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Divergence time estimation in Cichorieae (Asteraceae) using a fossil-calibrated relaxed molecular clock

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Abstract Knowing the age of lineages is key to understanding their biogeographic history. We aimed to provide the best estimate of the age of Cichorieae and its subtribes based on available fossil evidence and DNA sequences and to interpret their biogeography in the light of Earth history. With more than 1,550 species, the chicory tribe (Cichorieae, Asteraceae) is distributed predominantly in the northern Hemisphere, with centres of distribution in the Mediterranean region, central Asia, and SW North America. Recently, a new phylogenetic hypothesis of Cichorieae based on ITS sequences has been established, shedding new light on phylogenetic relationships within the tribe, which had not been detected so far. Cichorieae possess echinolophate pollen grains, on the surface of which cavities (lacunae) are separated by ridges. These lacunae and ridges show patterns characteristic of certain groups within Cichorieae. Among the fossil record of echinolophate pollen, the *Cichorium intybus*-type is the most frequent and also the oldest type (22 to 28.4 million years old). By using an uncorrelated relaxed molecular clock approach, the Cichorieae phylogenetic tree was calibrated with this fossil find. According to the analysis, the tribe originated no later than Oligocene. The species-rich core group originated no later than Late Oligocene or Early Miocene and its subtribes diversified no later than Middle/Late Miocene or Early Pliocene—an eventful period of changing geological setting and climate in the Mediterranean region and Eurasia. The first dispersal from Eurasia to North America, which resulted in the radiation of genera and species in North America (subtribe Microseridinae), also occurred no later than Middle or Late Miocene, suggesting the Bering land bridge as the route of dispersal.

Keywords Bering land bridge · Lactuceae · Miocene · Oligocene · Pollen evolution · Uncorrelated relaxed molecular clock

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Introduction

Knowing the age of lineages at all levels in the hierarchy can be an important tool with which to discriminate between competing biogeographic hypotheses, relate cladogenesis of lineages to environmental changes, and understand the pace of morphological change. Generations of botanists have

tried to correlate phases of evolutionary expansion and phases of stasis of species with Earth events based on general considerations about the sequence of geological events that might have promoted expansion or stasis. An outstanding example is Babcock's (1947) work on *Crepis* (Asteraceae), which integrated distributional, geological, and paleobotanical data as well as vegetational history. Recently, approaches implementing relaxed molecular clocks on phylogenetic trees using fossils or geological vicariance for calibration have become available for dating nodes of interest. However, molecular clock estimates are often associated with several sources of error (Graur and Martin 2004). Constraining nodes in a phylogenetic tree by geological events risks circularity in biogeographic analyses, because it already assumes that those events caused the divergence, rather than testing temporal coincidence (Renner 2005). Nevertheless, by using a critical approach to molecular dating, this approach can be relevant for discriminating between biogeographic hypotheses (Renner 2005). Recent dating studies carried out in animals and plants span tetrapods (Hugall et al. 2007), lissamphibians (Marjanović and Laurin 2007), caviomorph rodents and platyrrhine primates (Poux et al. 2006), thoracican barnacles (Pérez-Losada et al. 2004), *Gnetum* (Won and Renner 2006), Leguminosae (Lavin et al. 2005), asterids (Bremer et al. 2004), and *Pistia* (Araceae; Renner and Zhang 2004).

The chicory or dandelion tribe (Cichorieae, Asteraceae) comprises more than 1,550 species distributed in ~100 morphologically defined genera (Bremer 1994). Cichorieae are predominantly northern Hemisphere plants, with concentrations of genera and species in the Mediterranean area, central Asia, and, to a lesser extent, SW North America (Bremer 1994). Most Cichorieae occur in dry or moderately humid areas and some inhabit mountains, but they are mostly absent from the humid tropics (Bremer 1994). Kilian et al. (2009) have extended the traditional delimitation of Cichorieae on the basis of molecular data to accommodate the genera *Gundelia* and *Warionia*, hitherto variously placed, e.g. as sister to Cichorieae in their own tribe Gundelieae (Karis et al. 2001; Panero and Funk 2002; Funk et al. 2004, 2005). Cichorieae sensu Kilian et al. (2009), together with the tribes Arctoteae, Liabeae, and Vernonieae, form the clade Cichorioideae s. str., which is believed to have an African origin (Funk et al. 2004, 2005). In a parsimony optimization analysis of the Asteraceae supertree, Funk et al. (2005) also infer a N African-Mediterranean origin of Cichorieae, from where they are thought to have repeatedly spread into Eurasia. Nested in Cichorieae is a monophyletic radiation in North America (Funk et al. 2005; Lee et al. 2003).

A phylogenetic tree constructed from internal transcribed spacer (ITS) sequences has been adopted as a guide for the new Cichorieae classification by Kilian et al. (2009). ITS sequences are known to be impacted by a number of molecular genetic processes in ways that may mislead phylogenetic inference (Álvarez and Wendel 2003). The main problem is the existence of paralogous loci in many plant genomes, which are derived from a duplication event (Álvarez and Wendel 2003). For example, Fehrer et al. (2007) have found incongruences in the topologies of the ITS tree and the chloroplast *matK* and *trnT–trnL* trees of *Pilosella* hawkweeds (Cichorieae), which suggest intergeneric hybridization events between ancestral lineages that resulted in cytoplasmic transfer (chloroplast capture; Rieseberg and Soltis 1991). Such events might have happened in other groups of Cichorieae as well. Because ITS is not affected by chloroplast capture, it should reflect true phylogenetic relationships better than a chloroplast sequence in the case of frequent hybridization, though concerted evolution is known to homogenize ITS copies, making them more similar to one or the other parent after hybridization (Álvarez and Wendel 2003; Baldwin et al. 1995). However, ITS provides the best hypothesis of phylogenetic relationships in Cichorieae at the moment. In many cases, previous taxonomic groupings have been confirmed by ITS (e.g. the *Scorzonera* alliance; Kilian et al. 2009). When different relationships are revealed, morphological, anatomical, or other evidence that corroborates the inferred relationships can often be found (e.g. the presence of oil ducts and latex canals in the roots of *Gundelia* and *Warionia*, which otherwise has been reported only from two Cichorieae genera; Kilian et al. 2009).

The aim of this study was to obtain the best estimate of the minimum age of the tribe Cichorieae and its subtribes, which, as a member of Asteraceae, represents one of the characteristic temperate plant groups, by employing the available fossil pollen record and ITS sequences. Concomitantly, we also explore the effect of topological uncertainties in dating. This study intends to provide a time framework for more detailed biogeographic analyses of the tribe and its subtribes and genera, such as the widely distributed *Hieracium*, *Lactuca*, *Sonchus* and *Taraxacum*, or genera with disjunct distributions in North America and Eurasia (e.g. *Askellia*, *Crepis*, *Nabalus*).

