Available online at www.sciencedirect.com
SCIENCE DIRECT
Molecular Phylogenetics and Evolution 39 (2006) 668–687
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Molecular evidence for the age, origin, and evolutionary history of the American desert plant genus *Tiquilia* (Boraginaceae)

Michael J. Moore *, Robert K. Jansen

Section of Integrative Biology and Institute of Cellular and Molecular Biology, University of Texas at Austin, 1 University Station A6700, Austin, TX 78712, USA

Received 30 August 2005; revised 11 January 2006; accepted 12 January 2006
Available online 21 February 2006

Abstract

Although the deserts of North America are of very recent origin, their characteristic arid-adapted endemic plant lineages have been suggested to be much older. Earlier researchers have hypothesized that the ancestors of many of these modern desert lineages first adapted to aridity in highly localized arid or semi-arid sites as early as the late Cretaceous or early Tertiary, and that these lineages subsequently spread and diversified as global climate became increasingly arid during the Cenozoic. No study has explicitly examined these hypotheses for any North American arid-adapted plant group. The current paper tests these hypotheses using the genus *Tiquilia* (Boraginaceae), a diverse North American desert plant group. A strongly supported phylogeny of the genus is estimated using combined sequence data from three chloroplast markers (*matK*, *ndhF*, and *rps16*) and two nuclear markers (ITS and *waxy*). Ages of divergence events within the genus are estimated using penalized likelihood and a molecular clock approach on the *ndhF* tree for *Tiquilia* and representative outgroups, including most of the major lineages of Boraginales. The dating analysis suggests that the stem lineage of *Tiquilia* split from its nearest extant relative in the Paleocene or Eocene (~59–48 Ma). This was followed by a relatively long period before the first divergence in the crown group near the Eocene/Oligocene boundary (~33–29 Ma), shortly after the greatest Cenozoic episode of rapid aridification. Divergence of seven major lineages of *Tiquilia* is dated to the early-to-mid Miocene (~23–13 Ma). Several major lineages show a marked increase in diversification concomitant with the onset of more widespread semi-arid and then arid conditions beginning in the late Miocene (~7 Ma). This sequence of divergence events in *Tiquilia* agrees well with earlier researchers' ideas concerning North American desert flora assembly.

Keywords: *Tiquilia*; Boraginales; Phylogeny; North American deserts; Molecular dating

1. Introduction

The warm deserts of the world are considered to be of a relatively young age, in most cases no older than the late Miocene/early Pliocene [7–5 Ma (million years ago); all dates follow Berggren et al., 1995, unless otherwise noted], and in some cases, as for example the North American deserts, as young as the early to late Quaternary (1.75 Ma to the present; Axelrod, 1979a,b; Wolfe, 1985; Graham, 1999). Despite the youth of these ecosystems, each of the warm desert regions contains a large endemic flora with many unique arid-adapted morphotypes, which suggests that such plants have evolved much earlier than the desert regions they inhabit (Axelrod, 1958, 1979a,b). The deserts of southwestern North America furnish numerous examples of characteristic genera that likely possess relatively great antiquity, including *Fouquieria* HBK. (Fouquieriaceae), *Simmondsia* Nutt. (Simmondsiaceae), *Koeberlinia* Zucc. (Koeberliniaceae), *Pachycormus* Coville (Anacardiaceae), *Agave* L. (Agavaceae), *Yucca* L. (Agavaceae), *Olneya* A. Gray (Fabaceae), and several genera of large columnar cacti (such as *Carnegiea* Britton and Rose; Cactaceae).

Axelrod (1950, 1979a,b) was the first researcher to fully recognize and attempt to account for the difference in age between the North American deserts (including the

* Corresponding author. Present address: University of Florida, Florida Museum of Natural History, P.O. Box 117800, Gainesville, FL 32611-7800, USA. Fax: +1 352 846 2154.
E-mail address: mjmoore1@ufl.edu (M.J. Moore).

