, reticulation, and lineage sorting in Mediterranean Senecio sect. Senecio (Asteraceae). Evolution 55:1943–1962.

Couper RA. 1960. New Zealand Mesozoic and Cainozoic plant micro-fossils. New Zealand Geological Survey Paleontological Bulletin 32:1–87.

Crepet W, Nixon K, Gandolfo MA. 2004. Fossil evidence and phylogeny: the age of major angiosperm clades based on mesofossil and macrofossil evidence from Cretaceous deposits. American Journal of Botany 91:1666–1682.

Cunningham C. 1997. Can three incongruence tests predict when data should be combined? Molecular Biology and Evolution 14:733–740.

Dale W, McNeal JR, Jacobsen TD. 2002. Allium. In: Kiger E, ed. Flora of North America, Vol. 26. Oxford: Oxford University Press, 244–275.

Davis JJ, Stevenson DW, Petersen G, Seeger O, Campbell LM, Breedenstein JV, Goldman DH, Hardy CR, Michangelii FA, Simmons MP, Specht CD, Vergara-Silva F, Gandolfo M. 2004. A phylogeny of the monocots, as inferred from rbcl and atpA sequence variation, and a comparison of methods for calculating jackknife and bootstrap values. Systematic Botany 29:467–510.

Dobes C, Mitchell-Olds T, Koch M. 2004. Extensive chloroplast haplotype variation indicates Pleistocene hybridization and radiation of North American Arabis drummondii, A. × divaricarpa, and A. holboellii (Brassicaceae). Molecular Ecology 13:349–370.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bulletin, Botanical Society of America 19:11–15.

Drummond AJ, Ho SYW, Phillips MJ, Rambaut A. 2006. Relaxed phylogenetics and dating with confidence. PLoS Biology 4:699–710.

Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7:214.

Druselmann S. 1992. Vergleichende Untersuchungen an Vertretern der Alliaceae Agardh. 1. Morphologie der Keimpflanzen der Gattung Allium L. Flora, Morphologie, Geobotanik, Oekophysiologie 186:37–52.

Farris J. 1989. The retention index and the rescaled consistency index. Cladistics 5:417–419.

Farris JS, Källersjö M, Kluge AG, Bult C. 1994. Testing significance of incongruence. Cladistics 10:315–319.

Fay MF, Chase MW. 1996. Resurrection of Themiadaceae for the Brodiaea alliance, and recircumscription of Alliaceae, Amaryllidaceae and Agapanthoideae. Taxon 45:441–451.

Fay MF, Rudall PJ, Sullivan S, Stobart KL, de Brujin AY, Reeves G, Qamaruze-Zaman F, Hong WP, Joseph J, Hahn WJ, Conran JG, Chase MW. 2000. Phylogenetic studies of Asparagales based on four plastid DNA loci. In: Wilson KL, Morrison DA, eds. Monocots – Systematics and Evolution, Vol. 1. Melbourne: CSIRO, 360–371.

Fehr J, Gemeinholzer B, Chrtek J Jr, Bräutigam S. 2007. Incongruent plastid and nuclear DNA phylogenies reveal ancient intergeneric hybridization in Pilosella hawkweeds (Hieracium, Cichorieae, Asteraceae). Molecular Phylogenetics and Evolution 42:347–361.

Felsenstein J. 1988. Phylogenies from molecular sequences: inference and reliability. Annual Review of Genetics 22:521–565.

Friesen N, Fritsch RM, Pollner S, Blattner FR. 2000. Molecular and morphological evidence for an origin of the aberrant genus Milula within Himalayan species of Allium (Alliaceae). Molecular Phylogenetics and Evolution 17:209–218.

Friesen N, Fritsch RM, Blattner FR. 2006. Phylogeny and new infrageneric classification of Allium (Alliaceae) based on nuclear ribosomal DNA ITS sequences. Aliso 22:372–395.

Fritsch RM. 1992a. Zur Wurzelanatomie in der Gattung Allium L. (Alliaceae). Beitraege Zur Biologie Der Pflanzen 67:129–160.

Fritsch RM. 1992b. Septal nectaries in the genus Allium L. In: Hanelt P, Hammer K, Knüppfer H, eds. The genus Allium: taxonomic problems and genetic resources. Proceedings of an international symposium held at Gatersleben, Germany, 11–13 Jun 1991. Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany, 77–85.