Materials and methods

DNA sequences

ITS sequences were generated according to the protocol described by Enke and Gemeinholzer (2008), using the primers ITS-A and ITS-C for ITS1 and ITS-D and ITS-B for ITS2 (Blattner 1999). The sequence of *Hypochaeris patagonica* was generated following the protocol described in Tremetsberger et al. (2005), i.e. using primers ITS4 and ITS5 (White et al. 1990), with modifications. Additional

sequences were extracted from EMBL/GenBank. In total, 49 ingroup sequences representing 49 species were included (see Appendix).

ITS1 and ITS2, but not the intermediately located conserved 5.8 S rDNA were used for phylogenetic analysis. Within each subtribe, we chose sequences of taxa in order to maximize the distance between them based on the study by Kilian et al. (2009), so that the deepest node for the most recent common ancestor (MRCA) of the respective subtribe would be obtained based on sequences of extant taxa. However, some genera of still doubtful affinities (e.g. *Prenanthes*, *Urospermum*, and *Robertia*) were not used. Within Hypochaeridinae, in which some of us have a specific interest, we sampled more taxa. For Crepidinae, we sampled two genera of the predominantly Asian group (*Heteracia* and *Soroseris*) and three genera of the predominantly Eurasian group (*Crepis*, *Rhagadiolus*, and *Taraxacum*; Kilian et al. 2009). As outgroup, we used taxa from throughout the Asteraceae. *Heterolepis* (Arctoteae) is the closest relative to Cichorieae out of the outgroup taxa chosen. Sequences were aligned with Clustal X (Thompson et al. 1997) and the alignment was examined and edited by hand in BioEdit ver. 7.0.5.3 (Hall 1999). Two regions in the loops of helices A and B of ITS2 (Goertzen et al. 2003) were removed due to alignment ambiguities. The final matrix (284 bp for ITS1; 192 bp for ITS2 without the two removed regions) is available from TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S11348). The best-fitting model of base substitution for ITS1 and ITS2—the general time reversible model with an estimated proportion of invariable sites and gamma distributed site-to-site rate variation (GTR+I+G)—was determined with PAUP* ver. 4.0b10 (Swofford 2003) and Modeltest ver. 3.7 (Posada and Crandell 1998) using the Akaike information criterion.

Fossil pollen record of Cichorieae

A literature survey was carried out to explore the utility of fossil pollen records of Cichorieae for molecular dating. Care was taken to ascertain correct systematic placement of fossil pollen finds. To estimate the first appearance of important finds, their abundance across periods in time was compiled.

The echinolophate pollen characteristic of, and attributed to, the tribe Cichorieae is found in the literature under different generic names, e.g. *Cichoreacidites* S. C. D. Sah, *Cichoraearumpollenites* Nagy and *Fenestrites* T. van der Hammen ex J. H. Germeraad, C. A. Hopping et J. Muller. According to Blackmore et al. (1986), however, *Fenestrites* pollen has a pattern of over 30 more or less isodiammetric lacunae characteristic of tribe Vernonieae, and should rather be attributed to this latter tribe. Pollen of the tribe Cichorieae is characterized by a smaller amount of lacunae (9–24 in Cichorieae vs 27–c. 40 in Vernonieae; Blackmore 1986). *Cichoreacidites* and *Cichoraearumpollenites* correspond to Blackmore et al.'s (1986) *Cichorium intybus*-type and in the following we merely refer to this latter type. Reports of Liguliflorae-type pollen without more detailed description and without accompanying photographs (e.g. from the Miocene of Spain; Rivas-Carballo et al. 1994) were not considered.

Following the nomenclature employed by Wodehouse (1935) and Blackmore (1984), the *Cichorium intybus*-type pollen has three poral lacunae, six abporal lacunae (three at each side of the equator), and six paraporal lacunae (three on each side of the equatorial ridge). Cichorieae exine stratification follows the pattern typical of Compositae in having a tripartite ektexine consisting of a foot layer, columellae, and tectum (Skvarla and Larson 1965). The ektexine of *Cichorium*-type pollen is made up of several layers of extremely fine branching columellae below the ridges, giving the ektexine a spongy appearance (Blackmore 1981). There are several reports of *Cichorium*-type pollen from the Pliocene and Miocene from Europe and northern Africa. Reports cited by Muller (1981) and confirmed as *Cichorium intybus*-type by Blackmore et al. (1986) include those of Demarcq et al. (1976) from the Middle Miocene of Tunisia, and Nagy (1969) from the Middle Miocene of Hungary. Reports by Blackmore et al. (1986) are from the Late Miocene and Mid-Pliocene of Tunisia and Late Miocene of Spain, while those of Ivanov (1997) and Ivanov and Slavomirova (2000) are from the Middle to Late Miocene of Bulgaria. To our knowledge, the oldest unequivocal fossil report of *Cichorium intybus*-type pollen has been provided by Hochuli (1978) and dates back to the Early Miocene or Late Oligocene (Table 1). This fossil, from the Molasse of the central and western Paratethys, is at least 22 million years old and not more than 28.4 million years old (P. Hochuli, personal communication). Because the reports mentioned above occur rather continuously throughout Late Oligocene to Early Miocene (one report), Middle Miocene (three reports), Late Miocene (three reports), and Pliocene (one report), we believe that the fossil found by Hochuli (1978) rather reliably sets the age of earliest occurrence of *Cichorium intybus*-type pollen. Moreover, we could find no report of unspecified Liguliflorae-type pollen older than the *Cichorium*-type find of Hochuli (1978).

The *Sonchus oleraceus*-type pollen is derived from the *Cichorium intybus*-type. It has the same lacunae as the *Cichorium intybus*-type, but it also possesses three small lacunae within each polar area (polar lacunae), which make it readily distinguishable from the *Cichorium intybus*-type (Blackmore et al. 1986). *Sonchus oleraceus*-type pollen has been reported from the Late Miocene and Mid-Pliocene of Tunisia (at least 5.4 million years old; Blackmore et al. 1986). Because these are the only known records, there might well be older undiscovered fossil occurrences. Among extant taxa, only *Aetheorhiza* (= *Sonchus*; Kilian et al. 2009), *Hyoseris*, *Launaea*, and *Sonchus*, all members

Table 1 Fossil pollen types of Cichorieae along with their earliest occurrence and taxonomic affiliation

Pollen type	Oldest fossil occurrence	Taxonomic affiliation	Reference
Cichorium intybus-type (including *Cichoreacidites* and *Cichoraearumpollenites*)	Early Miocene: min. 22 mya, max. 28.4 mya	Distributed widely in all subtribes except Scorzonerinae; several other types are derived from the *Cichorium intybus*-type	Hochuli (1978, and personal communication)
Sonchus oleraceus-type	Late Miocene: min. 5.4 mya	*Aetheorhiza*, *Hyoseris*, *Launaea*, *Sonchus*	Blackmore et al. (1986)
Scorzonera hispanica-type	Middle Pliocene: min. 3.4 mya	*Scorzonera hispanica*, *S. suberosa*, and a few other *Scorzonera* species	Blackmore et al. (1986)

of the Hyoseridinae sensu Kilian et al. (2009), possess this pollen type (Blackmore 1986; Blackmore et al. 1986).