1055-7903/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2006.01.020

Mojave, Sonoran, and Chihuahuan Deserts) and their characteristic plant groups. He has developed a general evolutionary scenario, based on the analysis of fossil floras in conjunction with paleoclimatic reconstruction, that posits the autochthonous evolution of a drought-adapted flora drawn from local semi-arid or arid sites that existed among more mesic habitats (Axelrod, 1967, 1972, 1979a). Specifically, he suggests that the earliest adaptations to seasonally dry climates began as early as the late Cretaceous (99–65 Ma; Upper Cretaceous of Gradstein et al., 1995), possibly in areas of edaphic aridity, within the widespread tropical forests and seasonally dry woodlands that then characterized the continent (Axelrod, 1950, 1958, 1972, 1979a). By the late Eocene (approximately 37–33.5 Ma), plants typical of drier vegetation types such as sclerophyll woodland and arid tropic scrub had arisen and occupied at least some portion of the interior of southwestern North America (Axelrod, 1958, 1979a; Leopold et al., 1992). A series of global climatic changes near the Eocene/Oligocene boundary (~33.5 Ma), however, resulted in a dramatic cooling and drying of the Earth, greatly increasing the extent of woodland and savanna habitats (although probably not grass-dominated savanna as is common at present) in western North America (Axelrod, 1979a; Graham, 1999; Wolfe, 1997). While climate conditions fluctuated somewhat between the early Oligocene and the mid-Miocene (approximately 33.5–15 Ma; Graham, 1999; Wolfe, 1997), they remained wetter than at present; there is no evidence of widespread arid or semi-arid habitats during this time period, although they probably existed very locally under certain topographic or edaphic conditions (Axelrod, 1979a). The mid-Miocene (~15 Ma) ushered in another period of global cooling and drying, and it is in this era that evidence of grass-dominated habitats first appears in North America (Jacobs et al., 1999; Leopold et al., 1992; Retallack, 1997). Drier environments such as these spread gradually throughout the late Miocene, until another sharp drop in temperature and concomitant increase in aridity near the end of the Miocene (~5 Ma) caused the spread of semi-desert conditions into at least some of the areas of the modern North American deserts (Axelrod, 1950, 1979a; Graham, 1999). However, true desert vegetation was likely no more than a local phenomenon during the late Miocene, and probably remained so even during the relatively dry periods of the Pliocene (approximately 5–1.75 Ma; Axelrod, 1979a). It was not until the glacial climate upheavals of the Pleistocene that arid-adapted plants became widespread, and then only during the drier interglacials; there is abundant evidence that desert vegetation was greatly restricted during the pluvial periods (Axelrod, 1979a; Betancourt et al., 1990; Lowenstein et al., 1999; Thompson and Anderson, 2000; Wells, 1966). The modern extent of desert vegetation in North America may therefore be the widest it has ever been (Axelrod, 1979a; Betancourt et al., 1990).

Recent years have witnessed a remarkable increase in the use of fossil information to calibrate the ages of plant groups in molecular-based phylogenies (Sanderson et al., 2004), and while many of these studies have focused on correlating past cladogenetic events with significant climate changes or plate movements, very few have attempted to examine the evolution of aridland plant groups. For instance, a number of studies have attempted to test important biogeographic hypotheses, such as Gondwanan vicariance (examples include Davis et al., 2004 and Weeks et al., 2005) and the ages of the vicariant plant groups of the northern temperate forests (Donoghue and Smith, 2004; Wen, 1999), using molecular-based phylogenies in groups with good fossil records. Such studies have been completed for a few plant taxa in various arid regions of the world, such as in South Africa (Caujapé-Castells et al., 2001; Klak et al., 2004; Linder, 2003; Linder and Hardy, 2004) and Australia (Crisp et al., 2004), and for certain special problems such as the evolution of C_4 photosynthesis (Kadereit et al., 2003; Sage, 2004). However, no studies have explicitly attempted to infer dates for evolutionary events in any North American arid-adapted plant lineages, despite the presence of molecular-based phylogenies for several such groups, including Cactaceae (Butterworth et al., 2002; Nyffeler, 2002), *Fouquieria* (Schultheis and Baldwin, 1999), *Simmondsia* (Cuénoud et al., 2002), *Koeberlinia* (Hall et al., 2004), *Olneya* (Lavin et al., 2003), and Agavaceae (Bogler and Simpson, 1995, 1996).

How could we recognize that the evolutionary history of a given North American arid-adapted plant lineage conformed to the general scenario envisioned above by Axelrod, in the absence of fossil information? In other words, what kinds of patterns might we expect in a molecular-based phylogeny of such a desert plant group? Certain features of the phylogeny could be predicted given Axelrod's hypotheses in conjunction with the record of Tertiary climate change outlined above. First, the stem lineage of such a desert group should be relatively old and isolated phylogenetically with respect to its outgroups, due to its restriction to local arid pockets throughout much of its history. Second, it is possible that at least some of the deeper cladogenetic events within the ingroup would be correlated with one or more of the major episodes of aridification that occurred during the Tertiary. Each of these rapid periods of drying would have favored the spread (even if only in a limited fashion) of arid or semi-arid adapted plant lineages, providing new opportunities for diversification in these groups. Third, the species- or population-level diversity within these groups should be much younger, representing recent radiations in response to the spread of semi-arid and arid climates since the late Miocene.