Fritsch RM. 2001. Taxonomy of the genus Allium: Contribution from IPK Gatersleben. Herbaria 56:19–50.

Fritsch RM, Friesen N. 2002. Evolution, domestication and taxon. In: Robinowitch HD, Currah L, eds. Allium crop science: recent advances. Wallingford, UK: CABI Publishing, 5–30.

Funk DJ, Omland KE. 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. Annual Review of Ecology, Evolution, and Systematics 34:397–423.

Gladenkov AY, Oleinik AE, Marincovich L, Barinov KB. 2002. A refined mitochondrial DNA. Annual Review of Ecology, Evolution, and Systematics 34:397–423.

Good-Avila SV, Souza V, S Gaut B., Eguiarte LE, 2006. Timing and rate of speciation in Agave (Agavaceae). Proceedings of the National Academy of Sciences of the United States of America 103:9124–9129.

Gurushidze M, Fritsch RM, Blattner FR. 2008. Phylogenetic analysis of Allium subg. Melanocormyrum infers cryptic species and
demands a new sectional classification. Molecular Phylogenetics and Evolution 49:997–1007.

Gurushidze M, Fritsch RM, Blattner FR. 2010. Species-level phylogeny of Allium subgenus Melanocormyrum: incomplete lineage sorting, hybridization and trnF gene duplication. Taxon 59: 829–840.

Hanlet P. 1992. Ovule number and seed weight in the genus Allium L. In: Hanlet P, Hammer K, Knüppf er H, eds. The genus Allium: taxonomic problems and genetic resources. Proceedings of an international symposium held at Gatersleben, Germany, 11–13 Jun 1991. Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany, 99–105.

Hamilton MB. 1999. Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. Molecular Ecology 8:521–523.

Hardig TM, Solits PS, Solits DE. 2000. Diversification of the North American shrub genus Geoanthus (Rhamnaceae): conflicting phylogenies from nuclear ribosomal DNA and chloroplast DNA. American Journal of Botany 87:108–123.

Herendeen PS, Crane PR. 1995. The fossil history of the monocotyledons. In: Rudall PJ, Cribb PJ, Cutler DF, Humphries CJ, eds. Monocotyledons: systematics and evolution. Kew: Royal Botanic Gardens, 570.

Hewitt GM. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Botanical Journal of the Linnean Society 88:247–276.

Hewitt GM. 2000. The genetic legacy of the Quaternary ice ages. Nature 405:907–913.

Hewitt GM. 2004. Genetic consequences of climatic oscillations in the Quaternary. Philosophical Transactions of the Royal Society of London B Biological Sciences 359:183–195.

Jakob SS, Blattner FR. 2006. A chloroplast genealogy of Hordeum (Poaceae): Long-term persisting haplotypes, incomplete lineage sorting, regional extinction, and the consequences for phylogenetic inference. Molecular Biology and Evolution 23: 1602–1612.

Janssen T, Bremer K. 2004. The age of major monocot groups inferred from 800-rbcL sequences. Botanical Journal of the Linnean Society 146:385–398.

Jeanninou F, Thompson JD, Gouy M, Higgins DG, Gibson TJ. 1998. Multiple sequence alignment with Clustal X. Trends in Biochemical Sciences 23:403–405.

Jing WC, Xu JM, Yang L. 1999. A study on cytotaxonomy of Sect. Anguinum of Allium. Acta Phytotaxonomica Sinica 37:20–34.

Kim ST, Donoghue MJ. 2008. Incongruence between cpDNA and mrTS trees indicates extensive hybridization within Eupersicaria (Polygonaceae). American Journal of Botany 95:1122–1135.

Kim JH, Kim DK, Forest F, Fay MF, Chase MW. 2010. Molecular phylogenetics of Ruscaceae sensu lato and related families (Asparagales) based on plastid and nuclear DNA sequences. Annals of Botany 106:775–790.

Kimura M. 1980. A simple method for estimating evolutionary rates. Evolutionary Biology 16:111–120.

Kluge A, Farris J. 1969. Quantitative phylectics and the evolution of anurans. Systematic Zoology 18:1–32.

Kruse J. 1984. Rasterelektronenmikroskopische Untersuchungen an Samen der Gattung Allium L. Kulturpflanze 32:89–101.