The *Scorzonera hispanica*-type pollen is rather distinct from the other two types. Like all pollen types found in Scorzonerinae it has just two lacunae per ectoaperture. Six equatorial lacunae are situated along the equator. There are a total of six interporal lacunae (three on each side of the equator), and each pole is covered by one hexagonal polar lacuna. *Scorzonera hispanica*-type pollen has been reported from the Mid-Pliocene of Tunisia (at least. 3.4 million years old; Blackmore et al. 1986). As with the *Sonchus oleraceus*-type, because this is the only known record, there might also well be older undiscovered fossil occurrences. The *Scorzonera hispanica*-type is found only in certain species of *Scorzonera*, including *S. hispanica*, *S. suberosa*, and a few others (Blackmore 1982c).

Calibration and molecular clock

Correct assignment of fossil pollen types to branches of the phylogenetic tree is a crucial step of molecular clock calibrations. In the case of Cichorieae it is complicated by lack of support for basal nodes and conflicting topologies, which each support origin of *Cichorium intybus*-type pollen at different branches of the tree. Figure 1 shows two hypothetical pathways of evolution of echinolophate pollen types in Cichorieae corresponding to (a) the topology obtained in this study and (b) the topology obtained by Kilian et al. (2009).

Two main types of echinolophate pollen grains are found in Cichorieae in addition to echinate pollen grains, which occur scattered through many genera (Blackmore 1982a) and are most likely interpreted as reversals to a primitive state. In the *Cichorium intybus*-type pollen, each colpus is divided into three lacunae: two abapertural and one apertural (Blackmore 1982a). The *Cichorium intybus*-type pollen can have two different types of exine stratification, the *Cichorium*- and the *Scolymus*-type (with more massive columellae within the ridges than the *Cichorium*-type; Blackmore 1981). In the second type of echinolophate pollen grains, which is restricted to subtribe Scorzonerinae, each colpus is divided into just two abapertural lacunae. In addition to the distinct morphology of pollen grains of subtribe Scorzonerinae, the ektexine of Scorzonerinae pollen has a single layer of more or less unbranched columellae, whereas it is made up of extremely fine branching columellae in the *Cichorium*-type pollen (Blackmore 1981).

Cichorium intybus-type pollen with *Cichorium*-type exine stratification occurs in representatives of all present-day subtribes of Cichorieae except Scorzonerinae and Scolyminae, i.e. in all subtribes of the core group (Blackmore 1986). Within Scolyminae, the *Cichorium intybus*-type pollen also occurs, but with a different exine stratification (*Scolymus*-type; Blackmore 1981). The pollen of *Scolymus* represents the most derived condition within Cichorieae. It is identical to the *Cichorium intybus*-type pollen in its pattern of lacunae and ridges, but the ridges are fused completely, thus entirely prohibiting dehiscence of the germinating pollen (Blackmore 1982b, 1986). *Scolymus*-type exine stratification resembles the *Cichorium*-type stratification, but the massive columellae within the ridges and the extreme thickness of the foot layer make it quite distinctive from the *Cichorium*-type (Blackmore 1981; Tomb 1975). Also within Scolyminae, *Catananche* and *Gundelia* have simple echinate pollen grains representing the primitive condition in Asteraceae (Blackmore 1986; Robinson 1994). Exine stratification of *Catananche* is unique in the tribe, with fewer columellae under the spines than in most Cichorieae, and the presence of internal foramina (Tomb 1975). *Warionia* also has echinate pollen grains (Zhao et al. 2006).

Clearly, two distinct evolutionary lines lead to colpi with either three lacunae each (*Cichorium*-type pollen and related types; synapomorphy 1 in Fig. 1) or two lacunae each (several types all found in Scorzonerinae; synapomorphy 2 in Fig. 1) with no intermediates occurring between the three lacunate and two lacunate conditions (Blackmore 1982a). Moreover, the two lacunate pollen grains of Scorzonerinae have an exine stratification very different from the three lacunate pollen grains.

In accordance with the topology obtained in this study (Fig. 1a), the *Cichorium*-type pollen of *Hymenonema* and *Scolymus* with *Scolymus*-type exine stratification (Blackmore 1981) is interpreted as a parallel evolution to the *Cichorium*-type pollen of the core group with *Cichorium*-type exine

