SHORT COMMUNICATION

Susumu Takamatsu · Sanae Matsuda

Estimation of molecular clocks for ITS and 28S rDNA in Erysiphales

Abstract Based on a comparative phylogenetic analysis of *Goloviomyces* and their host tribes of the Asteraceae, we speculate that *Golovinomyces* first acquired parasitism to the Asteraceae after migration of the family into the Northern Hemisphere and before the divergence of the tribe Carduaeae. The divergence time of the Carduaeae is estimated to be 25.2 Myr ago based on the molecular clock of *rbcL* sequences of the Asteraceae. When 25.2 Myr is given at the node of the first split of the phylogenetic tree of *Golovinomyces*, nucleotide substitution rates of the Erysiphales are calculated to be 2.52×10^{-9} per site per year (0.01D = 3.97 Myr) in the ITS region and 6.5×10^{-10} per site per year (0.01D = 15.4 Myr) in the D1 and D2 regions of the 28S rDNA.

Key words Erysiphaceae · Evolution · *Golovinomyces* · Powdery mildew · Phylogeny

Estimation of timing of evolutionary events provides important information on the history of life on Earth. The only direct evidence for the age of a lineage is the fossil record. In the fungi, fossil records are extremely rare compared with those of animals or plants. In particular, there is no reliable fossil record of the Erysiphales (Tiffney and Barghoorn 1974; Pirozynski 1976; Braun 1987), which makes it almost impossible to estimate timing of evolutionary events by fossil records in this group of fungi. An alternative method for dating evolutionary events is the use of a molecular clock. The molecular clock approach has been used to provide independent estimates of divergence time for testing evolutionary hypotheses in many recent studies

(Baldwin and Sanderson 1998; Bremer and Gustafsson 1997; Francisco-Ortega et al. 1997; Kim et al. 1998; Kumar and Hedges 1998; Xiang et al. 1998, 2000). Mori et al. (2000b) estimated the divergence time of the Erysiphales based on molecular clocks (Berbee and Taylor 1993) and 18S rDNA sequences of Erysiphales. They reported that the first radiation of the Erysiphales occurred in the Cretaceous. However, dating of evolutionary events after the first radiation was difficult because the 18S rDNA region is too conserved to estimate timing of events that occurred recently. Calibration of molecular clocks using DNA sequences that have a more rapid substitution rate, such as variable regions of the 28S rDNA or the internal transcribed spacer (ITS) region, is required.

To estimate calibration points of molecular clocks, fossil records or geological events often have been used (Baldwin and Sanderson 1998; Berbee and Taylor 1993; Bremer and Gustafsson 1997; Kumar and Hedges 1998; Su et al. 1998; Xiang et al. 1998, 2000). A reliable fossil record, however, is not available in the Erysiphales, as already described. Geological events are useful for calibration of molecular clocks in organisms having low dispersal capacity, such as carabid ground beetles (Su et al. 1998). In the case of the Erysiphales, they can disperse long distances with airborne spores. Calibration of a molecular clock by geological events is thus not practical, although not impossible, in this group of fungi. The Erysiphales are a group of obligately parasitic fungi of plants. Their life cycle completely depends on living hosts, from which they obtain nutrients without killing the host cells and without which they are unable to survive. It is therefore expected that the association between the Erysiphales and their hosts would have been conserved during the course of their evolution (Braun 1987, 1995). If fungal lineages remain associated with their hosts over a long time, events that isolate the host populations may also isolate the populations of their associated fungi, which may eventually result in cospeciation of the parasites and their hosts. In this context, if there was any cospeciation between the Erysiphales and their hosts, it could provide a useful criterion to calibrate a molecular clock of the Erysiphales.

S. Takamatsu (✉) · S. Matsuda
Faculty of Bioresources, Mie University, 1515 Kamihama,
Tsu 514-8507, Japan
Tel. +81-59-231-9497; Fax +81-59-231-9540
e-mail: takamatu@bio.mie-u.ac.jp