Kruse J. 1988. Rasterelektronenmikroskopische Untersuchungen an Samen der Gattung Allium L. III. Kulturpflanze 36:355–368.

Leebens-Mack J, Rauson LA, Cui L, Kuehl JV, Fourcade MH, Chumley TW, Boone JL, Jansen RK, de Pamphilis CW. 2005. Identifying the basal angiosperm node in chloroplast genome phylogenies: sampling one’s way out of the Felsenstein zone. Molecular Biology and Evolution 22:1948–1963.

Li QQ, Zhou SD, He XJ, Yu Y, Zhang YC, Wei XQ. 2010. Phylogeny and biogeography of Allium (Amaryllidaceae: Allieae) based on nuclear ribosomal internal transcribed spacer and chloroplast rps16 sequences, focusing on the inclusion of species endemic to China. Annals of Botany 106:709–733.

Li JJ, Wen SX, Zhang QS, Wang FB, Zheng BX, Li BY. 1979. A discussion on the period, amplitude and type of the uplift of the Qinghai–Xizang Plateau. Scientia Sinica 22:1314–1328.

Lu L, Fritsch PW, Cruz BC, Wang H, Li DZ. 2010. Reticulate evolution, cryptic species, and character convergence in the core East Asian clade of Gautheria (Ericaceae). Molecular Phylogenetics and Evolution 57:364–379.

Maddison WP. 1997. Gene trees in species systems. Systematic Biology 46:523–536.

Magallón S, Castilla A. 2009. Angiosperm diversification through time. American Journal of Botany 96:349–365.

Marazzi B, Endress PK, de Queiroz LP, Conli E. 2006. Phylogenetic relationships within Senna (Leguminosae, Cassiinae) based on three chloroplast DNA regions: patterns in the evolution of floral symmetry and extrafloral nectarines. American Journal of Botany 93:288–303.

Meng Y, Wen J, Nie ZL, Sun H, Yang YP. 2008. Phylogeny and biogeographic diversification of Maianthemum (Ruscaceae: Polygonatae). Molecular Phylogenetics and Evolution 49: 424–434.

Mes THM, Friesen N, Fritsch RM, Kloas M, Bachmann K. 1997. Criteria for sampling in Allium based on chloroplast DNA PCR-RFLPs. Systematic Botany 22:701–712.

Milne RJ, Abbott RJ. 2002. The origin and evolution of Tertiary relict floras. Advances in Botanical Research 38:281–314.

Milne RJ. 2006. Northern hemisphere plant disjunctions: a window on Tertiary land bridges and climate change? Annals of Botany 98:465–472.

Mitchell JM. 1976. An overview of climatic variability and its causal mechanisms. Quaternary Research 4:481–493.

Moore WS. 1995. Inferring phylogenies from mtDNA variation: Mitochondrial-gene trees versus nuclear-gene trees. Evolution 49:718–726.

Muller J. 1981. Fossil pollen of extant angiosperms. Botanical Review 47:1–142.

Müller KF, Borsch T, Hilu KW. 2006. Phylogenetic utility of rapidly evolving DNA at high taxonomical levels: contrasting matK, trnF, and rbcL in basal angiosperms. Molecular Phylogenetics and Evolution 41:99–117.

Nguyen NH, Driscoll HE, Specht CD. 2008. A molecular phylogeny of the wild onions (Alliaceae) with a focus on the western North American center of diversity. Systematic Botany 33:1157–1172.

Nie ZL, Sun H, Beardsley PM, Olmstead RG, Wen J. 2006. Evolution of biogeographic disjunction between eastern Asia and eastern North America in Phryma (Phrymaceae). American Journal of Botany 93:1343–1356.
Noyes RD. 2006. Intraspecific nuclear ribosomal DNA divergence and reticulation in sexual diploid Erigeron strigosus (Asteraceae). American Journal Botany 93:470–479.

Nylander JAA. 2004. MrModeltest, version 2.2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden (http://www.abcc.se/~nylander/)

Oxelman B, Liden M, Berglund D. 1997. Chloroplast rpl16 intron phylogeny of the tribe Sileneae (Caryophyllaceae). Plant Systematics and Evolution 206:393–410.