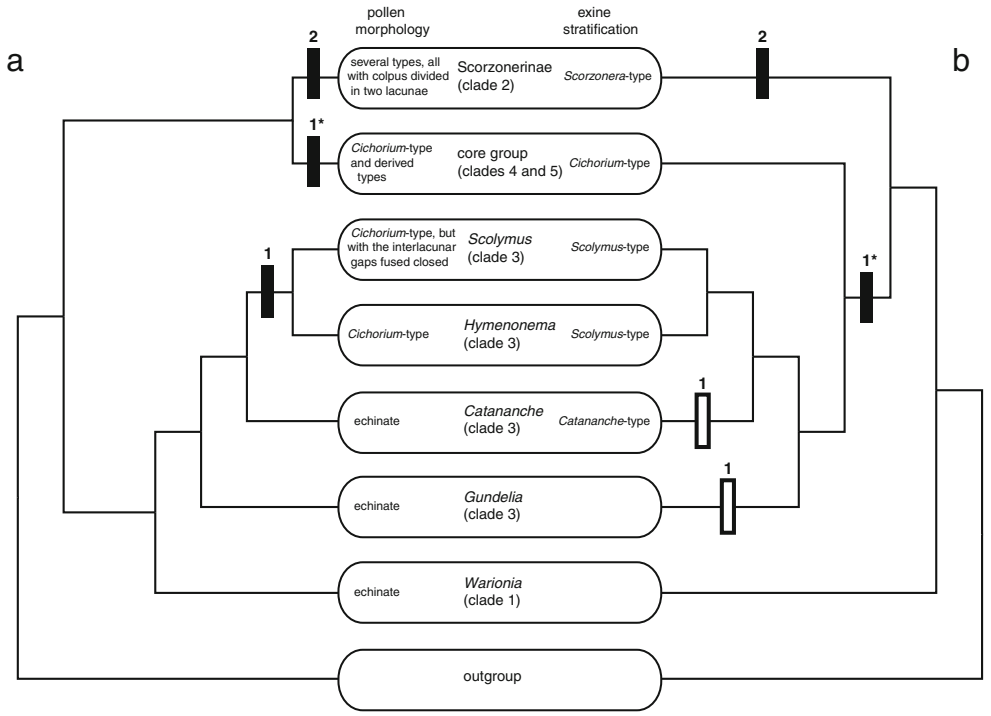

Fig. 1 Two hypothetical evolutionary pathways from echinate to echinolophate pollen types in Cichorieae in accord with (**a**) the topology obtained in this study, and (**b**) the topology obtained by Kilian et al. (2009). The most recent common ancestor (MRCA) of Cichorieae is assumed to have had echinate pollen. *Black bars* Synapomorphies: *1* echinolophate pollen with colpus divided in three lacunae (*Cichorium*-type pollen and related types); *2* echinolophate pollen with colpus divided in two lacunae (several types all found in Scorzonerinae). *White bars* Reversals. The branch used for calibration with the *Cichorium*-type pollen found of Hochuli (1978) is indicated with a *star*. Terminology of pollen morphology and exine stratification follows Blackmore (1981)

stratification. This hypothesis suggests three evolutionary lines in total leading to echinolophate pollen grains, namely in Scolyminae, in Scorzonerinae, and in the core group. The *Cichorium*-type pollen found of Hochuli (1978) is best assigned to the branch leading to the core group in this scenario as this large clade contains taxa distributed in the former Paratethys region, where the fossil was found (not the case for *Hymenonema* and *Scolymus*).

In contrast, the topology obtained by Kilian et al. (2009; Fig. 1b) necessitates only two evolutionary lines leading to echinolophate pollen grains, namely in Scorzonerinae and in the core group plus Scolyminae, and is thus more parsimonious. In this scenario, the *Cichorium*-type pollen originated only once and developed two resembling types of exine stratification (*Cichorium*-type and *Scolymus*-type). The *Cichorium*-type pollen found by Hochuli (1978) must here be assigned to the branch leading to the core group plus Scolyminae. Because we do not have enough evidence to rule out one of the two hypotheses, both were used for calibration and molecular clock calculations.

Assignment of *Sonchus oleraceus*- and *Scorzonera hispanica*-type pollen to branches of the phylogenetic tree is unequivocal. The *Sonchus oleraceus*-type is found exclusively in Hyoseridinae and must therefore be assigned to the basal branch of this clade. The *Scorzonera hispanica*-type occurs exclusively in all members of the clade containing *S. hispanica* and *S. suberosa* and must therefore be assigned to the basal branch of that clade.

The Bayesian approach to the construction of phylogenetic trees, in which phylogenetic uncertainty is incorporated in the HPD interval and the rates on each branch of the tree are drawn independently from an underlying rate distribution so as to account for lineage-specific rate heterogeneity (uncorrelated relaxed molecular clock; Drummond et al. 2006), was adopted. It is implemented in the software package BEAST (Drummond and Rambaut 2007), which estimates the phylogenetic tree and divergence times simultaneously. The programs BEAUti ver. 1.6.2 (Drummond et al. 2002–2010) and BEAST ver. 1.6.2 (Drummond et al. 2002–2010) were used to implement the uncorrelated relaxed clock on ITS1 and ITS2 sequences with different parameters of the GTR+I+G model of base substitution for each of the two data partitions, a Yule tree prior, and a randomly generated starting tree.

For calibration, we used the three fossil pollen types described above. The most important fossil for calibration is the *Cichorium*-type pollen. To account for the possibility that the oldest fossils of the *Cichorium*-type pollen have not yet been found, we implemented a lognormal calibration prior in a way that 95 % of the prior probability are contained within the age

range of the deposit of the oldest reported fossil find [22–28.4 million years ago (mya); Table 1] and 5 % are contained in the tail (older than 28.4 mya). The lower bound of the lognormal distribution was set to 22, the logarithm of the mean was set to 1.163151 and the logarithm of the standard deviation was set to 0.421404. To account for the greater uncertainty associated with the *Sonchus*- and *Scorzonera*-type pollen finds, we used them as minimum age constraints by implementing uniform priors (3.4–100 mya for the *Scorzonera*-type and 5.4–100 mya for the *Sonchus*-type pollen). Because the pollen types could have evolved anywhere along the stem lineage of the group they define (Renner 2005), the calibrations were performed on the crown group as well as stem group nodes in independent analyses, respectively.

The uncorrelated lognormal relaxed clock model was used to describe rate variation among branches. We sampled 100 million states of each of three independent Markov chain Monte Carlo (MCMC) runs. Trees and associated parameter values were logged every 10,000 states resulting in 10,001 sampled trees per independent MCMC run. Results of the MCMC runs were analysed with Tracer ver. 1.5.0 (Rambaut and Drummond 2003–2009). To this aim, the first 10 million states (i.e., 1,000 sampled trees) of each of the three independent MCMC runs were discarded as burn-in. After verification that the independent MCMC runs had converged on the same distribution, the remaining 9,001 sampled trees of each of the runs were combined. LogCombiner v. 1.6.2 was used to combine the trees created in the three independent MCMC runs by removing the first 1,000 sampled trees of each run as burn-in (i.e. 9,001 trees per run were kept). The maximum clade credibility tree was then obtained from the remaining 27,003 trees using TreeAnnotator v. 1.6.2 (Rambaut and Drummond 2002–2010) and graphed with FigTree v. 1.3.1.

The first set of analyses was carried out without topological constraints. The tree obtained by stem group node calibration (Fig. 2) was submitted to TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S11348). In a second set of analyses, we constrained the topology to that obtained by Kilian et al. (2009), i.e. clades 3, 4, and 5 (core group plus Scolyminae) and clades 2, 3, 4, and 5 (core group, Scolyminae, and Scorzonerinae) were constrained to be monophyletic. In this case, the constrained group (clades 3, 4, and 5) was used for calibration with the *Cichorium*-type pollen (Fig. 1b).

Results

In the first set of unconstrained analyses, the mean posteriors of the runs were −11,735 for stem group node calibration, and −11,749 for crown group node calibration. The standard deviation of the uncorrelated lognormal relaxed clock was ~0.43 for stem group node calibration, and ~0.42 for crown group node calibration. The effective sample sizes (ESS) of all parameters were far beyond 250 for both calibrations resulting in reasonable-looking bell-shaped posterior probability density curves.

In the second set of constrained analyses, the mean posteriors of the runs were −11,736 for stem group node calibration, and −11,739 for crown group node calibration. Similarly to the unconstrained analysis, the standard deviation of the uncorrelated lognormal relaxed clock was ~0.43 for both calibrations and the ESS of all parameters were far beyond 250 for both calibrations.