In a previous study (Matsuda and Takamatsu 2003), we reported cospeciation between *Golovinomyces* (Erysiphaceae: Golovinomyceteae) and their host tribes of the Asteraceae. *Golovinomyces* has been reported to occur on 2283 plant species from 58 families (Amano 1986). More than half of the host species (1264 species) belong to the Asteraceae, especially to tribes distributed in the Northern Hemisphere such as the Cardueae, Astereae, Heliantheae, Anthemideae, and Lactuceae. Of the five tribes, *Golovinomyces* isolates from the Cardueae occupy the basal position in phylogenetic trees constructed from nucleotide sequences of the rDNA ITS region and the 28S rDNA. The Asteraceae is known to have its geographic origin in South America, and it then dispersed in the Northern Hemisphere (Bremer 1994). The tribe Cardueae is considered to be the first major group of the Asteraceae that dispersed throughout the Northern Hemisphere. From these results, we speculate that *Golovinomyces* first acquired parasitism to the Asteraceae after migration of the family into the Northern Hemisphere and before the divergence of the Carduaeae.

We used the *rbcL* gene for estimation of timing of the divergence of the Cardueae. Thirty-two *rbcL* sequences of the Asteraceae and its allies were obtained from the DDBJ DNA database. We performed the likelihood ratio test to check whether a molecular clock hypothesis is applicable for the data set, and 16 sequences with significantly long or short branches were removed from the data set. The remaining 16 sequences were used to construct an unweighted pair-group method with arithmetic averages (UPGMA) tree (Fig. 1) using PAUP* 4.0b10 (Swofford 2002). The uncorrected p-distance was used for calculation of pairwise genetic distances. The mean nucleotide substitution is 37.25 between *Carthamus* (Cardueae) and its sister taxa (*Stokesia*, *Vernonia*, *Gazania*, and *Tragopogon*). Based on the molecular clock of the *rbcL* gene [0.74 ± 0.17 nucleotide substitutions per million year (Myr)] reported by Bremer and Gustafsson (1997), the divergence time of the Cardueae is estimated to be 25.2 Myr ago. This result is consistent with the report of Kim et al. (1998) that the Asteraceae underwent an explosive radiation and dispersal during or near the Oligocene/Miocene transition.

We then constructed UPGMA trees using 21 ITS sequences (Fig. 2A) and 14 28S rDNA sequences (D1 and D2 regions, 800 nucleotide length) (Fig. 2B) of *Golovinomyces* species isolated from the Asteraceae. Kimura's two-parameter criterion (Kimura 1980) was used for calculation of pairwise genetic distances. *Arthrocladiella mougeotii* was used as the outgroup taxon. The likelihood ratio test could not reject a molecular clock hypothesis for both data sets. As reported previously (Matsuda and Takamatsu 2003), the first split within *Golovinomyces* occurred between the isolates parasitic to the Cardueae and isolates parasitic to other tribes of the Asteraceae in both the ITS and 28S trees. Mean genetic distances between Cardueae-parasitic isolates and other isolates of *Golovinomyces* were $12.68\% \pm 0.56\%$ in the ITS region and $3.27\% \pm 0.21\%$ in the D1 and D2 regions of the 28S rDNA. When 25.2 Myr is given at the node of the first split, nucleotide substitution rates are calculated to be $2.52 \pm 0.11 \times 10^{-9}$ per site per year (0.01D = 3.97 Myr) in the ITS region and $6.5 \pm 0.4 \times 10^{-10}$ per site per year (0.01D = 15.4 Myr) in the D1 and D2 regions of the 28S rDNA. This substitution rate at the ITS region agrees well with those of a wide range of plants (1.72–7.83×10^{-9}