Pastor J, Valdes B. 1985. Bulb structure in some species of Allium (Liliaceae) of Iberian Peninsula. Annales Musei Goul andrae 7:249–261.

Pelser PB, Kennedy AH, Tepe EJ, Shidler JB, Nordenstam B, Kaderet JW, Watson LE. 2010. Patterns and causes of incongruence between plastid and nuclear Seneconideae (Asteraceae) phylogenies. American Journal of Botany 97:856–873.

Pistrik K. 1992: Phenological variability in the genus Allium L. In: Ramdhani S, Barker NP, Baijnath H. 2009. Rampant non-monophyly. Rambaut A, Drummond AJ. 2007. Tracer v 1.4, Available from: Rambaut A. 2009. FigTree ver. 1.3.1. Available at: http://tree.bio.ed.ac.uk/software/figtree.

Rambaut A, Drummond AJ. 2007. Tracer v 1.4, Available from: http://beast.bio.ed.ac.uk/Tracer.

Ramdhani S, Barker NP, Bajnath H. 2009. RAMPANT non-monophyly of species in Kniphofia Moench (Asphodelaceae) suggests a recent Afromontane radiation. Taxon 58:1141–1152.

Rautenberg A, Hathaway L, Oxelman B, Prentice HC. 2010. Geographic and phylogenetic patterns in Silene section Melandrium (Caryophyllaceae) as inferred from chloroplast and nuclear DNA sequences. Molecular Phylogenetics and Evolution 57:978–991.

Ree RH, Moore BR, Webb CO, Donoghue MJ. 2005. A likelihood framework for inferring the evolution of geographic range on phylogenetic trees. Evolution 59:2299–2311.

Ree RH, Smith SA. 2008. Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis. Systematic Biology 57:4–14.

Riesenberg LH, Wendel JF. 1993. Intronpression and its consequences in plants. In: Harrison RG, ed. Hybrid zones and the evolutionary process. New York: Oxford University Press, 70–109.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference using MrModeltest (*and other methods), version 4.0.b10. Sinauer Associates, Sunderland, Massachusetts, USA.

Soltis PS, Soltis DE. 1995. Plant molecular systematics: inferences of phylogeny and evolutionary processes. Evolution 28:139–194.

Sun H. 2002. Evolution of Arctic-Tertiary Flora in Himalayan-Hengduan Mountains. Acta Botanica Yunnanica 24:671–688.

Swofford DL. 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sinauer Associates, Sunderland, Massachusetts, USA.

Sun H. 2002. Evolution of Arctic-Tertiary Flora in Himalayan-Hengduan Mountains. Acta Botanica Yunnanica 24:671–688.

Tuffney BH. 1985a. Perspectives on the origin of the floristic similarity between eastern Asia and Northern America. Journal of the Arnold Arboretum 66:73–94.

Tuffney BH. 1985b. The Eocene North Atlantic Land Bridge: its importance in tertiary and modern phytogeography of the Northern Hemisphere. Journal of the Arnold Arboretum 66:243–273.

Tuffney BH. 2000. Geographic and climatic influences on the Cretaceous and Tertiary history of Euramerican floristic similarity. Acta Universitatis Carolinae Geologica 44:5–16.

Tuffney BH, Manchester SR. 2001. The use of geological and paleontological evidence in evaluating plant phylogeographic hypotheses in the North Hemisphere Tertiary. International Journal of Plant Sciences 162:53–517.

van Donk J. 1976. O18 of the Atlantic Ocean for the entire Pleistocene Epoch Memior. Geological Society of America 145:147–164.

Sanmartin I. 2007. Event-based biogeography: integrating patterns, processes, and time. In: Ebach MC, Tanger JS, eds. Biogeography in a Changing World. Oxon, UK: CRC Press, 135–159.

Sanmartin I, Van Der Mark P, Ronquist F. 2008. Inferring dispersal: a Bayesian approach to phylogeny-based island biogeography, with special reference to the Canary Islands. Journal of Biogeography 35:428–449.

Seehausen O. 2004. Hybridization and adaptive radiation. Trends in Ecology and Evolution 19:198–207.

Seregin AP, Anackov G, Friesen N. 2015. Molecular and morphological revision of the Allium saxatile group (Amaryllidaceae): geographical isolation as the driving force of underestimated speciation. Botanical Journal of the Linnean Society 178:67–101, with 10 figures.