In accord with the phylogenetic hypothesis presented in Kilian et al. (2009), five major clades can be discerned among Cichorieae also in our unconstrained analysis (Fig. 2). Clade 1, corresponding to Warioniinae, contains only *Warionia saharae*. Clade 2 corresponds to Scorzonerinae. Clade 3, corresponding to Scolyminae, includes *Catananche*, *Gundelia*, *Hymenonema*, and *Scolymus*. Clade 4 comprises Chondrillinae, Crepidinae, Hypochaeridinae, Hyoseridinae, and Lactucinae (i.e. roughly two-thirds of the species of the entire tribe). Finally, clade 5 comprises Cichoriinae, Hieraciinae, and Microseridinae. In the following, we denote clades 4 and 5 as the "core group". However, the backbone of the tree, i.e. the basal nodes among clades 1, 2, 3 and the core group, is essentially unresolved as indicated by low posterior probabilities and inconsistencies between the topologies obtained in this study and in that of Kilian et al. (2009). Most importantly, the core group groups with clade 2 (Scorzonerinae) in the present study, whereas it groups with clade 3 (Scolyminae) in that of Kilian et al. (2009).

The mean number of substitutions site^{-1} year^{-1} obtained in the unconstrained analysis is 8.7×10^{-9} and 10.7×10^{-9} (obtained by calibrating the crown and stem group nodes, respectively). It is 10.7×10^{-9} and 10.8×10^{-9} in the constrained analysis (again obtained by calibrating the crown and stem group nodes, respectively). Table 2 shows estimated ages of Cichorieae and subtribes.

Discussion

Estimated substitution rates in a broader context

The mean inferred ITS substitution rate ($8.7–10.7\times10^{-9}$ substitutions site^{-1} year^{-1} for the unconstrained topology; $10.7–10.8\times10^{-9}$ substitutions site^{-1} year^{-1} for the constrained topology) is slightly faster than the fastest rate reported for herbaceous taxa in a survey by Kay et al. (2006; $1.7–8.3\times10^{-9}$ substitutions site^{-1} year^{-1}), who noted a life history-effect but no effect of phylogenetic relatedness on substitution rates. Calibrations with fossils usually provide a minimum age of a particular lineage and might thus overestimate a substitution rate. However, Kay et al. (2006) did not find any effect of the type of calibration used (fossil,

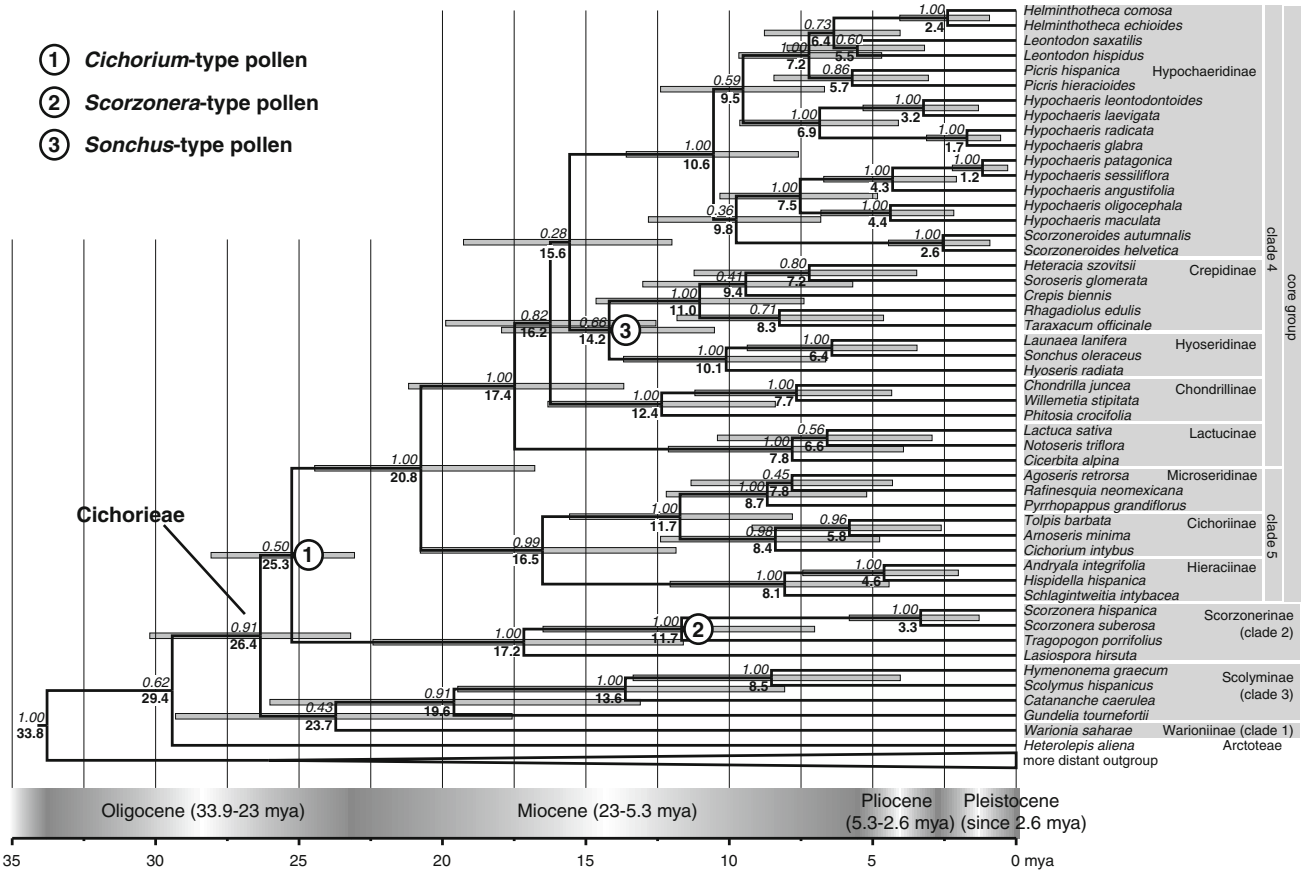

Fig. 2 Chronogram of Cichorieae produced by the program BEAST based on ITS1 and ITS2 sequences (unconstrained topology; maximum clade credibility tree with mean node heights obtained by stem group node calibration). Posterior probabilities of nodes are shown above branches. Mean age estimates are shown below branches. *Bars* show 95 % HPD intervals of age estimates. The three calibration nodes are also indicated

geographic vicariance or molecular clock from a different locus) on substitution rates.