Tiquilia Pers. (Boraginaceae) provides a good opportunity to test these assumptions regarding evolution of North American aridland plants. The genus comprises approximately 30 species of prostrate herbs and subshrubs that inhabit the deserts of both North and South America (Richardson, 1977). There are a number of reasons to suspect that *Tiquilia* may conform to the predictions outlined above. Although *Tiquilia* is clearly a member of subfamily Ehretioideae of Boraginaceae, its adaptation to aridity and

its other unique morphological characters have left its position within the subfamily unclear (Al-Shehbaz, 1991; Miller, 2003). *Tiquilia* is also highly diverse. The genus is divided into two subgenera that differ in a number of features, including morphology, chromosome number, substrate preference, and geographic distribution. Subgenus *Tiquilia* (~20 spp.) is found on sandy soils in the Sonoran, Mojave, and Great Basin Deserts of North America, as well as in the coastal deserts of Peru and the deserts of western Argentina, while subg. *Eddya* A.T. Richardson (~10 spp.) is entirely North American and is restricted to calcareous and gypseous substrates, with most species in the Chihuahuan Desert Region (Richardson, 1977). Furthermore, the North American species of *Tiquilia* fall into several groups that are quite distinct morphologically; all of these species groups are common in desert habitats and in many cases occur sympatrically without any evidence of hybridization. At first glance, then, *Tiquilia* would appear to be a good candidate group in which to test the hypotheses implied in Axelrod's work: it is almost certainly of North American origin; its isolated position within the Boraginaceae combined with its tremendous morphological diversity suggest a possible ancient age for the genus; and its fidelity to arid habitats together with its widespread and common nature in deserts today suggest that it has benefited from the recent spread of the desert biome.

The purpose of the present paper, then, is to use several markers from the chloroplast and nuclear genomes to construct a well-supported phylogeny of *Tiquilia* and its potential outgroups, and to apply reasonable dates to the cladogenetic events in the resulting tree. From this information we can then test the predictions outlined above: (1) is *Tiquilia* a relatively old arid-adapted lineage that evolved in North America? (2) when did the majority of diversification within the genus occur, and is any of this diversification correlated with major episodes of aridification during the Cenozoic? and (3) is there any evidence of increased species- or population-level diversity with the onset of semi-arid and arid conditions since the late Miocene? Answers to such questions require both a well-supported phylogeny as well as reasonable calibration points to help date diversification events in *Tiquilia*. In many molecular-based studies that seek to apply dates to specific events on phylogenetic trees, well-dated ingroup fossils with reasonably clear affinities to modern taxa are used as constraints on the ages of nodes. However, such an approach for *Tiquilia* is currently impossible because of the complete absence of a known fossil record for the genus. Such a situation is common in plant taxa of arid regions because the conditions required for fossil formation are usually not encountered in such areas (Axelrod, 1979a,b). Nevertheless, it is still possible, although less desirable, to use other types of information, such as fossil-based ages from outgroups as well as known ages of past climatic or geologic events, to constrain the taxon ages in the phylogeny of a desert group. This approach has been used profitably in at least one other study involving the radiation of a drought-adapted plant group [in *Moraea* Mill. (Iridaceae); Goldblatt et al., 2002], and we have used a similar approach in the current study.

2. Methods

2.1. Taxon and marker selection—Tiquilia

Preliminary phylogenetic analyses of 202 accessions representing 27 species of *Tiquilia* (see Supplemental materials) using sequences from the chloroplast *rps16* intron (Kelchner, 2002; Oxelman et al., 1997), the nuclear ribosomal internal transcribed spacer (ITS; Álvarez and Wendel, 2003; Baldwin et al., 1995), and part of the nuclear granule-bound starch synthase gene (GBSSI, or *waxy*; Mason-Gamer et al., 1998) allowed the selection of a subset of 25 accessions representing all of the major lineages of *Tiquilia* for sequencing of two additional chloroplast markers, *ndhF* (Olmstead and Sweere, 1994) and *matK* (including the 3′ portion of the *trnK* intron; Johnson and Soltis, 1994; Kelchner, 2002; Steele and Vilgalys, 1994). Using sequence data for all five markers for these 25 accessions, the incongruence length difference (ILD) test (Farris et al., 1994) was used to assess data combinability using each marker as a separate partition. Initial results of the ILD test suggested incongruence among three partitions ($p = 0.01$): chloroplast DNA (cpDNA), ITS, and *waxy*. The taxa causing the incongruence were identified and removed so that a reduced data set including just 14 species of *Tiquilia* (Table 1), but still representing every major lineage in the genus, exhibited congruence among all data partitions. These 14 species were then included in the SH test of alternate topologies (see below).

The *ndhF* data set was selected for analyses involving dating of divergence events because the maximum likelihood topologies of the combined data tree and the *ndhF* tree were congruent with nearly identical relative branch lengths, and because a previous study of the Hydrophyllaceae had sampled the Boraginales widely for *ndhF* (Ferguson, 1999). The *ndhF* analyses included all 14 species of *Tiquilia* that were included in the combined analysis as well as an additional five species that were added to increase coverage within the more species-rich main lineages of *Tiquilia*, without adding zero length terminal branches (see Table 1 for a list of all taxa included in these analyses). The addition of these five taxa did not significantly alter the topology or branch lengths with respect to the major lineages of *Tiquilia*.