Fig. 1. An unweighted pair-group method with arithmetic averages (UPGMA) tree of the Asteraceae constructed from nucleotide sequences of the *rbcL* gene. The uncorrected p-distance was used for calculating pairwise genetic distances. Based on the molecular clock of the *rbcL* gene (0.74 ± 0.17 nucleotide substitutions/Myr) reported by Bremer and Gustafsson (1997), the divergence time of the tribe Cardueae is estimated to be 25.2 Myr ago. Percent bootstrap support (1000 replications) is indicated *above nodes*
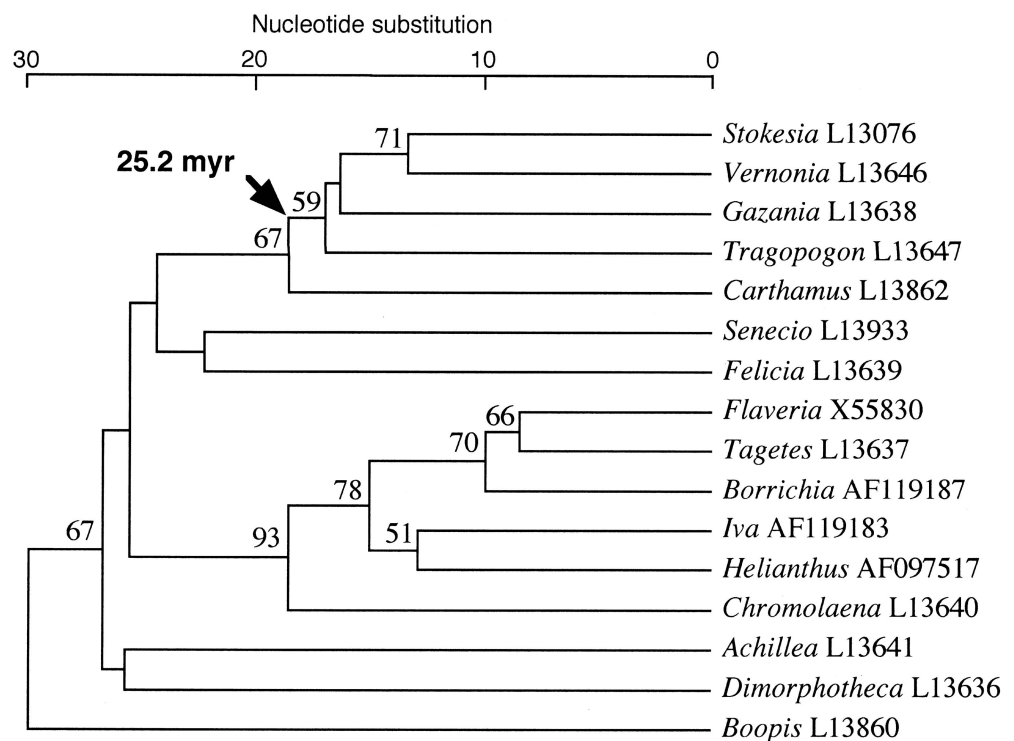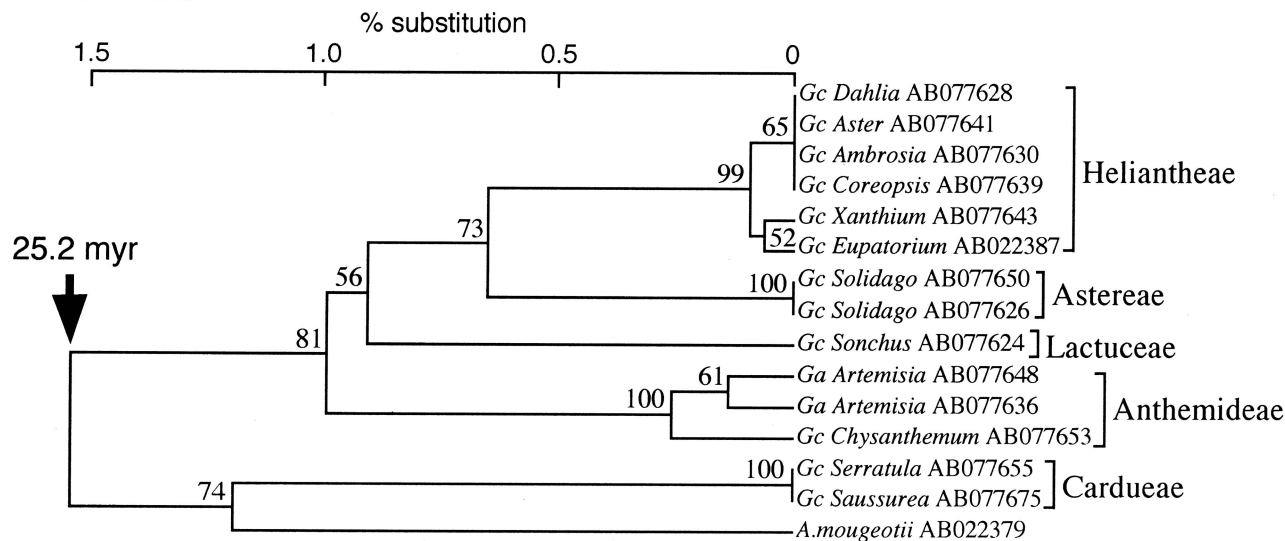
A. ITS tree
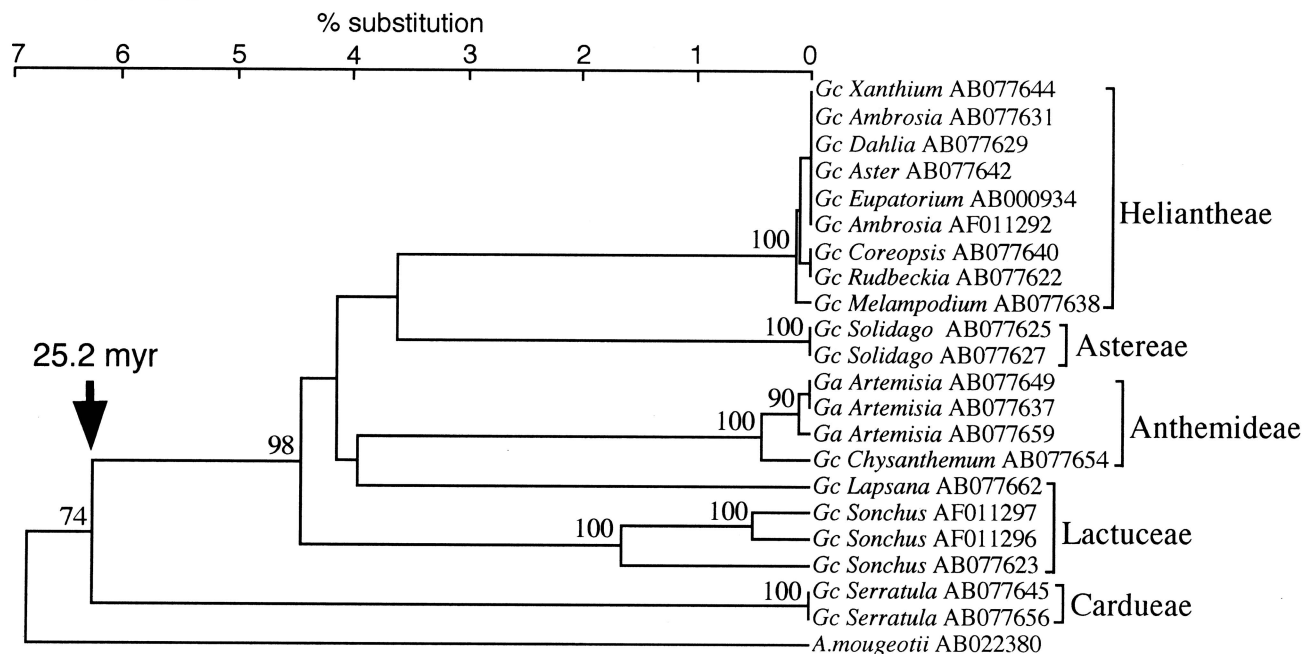
B. 28S tree