Topological effects on estimated substitution rates

Constraining the topology to that obtained by Kilian et al. (2009) returns similar estimates of substitution rates to those obtained with stem group node calibration of the unconstrained topology. The basal nodes connecting Scolyminae, Scorzonerinae and the core group obtained here without constraining the topology, i.e. [Scolyminae, (Scorzonerinae, core group)], are also supported by analysis of chloroplast restriction sites (Whitton et al. 1994) and chloroplast *ndhF* sequences (Karis et al. 2001). These studies independently suggest that the *Cichorium intybus*-type pollen could have evolved in parallel in Scolyminae and in the core group (Fig. 1a). Also within Barnadesieae and Cichorioideae, there seems to have been a repeated tendency from echinate to echinolophate (or psilolophate) pollen (independently in Arctoteae, Barnadesieae, Cichorieae, and Vernonieae), which may be related to specific functional attributes (Blackmore 1986).

Biogeographic interpretation of age estimates

For the interpretation of age estimates in the light of Earth history, one needs to keep in mind that all estimates are minimum estimates due to the nature of our molecular clock calibration using the fossil record of Cichorieae pollen. As such, age estimates can be used to rule out only later events as relevant, but not earlier ones (Heads 2011). However, the lognormal calibration prior used incorporates a 5 % probability that older *Cichorium*-type fossil pollen grains might be found. In the following, we always refer to minimum estimates obtained by stem group node calibration. We report the age estimates of the unconstrained as well as constrained topology. The age estimates obtained by crown group node calibration might provide maximum estimates only in the case that no older *Cichorium*-type fossil pollen grains are found. Therefore, they are not considered in the discussion.

Table 2 Estimated minimum ages of the most recent common ancestor (MRCA) of Cichorieae and its subgroups based on the relaxed molecular clock approach using BEAST (uncorrelated Bayesian inference; median and 95 % HPD interval shown). The core group (i.e. clades 4 and 5) was used for calibration with the *Cichorium intybus*-type pollen in the unconstrained topology, and the constrained clade (core group plus Scolyminae, i.e. clades 3, 4, and 5) in the constrained topology. Age estimates were obtained by calibrating stem group (*S*) and crown group (*C*) nodes, respectively

	Age estimates (mya)	
	Unconstrained topology	Constrained topology
Cichorieae	S: 26.0 (23.2-30.3)	S: 25.9 (23.5-29.5)
	C: 31.7 (26.9-38.3)	C: 26.9 (24.0-31.2)
Scolyminae: *Catananche*, *Gundelia*, *Hymenonema*, *Scolymus*	S: 19.9 (13.1-26.4)	S: 19.6 (13.2-24.9)
	C: 24.2 (15.9-32.5)	C: 20.5 (14.1-26.0)
Scorzonerinae: *Lasiospora, Scorzonera, Tragopogon*	S: 17.2 (11.6-22.4)	S: 17.5 (12.4-22.4)
	C: 21.1 (14.5-28.0)	C: 18.5 (13.3-23.5)
Clades 4 and 5: "core group"	S: 20.8 (16.8-24.4)	S: 20.2 (16.8-23.8)
	C: 25.1 (23.1-28.1)	C: 21.1 (17.7-24.7)
Clade 4: Chondrillinae, Crepidinae, Hypochaeridinae, Lactucinae, Hyoseridinae	S: 17.5 (13.7-21.2)	S: 17.1 (13.8-20.7)
	C: 21.4 (17.8-24.9)	C: 17.9 (14.6-21.4)
Lactucinae: *Cicerbita*, *Lactuca*, *Notoseris*	S: 7.6 (3.9-12.1)	S: 7.5 (4.0-12.0)
	C: 9.4 (5.0-14.7)	C: 7.9 (4.2-12.5)
Chondrillinae: *Chondrilla*, *Phitosia*, *Willemetia*	S: 12.3 (8.4-16.3)	S: 12.1 (8.5-15.8)
	C: 15.2 (11.0-19.7)	C: 12.6 (8.9-16.5)
Hyoseridinae: *Hyoseris*, *Launaea*, *Sonchus*	S: 10.1 (6.6-13.7)	S: 9.8 (6.6-13.1)
	C: 12.3 (8.5-16.4)	C: 10.3 (7.2-14.0)
Crepidinae: *Crepis*, *Heteracia*, *Rhagadiolus*, *Soroseris*, *Taraxacum*	S: 11.0 (7.4-14.7)	S: 10.8 (7.4-14.1)
	C: 13.5 (9.6-17.6)	C: 11.3 (8.0-15.0)
Hypochaeridinae: *Helminthotheca*, *Hypochaeris*, *Leontodon*, *Picris*, *Scorzoneroides*	S: 10.5 (7.6-13.6)	S: 10.3 (7.6-13.2)
	C: 12.9 (9.7-16.4)	C: 10.8 (8.1-13.8)
Clade 5: Cichoriinae, Hieraciinae, Microseridinae	S: 16.5 (11.8-20.9)	S: 16.2 (12.2-20.7)
	C: 20.3 (15.3-25.0)	C: 17.0 (12.7-21.3)
Hieraciinae: *Andryala*, *Hispidella*, *Schlagintweitia*	S: 7.9 (4.4-12.1)	S: 7.8 (4.4-11.7)
	C: 9.7 (5.5-14.6)	C: 8.2 (4.7-12.4)
Cichoriinae: *Arnoseris*, *Cichorium*, *Tolpis*	S: 8.3 (4.6-12.4)	S: 8.2 (4.7-12.2)
	C: 10.3 (5.8-15.0)	C: 8.6 (5.1-12.8)
Microseridinae: *Agoseris*, *Pyrrhopappus*, *Rafinesquia*	S: 8.6 (5.2-12.2)	S: 8.5 (5.3-12.1)
	C: 10.6 (6.6-14.7)	C: 8.9 (5.6-12.6)

The geographic distribution of the Cichorieae fossil pollen record is from Europe and the Mediterranean region. This latter region is where the tribe is thought to have evolved in response to gradual cooling and the development of a temperate and Mediterranean climate during Miocene and Pliocene (Funk et al. 2005). Both the unconstrained and constrained analyses return a minimum median age of the MRCA of Cichorieae of ~26 mya (95 % HPD interval=~23–30 mya). Our results thus indicate that Cichorieae originated no later than Oligocene. From the inferred N African–Mediterranean ancestral area of Cichorieae (including Gundelieae; Funk et al. 2005), N Africa seems the most plausible region of origin. Africa, including Arabia, was isolated from contact with other continents at that time (Potts and Behrensmeyer 1992).

For the MRCA of Scolyminae, both the unconstrained and constrained analyses return a minimum median age of ~20 mya (95 % HPD interval=~13–26 mya), for the MRCA of Scorzonerinae ~17–18 mya (95 % HPD interval=~12–22 mya), and for the MRCA of the core group ~20–21 mya (95 % HPD interval=~17–24 mya). Scolyminae thus seem to have diversified no later than Late Oligocene or Early/Middle Miocene, Scorzonerinae no later than Early/Middle Miocene, and the core group no later than Late Oligocene or Early Miocene. The origin of these three groups might thus have been associated with the Early Miocene establishment of land bridges between the Afro-Arabian and Eurasian plates as a result of northward drift of the Afro-Arabian plate. Various lines of evidence, especially faunal interchange, suggest that definitive contact between Africa and Eurasia occurred by at least 20 mya, though this timing is under debate (Potts and Behrensmeyer 1992). The present-day distributions of Scolyminae, Scorzonerinae, and the core group in N Africa, Middle East, Turkey, and S Europe (with Scorzonerinae and the core group extending into Asia and Europe; Bremer 1994) substantiate this view.