2.2. Taxon selection—outgroups

Four outgroups were initially chosen from among the Boraginales based on their potential affinities to *Tiquilia*: *Coldenia procumbens* was chosen because of earlier morphological hypotheses regarding it as the closest relative of *Tiquilia* (Richardson, 1977); *Ehretia anacua* and *Bourreria succulenta* were chosen as representative taxa of the subfamily Ehretioideae, to which *Tiquilia* belongs (Miller, 2003; Richardson, 1977); and the holoparasite *Pholisma arenarium* Nutt.

Table 1
List of accessions included in 41-taxon *ndhF* analyses and 16-taxon, 5-marker combined phylogenetic analyses

Species	GenBank/collection Info.
Boraginales	
Boraginaceae subfam. Boraginoideae	
Borago officinalis L.	L36393 (*ndhF*; Olmstead and Reeves, 1995)
Cryptantha flavoculata Payson	AF047803 (*ndhF*; Ferguson, 1999)
Boraginaceae subfam. Cordioideae	
Cordia nodosa Lam.	AF047808 (*ndhF*; Ferguson, 1999)
Boraginaceae subfam. Ehretioideae	
Bourreria costaricensis (Standl.) A.H. Gentry	AF047797 (*ndhF*; Ferguson, 1999)
**Bourreria succulenta* Jacq.	Cuba: Pinar del Rio, *R. G. Olmstead 96-114* (WTU); DQ197285 (ITS); DQ197229 (*matK*); DQ197257 (*ndhF*); DQ197037 (*rps16*); DQ197599 (*waxy*)
**Coldenia procumbens* L.	Ghana: Bolgatanga, *Jongkind and Nieuwenhuis 1973* (MO) (cloned for *waxy*); DQ197284 (ITS); DQ197227 (*matK*); DQ197255 (*ndhF*); DQ197036 (*rps16*); DQ197597 (*waxy*)
Ehretia acuminata R.Br. (in GenBank as *E. ovalifolia* Hassk.)	AF047800 (*ndhF*; Ferguson, 1999)
Ehretia anacua I.M. Johnst.	USA, Texas: Travis County, *M. J. Moore s.n.*; DQ197286 (ITS); DQ197228 (*matK*); DQ197256 (*ndhF*); DQ197038 (*rps16*); DQ197600 (*waxy*)
**Tiquilia canescens* (DC.) A.T. Richardson	USA, Nevada: Clark County, *M. J. Moore 239*; DQ197312 (ITS); DQ197230 (*matK*); DQ197258 (*ndhF*); DQ197068 (*rps16*); DQ197630 (*waxy*)
Tiquilia conspicua (I.M. Johnst.) A.T. Richardson	Perú: Dpto. Arequipa, *M. J. Moore 294* (cloned for *waxy*); DQ197586 (ITS); DQ197250 (*matK*); DQ197278 (*ndhF*); DQ197216 (*rps16*); DQ197817 (*waxy*)
**Tiquilia cuspidata* (I.M. Johnst.) A.T. Richardson	México: Baja California Sur, *M. J. Moore 223*; DQ197540 (ITS); DQ197247 (*matK*); DQ197277 (*ndhF*); DQ197192 (*rps16*); DQ197795 (*waxy*)
Tiquilia darwinii (Hook.f.) A.T. Richardson	Galápagos Islands: Isla Santiago, *A. Tye 573*; DQ197541 (ITS); DQ197248 (*matK*); DQ197276 (*ndhF*); DQ197194 (*rps16*); DQ197797 (*waxy*)
**Tiquilia* "Durango" (undescribed species)	México: Durango, *M. J. Moore 260*; DQ197331 (ITS); DQ197232 (*matK*); DQ197260 (*ndhF*); DQ197141 (*rps16*); DQ197649 (*waxy*)
**Tiquilia elongata* (Rusby) A.T. Richardson	Perú: Dpto. Arequipa, *M. J. Moore 289* (cloned for *waxy*); DQ197588 (ITS); DQ197251 (*matK*); DQ197279 (*ndhF*); DQ197218 (*rps16*); DQ197819 (*waxy*)
**Tiquilia gossypina* (Wooton and Standl.) A.T. Richardson	*Accession 134—USA, Texas: Brewster County, *M. J. Moore 134*; DQ197337 (ITS); DQ197233 (*matK*); DQ197267 (*ndhF*); DQ197146 (*rps16*); DQ197653 (*waxy*); Accession 263—México: Coahuila, *M. J. Moore 263*; DQ197353 (ITS); DQ197234 (*matK*); DQ197263 (*ndhF*); DQ197158 (*rps16*); DQ197667 (*waxy*)
**Tiquilia greggii* (Torr. and A. Gray) A.T. Richardson	USA, Texas: Brewster County, *M. J. Moore 133*; DQ197325 (ITS); DQ197231 (*matK*); DQ197259 (*ndhF*); DQ197083 (*rps16*); DQ197644 (*waxy*)
**Tiquilia hispidissima* (Torr. and A. Gray) A.T. Richardson	Accession 131—USA, Texas: Brewster County, *M. J. Moore 131*; DQ197423 (ITS); DQ197240 (*matK*); DQ197268 (*ndhF*); DQ197098 (*rps16*); DQ197727 (*waxy*); *Accession 154—USA, Texas: Culberson County, *M. J. Moore 154*; DQ197442 (ITS); DQ197241 (*matK*); DQ197269 (*ndhF*); DQ197101 (*rps16*); DQ197734 (*waxy*)
**Tiquilia latior* (I.M. Johnst.) A.T. Richardson	*Accession 211—USA, Arizona: Navajo County, *M. J. Moore 211*; DQ197535 (ITS); DQ197243 (*matK*); DQ197271 (*ndhF*); DQ197136 (*rps16*); DQ197777 (*waxy*); Accession 216—USA, Utah: Wayne County, *M. J. Moore 216*; DQ197538 (ITS); DQ197244 (*matK*); DQ197272 (*ndhF*); DQ197037 (*rps16*); DQ197786 (*waxy*)
Tiquilia mexicana (S. Watson) A.T. Richardson	USA, Texas: Terrell County, *M. J. Moore 117*; DQ197368 (ITS); DQ197235 (*matK*); DQ197261 (*ndhF*); DQ197167 (*rps16*); DQ197682 (*waxy*)
**Tiquilia nuttallii* (Benth.) A.T. Richardson	USA, Washington: Grant County, *M. J. Moore 218*; DQ197579 (ITS); DQ197254 (*matK*); DQ197282 (*ndhF*); DQ197190 (*rps16*); DQ197814 (*waxy*)
**Tiquilia palmeri* (A. Gray) A.T. Richardson	*Accession 197—USA, California: Riverside County, *M. J. Moore 197*; DQ197581 (ITS); DQ197252 (*matK*); DQ197280 (*ndhF*); DQ197210 (*rps16*); DQ197820 (*waxy*); Accession 202—USA, Arizona: Yuma County, *M. J. Moore 202*; DQ197583 (ITS); DQ197253 (*matK*); DQ197281 (*ndhF*); DQ197212 (*rps16*); DQ197822 (*waxy*)
**Tiquilia paronychioides* (Phil.) A.T. Richardson	Perú: Dpto. Arequipa, *M. J. Moore 300* (cloned for ITS, *waxy*); DQ197564 (ITS); DQ197249 (*matK*); DQ197275 (*ndhF*); DQ197201 (*rps16*); DQ197800 (*waxy*)
**Tiquilia plicata* (Torr.) A.T. Richardson	USA, California: Riverside County, *M. J. Moore 196*; DQ197246 (*matK*); DQ197274 (*ndhF*); DQ197205 (*rps16*); DQ197806 (*waxy*)
**Tiquilia purpusii* (Brandegee) A.T. Richardson	México: San Luís Potosí, *M. J. Moore 109*; DQ197409 (ITS); DQ197245 (*matK*); DQ197273 (*ndhF*); DQ197090 (*rps16*); DQ197789 (*waxy*)
**Tiquilia turneri* A.T. Richardson	México: Coahuila, *M. J. Moore 89*; DQ197398 (ITS); DQ197237 (*matK*); DQ197262 (*ndhF*); DQ197181 (*rps16*); DQ197706 (*waxy*)
Boraginaceae subfam. Heliotropioideae	
Tournefortia acutiflora M. Martens and Galeotti	AF047813 (*ndhF*; Ferguson, 1999)
Heliotropium arborescens L.	AF014000 (*ndhF*; Ferguson, 1999)

(*continued on next page*)

Table 1 (*continued*)