Fig. 2. UPGMA trees of *Golovinomyces* constructed from nucleotide sequences of the rDNA internal transcribed spacer (ITS) region (**A**) and the 28S rDNA (**B**). Kimura's two-parameter criterion (Kimura 1980) was used for calculating pairwise genetic distances. The first split within *Golovinomyces* is assumed to have occurred 25.2 Myr ago based on the result shown in Fig. 1. *Gc*, *Golovinomyces cichoracearum*; *Ga*, *Golovinomyces artemisiae*. Percent bootstrap support (1000 replications) is indicated *above nodes*

per site per year; Richardson et al. 2001) and is somewhat higher compared with the rate of Eurotiomycete fungi (Kasuga et al. 2002).

To evaluate the molecular clock of the 28S rDNA, we obtained 33 nucleotide sequences of the D1 and D2 regions of the 28S rDNA of the Erysiphales and its allies reported by Mori et al. (2000a) from the DDBJ database. Using the likelihood ratio test, we removed 9 sequences from the data set and used the remaining 23 sequences to construct a UPGMA tree by PAUP* (Fig. 3). Kimura's two-parameter criterion was used to calculate the pairwise genetic distances. The first split within the Erysiphales occurred between the Erysipheae/Golovinomyceteae/Phyllactinieae clade and the Cystotheceae/Blumerieae clade. The genetic distance between the clades is 9.11% ± 0.87%. When the molecular clock of the 28S rDNA calibrated in this report is
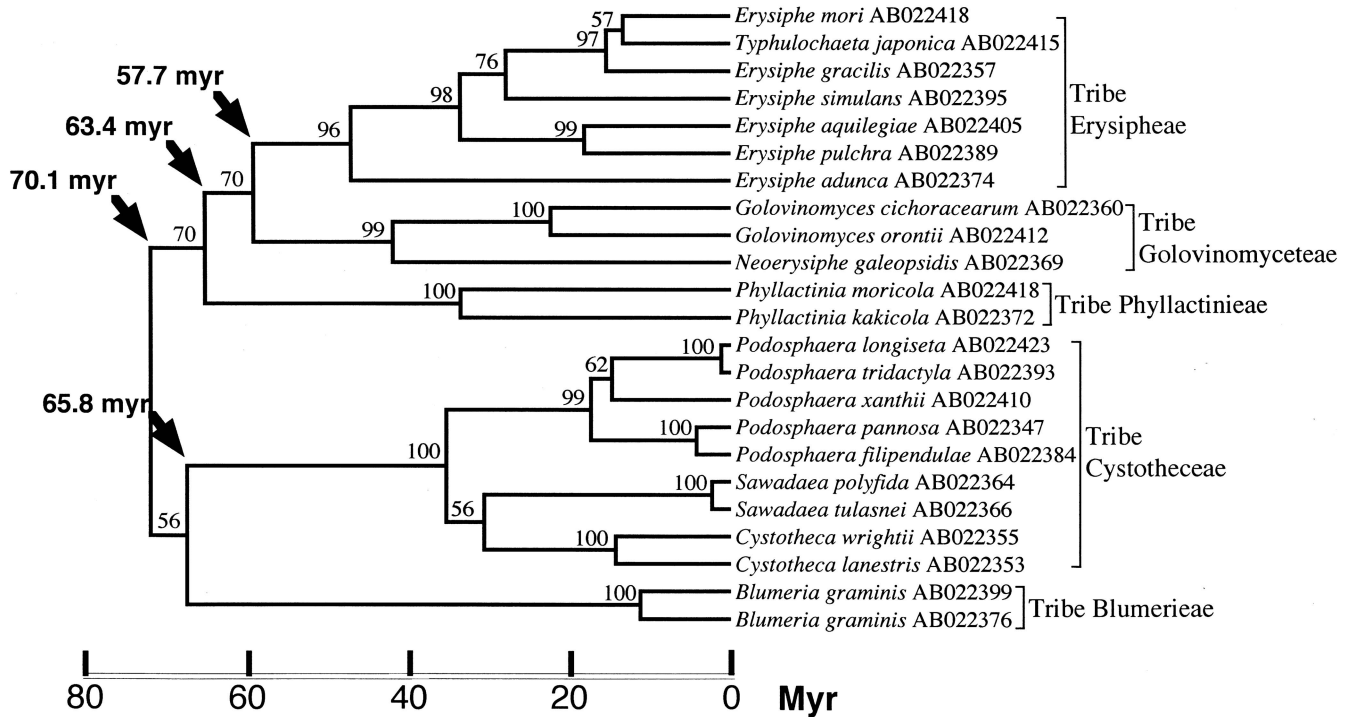
Fig. 3. An UPGMA tree of the Erysiphales constructed nucleotide sequences of the variable regions (D1 and D2) of the 28S rDNA. Kimura's two-parameter criterion (Kimura 1980) was used to calculate pairwise genetic distances. Divergence time of the tribes of the Erysiphales was calculated based on the molecular clock of the 28S rDNA estimated in this study and is shown at the respective node. Percent bootstrap support (1000 replications) is indicated *above nodes*

adopted in the calculation, timing of the first radiation within the Erysiphaceae is estimated to be 70.1 ± 6.7 Myr ago. Based on complete sequences of the 18S rDNA and the molecular clock (1%/100 Myr) reported by Berbee and Taylor (1993), Mori et al. (2000b) estimated the first radiation within the Erysiphales to be 92 Myr ago. Thereafter, Berbee and Taylor (2001) corrected the molecular clock to 1.26%/Myr based on a new fossil record (Taylor et al. 1999). When this new molecular clock is used for the calibration, the first radiation within the Erysiphales is calculated to be 73.4 Myr ago, which is congruent with the present calculation based on the molecular clock of the 28S rDNA sequences. Therefore, we concluded that the present molecular clock of the 28S rDNA is reliable enough to calculate timing of evolutionary events of the Erysiphales. We then estimated the timing of divergence of the five major tribes of the Erysiphales. The result shows that the radiation of the major tribes occurred within a short period near the Cretaceous/Tertiary boundary (Fig. 3).