The subtribes of the core group diversified no later than Middle/Late Miocene or Early Pliocene, an eventful period of changing geological setting and climate in the Mediterranean region and Eurasia. During the Miocene, cooling favoured the development of deciduous broad-leaved vegetation (replacing tropical evergreen vegetation; Mai 1995). The uplift of the Alps began in Middle Miocene and of the Himalayas and the Tibetan Plateau in the late Neogene (with varying estimates of the rate and timing of uplift; Mai 1995; Potts and Behrensmeyer 1992) allowing evolution of mountain taxa. European localities from the latter part of the Middle Miocene possess faunas and floras that appear to indicate greater habitat variation than existed earlier in the Miocene, including ecotones between forest, open vegetation, and marshy areas. The Paratethys, Mediterranean basin, and Tethys became disconnected (forming a number of land dispersal routes) culminating in an almost complete desiccation (Late Miocene; 6–5.5 mya), which led to a drier climate in S Europe and N Africa, the development of Mediterranean sclerophyllous vegetation, a considerable regional differentiation of the flora, and extensive interchanges between African and Eurasian biotas (Mai 1995; Potts and Behrensmeyer 1992).

Dispersal to North America and other parts of the world

A relationship of Microseridinae, which are distributed mainly in W and SW North America, with Cichoriinae is suggested by the ITS tree (Fig. 2; Kilian et al. 2009). Cichoriinae are found mainly in Europe, Africa, and SW Asia (Kilian et al. 2009). The minimum time frame for dispersal is given by the stem and crown group ages of Microseridinae. Both the unconstrained and constrained analyses suggest a minimum median crown group age of ~9 mya (95 % HPD=~5–12 mya) and a minimum median stem group age of Microseridinae (i.e. of the clade consisting of Cichoriinae and Microseridinae) of ~11–12 mya (95 % HPD=~8–16 mya). The ancestor of the North American clade is thus estimated to have dispersed from Eurasia to North America, where it subsequently radiated no later than Middle or Late Miocene, suggesting the Bering land bridge as route of dispersal. Physically, the Bering land bridge was open to terrestrial migration throughout the Miocene until approximately 4.8 to 7.3–7.4 mya (Marincovich and Gladenkov 1999; Tiffney and Manchester 2001). The North Atlantic land bridge, which was disrupted in Early Eocene by the sinking of the Iceland hot spot (Tiffney and Manchester 2001), is thus ruled out as a possible migration route unless much older *Cichorium*-type fossil pollen grains are found, which we deem rather unlikely.

Other age estimates for Beringian divergence between angiosperm and gymnosperm sister taxa reported so far (Donoghue et al. 2001 and literature cited therein; Wen 1999; Xiang et al. 2000) also focus on temperate taxa and infer a Miocene or more recent time of last contact between the taxa in Asia and North America, i.e. they span a long time period. Correlation of Beringian climate and vegetation at different times in the Neogene with the ecological requirements of the migrating taxa can be used to substantiate or dismiss molecular age estimates. Factors limiting plant migration across the Bering land bridge were temperature, increasing seasonality, and an extended period of winter darkness, but temperate taxa that could tolerate winter darkness through deciduousness or dieback, and ultimately boreal taxa could have crossed the Bering land bridge at almost any time in the Tertiary (Tiffney and Manchester 2001). Based on the habits and ecological requirements encountered in present-day Cichoriinae and species of its North American sister clade, we can infer their common ancestor to have been a herb confined to the understory of deciduous forests and/or open habitats. Miocene floras in NE Asia and Alaska were deciduous and rich in herbaceous taxa, but global cooling led to increasing dominance of hardy deciduous angiosperms and conifers (Betulaceae, *Larix*, *Pinus*) in the later Miocene and Pliocene (leading to a full taiga/tundra community; Tiffney and Manchester 2001), thus a Middle to Late Miocene migration of the ancestor of the North American clade across the Bering land bridge is supported.

Provided that there were no older extinct species in North America, radiation of Microseridinae in W and SW North America, from where they dispersed to E North America, Central and South America, and Australia and New Zealand, seems to have started no later than Late Miocene, not less than ~9 mya (95 % HPD interval=~5–12 mya), and was presumably associated with the emergence of mountain ranges along the west coast. In Late Miocene, the Sierra Nevadan–Cascadian orogeny brought increasing aridity to the intermontane region west of the Rocky Mountains and cool- and arid-adapted vegetation gradually displaced broad-leaved forests (Potts and Behrensmeyer 1992), thus offering an Eurasian immigrant adapted to cooler temperatures newly developing habitats to be colonised.

Other genera have also dispersed to other parts of the world (tropical and southern Africa, tropical Asia, Australia, New Zealand, and South America; Kilian et al. 2009). Only one of these (*Hypochaeris*) has yet been dated. A more complete taxon sampling of the others is needed for dating the dispersals. For instance, *Crepis* and *Hieracium* dispersed to North America, the latter also farther to South America, where these genera diversified involving hybridization, polyploidization and/or apomixis. *Hypochaeris* provides a peculiar example of long-distance dispersal from NW Africa, the home of *H. angustifolia*, which is sister to the South American clade, to South America, where it radiated (Tremetsberger et al. 2005). *Hypochaeris* is inferred to have dispersed from NW Africa to South America no later than Late Miocene, Pliocene or

Pleistocene. The earliest possible date of dispersal is set by the divergence between *H. angustifolia* and the South American clade represented here by *H. patagonica* and *H. sessiliflora* and is not less than ~4.1–4.2 mya (95 % HPD interval=~2.1–6.7 mya). Provided that there were no older extinct species in South America, radiation started no later than Pleistocene [not less than ~1.1 mya; 95 % HPD interval=~0.3–2.2 mya; see also Tremetsberger et al. (2005)]. The Late Pliocene and Pleistocene in South America are characterized by marked expansions and contractions of forest and other types of vegetation such as savanna and dry steppe following the interglacial-glacial cycles, and it seems highly probable that alternating contraction and expansion of different environmental conditions have had major effects on speciation (Potts and Behrensmeyer 1992). At the end of the Cenozoic, the Andes also experienced increased uplift and volcanism (Potts and Behrensmeyer 1992), also stimulating speciation.