Smiley TH, Bryson RA, King JE, Kukia GJ, Smith GI. 1991. Quaternary paleoclimates. In: Morrison RB, ed. Quaternary nonglacial geology: conterminous U.S. Geological Society of America, Boulder, Colorado, 13–44.

Soltis PS, Soltis DE. 1995. Plant molecular systematics: inferences of phylogeny and evolutionary processes. Evolution 28:139–194.

Sun H. 2002. Evolution of Arctic-Tertiary Flora in Himalayan-Hengduan Mountains. Acta Botanica Yunnanica 24:671–688.
Vargas P, Carrió E, Guzmán B, Amat E, Güemes J. 2009. A geographical pattern of Antirrhinum (Scrophulariaceae) speciation since the Pliocene based on plastid and nuclear DNA polymorphisms. *Journal of Biogeography* 36:1297–1312.

Vinnersten A, Bremer K. 2001. Age and biogeography of major clades in Liliales. *American Journal of Botany* 88:1695–1703.

Walker JD, Geissman JW. 2009. 2009 GSA Geologic Time Scale. *GSA Today* 19:60–61.

Waters JM, Rowe DL, Burridge CP, Wallis GP. 2010. Gene trees versus species trees: reassessing life-history evolution in a freshwater fish radiation. *Systematic Biology* 59:504–517.

Waterman RJ, Pauw A, Barraclough TG, Savolainen V. 2009. Pollinators underestimated: A molecular phylogeny reveals widespread floral convergence in oil-secreting orchids (sub-tribe Coryciinae) of the Cape of South Africa. *Molecular Phylogenetics and Evolution* 51:100–110.

Wen J. 1999. Evolution of eastern Asian and eastern North American disjunct distributions in flowering plants. *Annual Review of Ecology and Systematics* 30:421–455.

Wen J. 2000. Internal transcribed spacer phylogeny of the Asian and eastern North America disjunct *Aralia* sect. *Dimorphanthus* (Araliaceae) and its biogeographic implications. *International Journal of Plant Sciences* 161:959–966.

Wendel JF, Doyle JJ. 1998. Phylogenetic incongruence: window into genome history and molecular evolution. In: Soltis DE, Soltis PS, Doyle JJ, eds. *Molecular systematics of plants II: DNA sequencing*. Dordrecht: Kluwer, 265–296.

White TJ, Bruns TD, Lee SB, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, eds. *PCR protocols: a guide to methods and applications*. New Yorks: Academic Press, 315–321.

Whitfield JB, Lockhart PJ. 2007. Deciphering ancient rapid radiations. *Trends in Ecology and Evolution* 22: 258–265.

Wikström N, Savolainen V, Chase MW. 2001. Evolution of the angiosperms: calibrating the family tree. *Philosophical Transactions of the Royal Society of London B Biological Sciences* 268:2211–2220.

Xie L, Wagner WL, Ree RH, Berry PE, Wen J. 2009. Molecular phylogeny, divergence time estimates, and historical biogeography of Circaeae (Onagraceae) in the Northern Hemisphere. *Molecular Phylogenetics and Evolution* 53:995–1009.

Xu JM, Kamelin RV. 2000. *Allium* L. In: Wu ZY, Raven PH, eds. *Flora of China*, Vol. 24. Beijing: Science Press; St. Louis: Missouri Botanical Garden Press, 165–202.

Yu Y, Harris AJ, He XJ. 2010. S-DIVA (statistical dispersal-vicariance analysis): a tool for inferring biogeographic histories. *Molecular Phylogenetics and Evolution* 56:848–850.

Yu Y, Harris AJ, Blair C, He XJ. 2015. RASP (Reconstruct Ancestral State in Phylogenies): A tool for historical biogeography. *Molecular Phylogenetics and Evolution* 87:46–49.

Zachos J, Pagani M, Sloan L, Thomas E, Billups K. 2001. Trends, rhythms and aberrations in global climate 65 Ma to present. *Science* 292:686–693.

Zhou SZ, Wang XL, Wang J, Xu LB. 2006. A preliminary study on timing of the oldest Pleistocene glaciation in Qinghai–Tibetan Plateau. *Quaternary International* 154-155:44–51.