Species	GenBank/collection Info.
Hydrophyllaceae	
Codon schenckii Schinz	AF047776 (*ndhF*; Ferguson, 1999)
Eriodictyon californicum Greene	AF047820 (*ndhF*; Ferguson, 1999)
Hydrophyllum virginianum L.	AF019646 (*ndhF*; Ferguson, 1999)
Nama sericeum Willd.	AF047798 (*ndhF*; Ferguson, 1999)
Phacelia congesta Hook.	AF047780 (*ndhF*; Ferguson, 1999)
Romanzoffia californica Greene	AF047804 (*ndhF*; Ferguson, 1999)
Tricardia watsonii Torr. ex S. Watson	AF047775 (*ndhF*; Ferguson, 1999)
Wigandia urens Urb.	AF047763 (*ndhF*; Ferguson, 1999)
Loganiaceae	
Logania vaginalis (Labill.) F. Muell.	AJ235837 (*ndhF*; Backlund et al., 2000)
Rubiaceae	
Luculia gratissima Sweet	AJ011987 (*ndhF*; Oxelman et al., 1999)
Solanaceae	
Nicotiana tabacum L.	NC_001879 (*ndhF*; Shinozaki et al., 1986)
Vahliaceae	
Vahlia capensis Thunb.	AJ429112 (*ndhF*; Bremer et al., 2002)

Species and accessions included in the 16-taxon combined analyses are denoted with an asterisk (∗). All vouchers are deposited at TEX, unless otherwise noted (standard herbarium acronyms are used).

(Lennoaceae) was chosen because of some recent molecular work indicating a possible close relationship between it and *Tiquilia* (Olmstead and Ferguson, 2001; Smith, 1998; Smith et al., 2000). However, sequences derived from *P. arenarium* were always the most distant from *Tiquilia* in preliminary analyses (see Figure S1 in supplementary materials for an example), and the amount of divergence present in these sequences was suggestive of an accelerated rate of evolution, a common feature of holoparasitic genomes (Barkman et al., 2004; Nickrent et al., 1998). Because of the problems such divergent sequences can pose in phylogenetic analysis (Nickrent et al., 1998; Stefanovic and Olmstead, 2004), *Pholisma* was excluded from all analyses (although the sequences derived from *Pholisma* have been deposited in GenBank). The remaining three outgroups were included in the *ndhF* analyses, while only *Coldenia* and *Bourreria* were included in the combined analyses (also called the "16-taxon" analyses) and in the SH tests to simplify the analyses.

In addition to these three outgroups, *ndhF* sequences of 15 additional outgroups were selected from earlier work on Boraginales [mainly from Ferguson (1999); Table 1]. These taxa are representative of all the major groups of Boraginales, except Lennoaceae [although we had material of *Pholisma*, it lacks *ndhF* (Bremer et al., 2002)]. In addition, *ndhF* sequences representing four families of euasterids closely related to Boraginales were selected to provide a means for calibrating the stem lineage of Boraginales in the r8s analysis using the dates of Bremer et al. (2004; Table 1). These *ndhF* analyses will also be referred to as the "41-taxon" analyses.

2.3. DNA extraction, amplification, sequencing, and alignment

All accessions of *Tiquilia* were field collected in silica gel, and vouchers deposited in TEX. Of the outgroups, *E. anacua* was collected and extracted from fresh material at the University of Texas, while whole genomic DNA of *Coldenia*, *Pholisma*, and *Bourreria* was provided by other labs (Table 1). All accessions of *Tiquilia* and *Ehretia* were extracted using the 0.1 g protocol of the Nucleon Phytopure DNA extraction kit (Amersham Biosciences), with 10 μL of 2-mercaptoethanol added to each extraction.

Amplification of the five molecular markers utilized the primers listed in Table 2. The total *ndhF* region amplified includes the entire gene sequence with the exception of the first 23 bases at the 5′-end, and the last 146 bases at the 3′-end (relative to the sequence for *Nicotiana tabacum*). The *matK* primers amplified a region including the entire *matK* gene as well as the 3′-end of the *trnK* intron. Both *ndhF* and *matK* were amplified in two separate, overlapping fragments, and were sequenced using 8 and 7 primers, respectively. All other markers were sequenced using the same primers used for amplification. *Waxy* is present in a single copy in most angiosperms (Mason-Gamer et al., 1998), although it is present in multiple copies in some groups (Evans et al., 2000; Winkworth and Donoghue, 2004). It has been found to be useful for reconstructing phylogenies at the species level in several plant families, notably in Poaceae (Ingram and Doyle, 2004; Mason-Gamer et al., 1998; Mathews et al., 2002) and Solanaceae (Peralta and Spooner, 2001; Walsh and Hoot, 2001). This study represents the first use of this marker in the Boraginales. The *waxy* primers used in this study amplified a region approximately 600 bp long that is homologous to the region from exon 9 to exon 11 in *Ipomoea* (Convolvulaceae; Miller et al., 1999). However, the primers designed specifically for this study were located immediately inside the 5′ and 3′-primers of Miller et al. (1999).