References

Amano (Hirata) K (1986) Host range and geographical distribution of the powdery mildew fungi. Japan Scientific Societies, Tokyo

Baldwin BG, Sanderson MJ (1998) Age and rate of diversification of the Hawaiian silversword alliance (Compositae). Proc Natl Acad Sci USA 95:9402–9406

Berbee ML, Taylor JW (1993) Dating the evolutionary radiation of the true fungi. Can J Bot 71:1114–1127

Berbee ML, Taylor JW (2001) Fungal molecular evolution: gene trees and geologic time. In: McLaughlin DJ, Mclaughlin EG, Lemke PA (eds) The Mycota. VII. Systematics and evolution, part B. Springer, Berlin, pp 229–245

Braun U (1987) A monograph of the Erysiphales (powdery mildews). Beih Nova Hedwigia 89:1–700

Braun U (1995) The powdery mildews (Erysiphales) of Europe. Fischer, Jena

Bremer K (1994) Asteraceae: cladistics and classification. Timber, Portland

Bremer K, Gustafsson MHG (1997) East Gondwana ancestry of the sunflower alliance of families. Proc Natl Acad Sci USA 94:9188–9190

Francisco-Ortega J, Santos-Guerra A, Hines A, Jansen RK (1997) Molecular evidence for a Mediterranean origin of the Macaronesian endemic genus *Argyranthemum* (Asterceae). Am J Bot 84:1595–1613

Kasuga T, White TJ, Taylor JW (2002) Estimation of nucleotide substitution rates in Eurotiomycete fungi. Mol Biol Evol 19:2318–2324

Kim HG, Keeley SC, Vroom PS, Jansen RK (1998) Molecular evidence for an African origin of the Hawaiian endemic *Hesperomannia* (Asteraceae). Proc Natl Acad Sci USA 95:15440–15445

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kumar S, Hedges SB (1998) A molecular timescale for vertebrate evolution. Nature (Lond) 392:917–920

Matsuda S, Takamatsu S (2003) Evolution of host–parasite relationships of *Golovinomyces* (Ascomycete: Erysiphaceae) inferred from nuclear rDNA sequences. Mol Phylogenet Evol 27:314–327

Mori Y, Sato Y, Takamatsu S (2000a) Evolutionary analysis of the powdery mildew fungi (Erysiphales) using nucleotide sequences of the nuclear ribosomal DNA. Mycologia 92:74–93

Mori Y, Sato Y, Takamatsu S (2000b) Molecular phylogeny and radiation time of Erysiphales inferred from the nuclear ribosomal DNA sequences. Mycoscience 41:437–447

Pirozynski KA (1976) Fossil fungi. Annu Rev Phytopathol 14:237–246

Richardson JE, Pennington RT, Pennington TD, Hollingsworth PM (2001) Rapid diversification of a species-rich genus of neotropical rain forest trees. Science 293:2242–2245

Su ZH, Tominaga O, Okamoto M, Osawa S (1998) Origin and diversification of hindwingless *Damaster* ground beetles within the Japanese island as deduced from mitochondrial ND5 gene sequences (Coleoptera, Carabidae). Mol Biol Evol 15:1026–1039

Swofford DL (2002) PAUP*: phylogenetic analysis using parsimony (and other methods), 4.0b10. Sinauer, Sunderland, MA.

Taylor TN, Hass H, Kerp H (1999) The oldest fossil Ascomycetes. Nature (Lond) 399:648

Tiffney BH, Barghoorn ES (1974) The fossil record of the fungi. Occas Pap Farlow Herb Cryptogam Bot Harv 7:1–42

Xiang QY, Crawford DJ, Wolfe AD, Tang YC, DePamphilis CW (1998) Origin and biogeography of *Aesculus* L. (Hippocastanaceae): a molecular phylogenetic perspective. Evolution 52:988–997

Xiang QY, Soltis DE, Soltis PS, Manchester SR, Crawford DJ (2000) Timing the eastern Asian–eastern North American floristic disjunction: molecular clock corroborates paleontological estimates. Mol Phylogenet Evol 15:462–472