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Appendix

ITS sequences used with their EMBL/GenBank accession numbers and voucher information including collector(s) and number and herbarium accession number (only for newly provided sequences). The DNA Bank Network number (http://www.dnabank-network.org/; Gemeinholzer et al. 2011) is indicated, if available.

Ingroup: *Agoseris retrorsa* (Benth.) Greene AJ633461 (Bachmann A71, GAT bg65); *Andryala integrifolia* L. AJ633384[a]; *Arnoseris minima* (L.) Schweigg. & Körte AJ633445 (Schuster s/n, GAT bg155); *Catananche caerulea* L. AJ633466 (Romania, Hortus Botanicus Universitatis Iasi: 265-53/02-18/36, GAT bg26); *Chondrilla juncea* L. AJ633348 (IPK-Gatersleben-Expedition: ITA-81 no. 7628, GAT bg20); *Cicerbita alpina* (L.) Wallr. AJ633324 (Blattner & Jakob BJ02-067, GAT bg96); *Cichorium intybus* L. AJ633451[b]; *Crepis biennis* L. AJ633355 (Czech Republic, BG Brno 84 9/03, GAT bg223); *Gundelia tournefortii* L. AY504691[c]; *Helminthotheca comosa* (Boiss.) Holub AJ633323 (Zidorn 23.01.2003a-1, GAT bg274); *Helminthotheca echioides* L. (Holub) AJ633321 (France, Jardin Botanique de Dijon 19-103/02, GAT bg128); *Heteracia szovitsii* Fisch. & C.A.Mey. AJ633283 (Newodowski s/n, GAT bg81); *Hispidella hispanica* Lam. AJ633432 (Pizarro & Navarro 2460, GAT bg199); *Hymenonema graecum* (L.) DC. EU436694 (Jäth s/n, B 100209163, DB 467); *Hyoseris radiata* L. AF528494[d]; *Hypochaeris angustifolia* (Litard. & Maire) Maire AJ627257[e]; *Hypochaeris glabra* L. AJ627264[e]; *Hypochaeris laevigata* (L.) Ces. & al. AJ627265[e]; *Hypochaeris leontodontoides* Ball AJ627266[e]; *Hypochaeris maculata* L. AF528454[d]; *Hypochaeris oligocephala* (Svent. & Bramwell) Lack AJ627268[e]; *Hypochaeris patagonica* Cabrera AM932283 (Essl 6202, WU 59780); *Hypochaeris radicata* L. AJ627270[e]; *Hypochaeris sessiliflora* Kunth AF528482[d]; *Lactuca sativa* L. AJ633337 (Romania, BG Cluj-Napoca 681-7/03, GAT bg207); *Lasiospora hirsuta* (Gouan) Cass. AJ633479 (France, BG Montpellier 774-101-28/03, GAT bg213); *Launaea lanifera* Pau EU436699 (Vogt 14455/Oberprieler 8764, B 100355175, DB 7038); *Leontodon hispidus* L. DQ451770[f]; *Leontodon saxatilis* Lam. AJ633317 (Egli, Leuenberger & Arroyo-Leuenberger 3137b, B, GAT bg112); *Notoseris triflora* (Hemsl.) C.Shih EU436698 (Li Heng 13455, CAS 1031382); *Phitosia crocifolia* (Boiss. & Heldr.) Kamari & Greuter EU436695 (Strid & Papanikolaos 15261, herb. Greuter); *Picris hieracioides* L. AJ633320 (Germany, BG Bremen 117-86/02, GAT bg127); *Picris hispanica* (Willd.) P. D. Sell DQ451808[f]; *Pyrrhopappus grandiflorus* (Nutt.) Nutt. AJ633459 (Bachmann B05, GAT bg68); *Rafinesquia neomexicana* A.Gray AF473613[g]; *Rhagadiolus edulis* Gaertn. AF528495[d]; *Schlagintweitia intybacea* (All.) Griseb. AJ633426[a]; *Scolymus hispanicus* L. AJ633470 (Germany, Berlin, Arboretum Späth s/n, GAT bg25); *Scorzonera hispanica* L. AJ633472 (Denmark, BG Hauniensis 429-130-149/01, GAT bg12); *Scorzonera suberosa* K.Koch AY508199[h]; *Scorzoneroides autumnalis* (L.) Moench AF528486[d]; *Scorzoneroides helvetica* (Mérat) Holub DQ451767[f]; *Sonchus oleraceus* L. AJ633306 (Ochsmann 8192, GAT bg117); *Soroseris glomerata* (Decne.) Stebbins EU436696 (T.-N. Ho 1692, CAS 939054); *Taraxacum officinale* F.H.Wigg. L48337, L48338[i]; *Tolpis barbata* (L.) Gaertn. AJ633434 (France, Jardin Botanique de Dijon 19-229-103/02, GAT bg58); *Tragopogon porrifolius* L. AJ633496 (Romania, BG Cluj-Napoca 759 7/03, GAT bg219); *Warionia saharae* Benth. & Coss. AY190608 (Morocco, Lippat 25346, US)[j]; *Willemetia stipitata* (Jacq.) Dalla Torre EU436697 (Greece, Willing 11335, B 100209153, DB 462).

Outgroup: *Barnadesia arborea* Kunth AF412883[k]; *Brachylaena discolor* DC. AY826236[l]; *Cardopatium corymbosum* (L.) Pers. AY826238[l]; *Echinops exaltatus* Schrad. AY538649[m]; *Ericentrodea corazonensis* (Hieron.) S.F.Blake & Sherff AY429088[n]; *Ericentrodea decomposita* S.F.Blake & Sherff AY429089[n]; *Geigeria ornativa* O.Hoffm. U84774[o]; *Gerbera crocea* Kuntze AY504687[b]; *Heterolepis aliena* (L.f.) Druce AY504700[b]; *Mutisia grandiflora* Humb. & Bonpl. AF546081[p]; *Oldenburgia intermedia* P.Bond AY826303[l]; *Pluchea indica* (L.) Less. AF430795[q]; *Saussurea maximowiczii* Herder AY826324[l]; *Schlechtendalia luzulifolia* Less. AF412836[k].

[a] Fehrer et al. (2007); [b] Gemeinholzer & Bachmann (2005); [c] Funk et al. (2004); [d] Samuel et al. (2003); [e] Tremetsberger et al. (2005); [f] Samuel et al. (2006); [g] Lee et al. (2002); [h] Mavrodiev et al. (2004); [i] Kim et al. (1996); [j] J.L. Panero (unpublished data); [k] Gustaffson et al. (2001); [l] Susanna et al. (2006); [m] Garnatje et al. (2005); [n] Kimball & Crawford (2004); [o] Eldenäs et al. (1998); [p] H.-G. Kim (unpublished data); [q] C.H. Chou & F.T. Huang (unpublished data).

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