A few taxa exhibited clearly evident sequence polymorphism in directly sequenced PCR product for ITS and/or

Table 2
List of primers utilized in this study

Marker/primer name	Primer sequence (5′ → 3′)	Reference
ndhF		
ndhF 1F	ATG GAA CAK ACA TAT SAA TAT GC	Olmstead and Sweere (1994)
ndhF 536F*	TTG TAA CTA ATC GTG TAG GGG A	Olmstead and Sweere (1994)
ndhF 972F	GTC TCA ATT GGG TTA TAT GAT G	Olmstead and Sweere (1994)
ndhF T972F	GTC TCA GTT RGG TTA TAT GAT G	Designed for this study
ndhF 1318F*	GGA TTA ACY GCA TTT TAT ATG TTT CG	Olmstead and Sweere (1994)
ndhF T1318F*	GGA TTA ACT GCA TTT TAT ATG TTT CG	Designed for this study
ndhF T730R*	CAT ACA TGA AGT GGA AAT TGT GCA	Designed for this study
ndhF 972R*	CAT CAT ATA ACC CAA TTG AGA C	Olmstead and Sweere (1994)
ndhF T972R*	CAT CAT ATA ACC YAA CTG AGA C	Designed for this study
ndhF 1318R	CGA AAC ATA TAA AAT GCR GTT AAT CC	Olmstead and Sweere (1994)
ndhF T1318R	CGA AAC ATA TAA AAT GCA GTT AAT CC	Designed for this study
ndhF 1603R*	GCA TAG TAT TGT CCG ATT CAT RAG G	Olmstead and Sweere (1994)
ndhF T1603R*	ACA TAG TAT TAT CCG ATT CC	Designed for this study
ndhF 2110R	CCC CCT AYA TAT TGA TAC CTT CTC C	Olmstead and Sweere (1994)
matK		
matK 1F	ACT GTA TCG CAC TAT GTA TCA	Sang et al. (1997)
matK 230F*	GTT CAC TAA TTG TGA AAC GT	Sang et al. (1997) (=*matK* 2F)
matK T230F*	CAG TTT ACT AAT TGT GAA ACG T	Designed for this study
matK 590F	AAG ATG CCT CTT CTT TGC AT	Sang et al. (1997) (=*matK* 3F)
matK T590F	AAG ACC CCT CTT CTT TGC AT	Designed for this study
matK 1320F*	TCT CAT TAT CAC AGC GGA TC	Sang et al. (1997)
matK T1320F*	TCT CAT TAT TAT AGC GGA TC	Designed for this study
matK 1320R	GAT CCG CTG TGA TAA TGA GA	Sang et al. (1997) (=*matK* 3R)
matK T1320R	GAT CCG CTA TAA TAA TGA GA	Designed for this study
matK 1580R*	TTC ATG ATT GGC CAG ATC A	Sang et al. (1997) (=*matK* 2R)
matK T1580R*	TTG ATG ATT GGC CAG ATC A	Designed for this study
matK 1820R	GAA CTA GTC GGA TGG AGT AG	Sang et al. (1997) (=*matK* 1R)
rps16 intron		
rps16 F	GTG GTA GAA AGC AAC GTG CGA CTT	Oxelman et al. (1997)
rps16 R2	TCG GGA TCG AAC ATC AAT TGC AAC	Oxelman et al. (1997)
ITS		
ITS 1A (modified ITS 5)	GGA AGG AGA AGT CGT AAC AAG G	Downie and Katz-Downie (1996)
ITS 4	TCC TCC GCT TAT TGA TAT GC	White et al. (1990)
waxy		
waxy 9F	GAT ACC CAA GAG TGG AAC CC	Miller et al. (1999)
waxy T9F-alt	GCA ACT GAT AAA TAC ATT GAT GTT C	Designed for this study
waxy 11R	GTT CCA TAC GCA TAG CAT G	Miller et al. (1999)
waxy T11R-alt	CAA TTG AAT GAG ACC ACA AGG CTC	Designed for this study

Primers denoted with an asterisk (*) were used only for sequencing. Numbers in primer names for *ndhF* and *matK* primers refer to approximate base pair position downstream of the transcription start position. F, forward primer; R, reverse primer.

waxy. These accessions were cloned using the TOPO TA kit (Invitrogen) with vector pCR 2.1-TOPO using one-third the recommended reaction volumes. For each cloning reaction, from 5 to 15 positively transformed colonies were reamplified and the products sequenced. In all but one case, cloned sequences from a given accession formed a monophyletic group and in these cases one cloned sequence per accession was included in the combined phylogenetic analyses. The cloned *waxy* sequences of the allotetraploid *Tiquilia elongata* fell into two monophyletic groups, the first nearly identical in sequence to *T. palmeri*, and the second clearly more distant and basal to the preceding clade. Because the other four markers agree with the phylogenetic position implied for *T. elongata* by the second group of *waxy* sequences, one of these sequences was selected for the combined analyses in this paper. Cloned accessions are listed in Table 1.

PCR volumes of 25 μL included 10–100 ng of template DNA, 12.5 μL of FailSafe PCR 2X Premix J (Epicentre), 0.4 mM of each primer, and 0.5 U *Taq* polymerase. ITS and the *rps16* intron were amplified using an initial denaturation of 96 °C (3 min), followed by 36 cycles of 94 °C (1 min), 54 °C (1 min), and 72 °C (45 s + 3 sec/cycle), followed by a final extension at 72 °C (7 min). Amplification of *waxy* used the same program parameters but with a 50 °C annealing temperature. Amplification of *ndhF* and *matK* proceeded as follows: an initial denaturation of 96 °C (2 min, 30 sec), followed by 35 cycles of 94 °C (1 min), 50 °C (1 min), and 72 °C (2 min), followed by a final extension at 72 °C (15 min). PCR parameters were identical for cloned template except for the substitution of an initial 10 min hot start denaturation at 95 °C, followed by the addition of *Taq* polymerase.

PCR products were cleaned using QiaQuick columns (Qiagen) and the amount of product was quantified using agarose gel electrophoresis with a low mass DNA ladder. Cycle sequencing reactions included 20–40 ng of template DNA, 2 μL Big Dye terminator (Perkin-Elmer), and 0.5 mM of each primer per 20 μL reaction volume. The cycle sequencing program included an initial denaturation of 96 °C (2 min) followed by 26 cycles of 96 °C (10 s), 50 °C (5 s) and 60 °C (4 min). Samples were then cleaned using Centri-Sep columns (Princeton Separations) packed with G-50 Sephadex (Amersham Biosciences), and then sequenced using an MJ Research BaseStation automated sequencer.

Raw sequences were trimmed and edited using Sequencher v. 3.0 and v. 4.0 (GeneCodes). Sequences from each marker were aligned initially using ClustalX (Thompson et al., 1997), and the resulting alignments were adjusted manually using SeqApp (Gilbert, 1992), with the exception of the *ndhF* data set, which was aligned manually. Several short regions of ITS (amounting to 13.7% of the total ITS alignment) that were difficult to align among the major lineages of *Tiquilia* and/or the outgroups were eliminated from the combined analyses. All sequences have been deposited in GenBank (Table 1), and the final alignments are available in TreeBASE (study accession number S1426).

2.4. Phylogenetic analyses

Parsimony and maximum likelihood (ML) analyses were conducted on both the 5-marker, 16-taxon combined data set and the 41-taxon *ndhF* data set in PAUP* v. 4.0b10 (Swofford, 2002) using heuristic searches with TBR branch swapping, multrees in effect, and with gaps treated as missing data. Gaps were included in parsimony analyses, but were coded separately using the simple gap coding method of Simmons and Ochoterena (2000). Parsimony heuristic searches included 100 random sequence addition replicates, and clade support was assessed using nonparametric bootstrap analyses with 100 replicates. ML analyses incorporated the best fitting model of sequence evolution as selected by Modeltest v. 3.6 (Posada and Crandall, 1998) using the Akaike Information Criterion (Posada and Buckley, 2004), with the separate gap characters excluded and 10 random sequence addition replicates.

Bayesian analyses were also conducted on both data sets, using the Metropolis Coupled Markov Chain Monte Carlo simulation program, MrBayes v. 3.0b4 (Huelsenbeck and Ronquist, 2001). Modeltest selected GTR+I+Γ (Rodríguez et al., 1990) as the appropriate model for the combined data, and TVM+I+Γ (Posada and Crandall, 1998) for the *ndhF* data; because the latter model is not implemented in MrBayes, we set the model to GTR+I+Γ for the *ndhF* data. Three replicate analyses were run for 3 million generations each to ensure that the runs were converging on the appropriate posterior probability distribution. Analyses were conducted with four chains, with the heating set to 0.15 and proposal parameters adjusted to ensure acceptance rates between 10 and 70%. Trees were sampled every 100 generations, and the point of stationarity was determined by examining plots of the values of the estimated parameters against generation time in Microsoft Excel. All trees prior to reaching stationarity were discarded and the remaining trees were used to compute majority rule consensus trees.

To assess whether *Tiquilia* is of North or South American origin, the current distributions of the various taxa within *Tiquilia* were included as separate characters in the 5-marker combined data set. The distribution of the outgroup *B. succulenta* was defined as the composite distribution of the monophyletic group composed of the sister genera *Bourreria* P. Browne (North, South, and Central America, and the Caribbean) and *Hilsenbergia* Tausch ex Meisn. (Africa; Gottschling and Hilger, 2001; Miller, 2003). The resulting distribution character was mapped using MacClade v. 4.06 (Maddison and Maddison, 2003) onto the tree found from the ML search of the combined data set.

2.5. Test of alternate topologies

Preliminary analyses of each data partition indicated potential incongruence in the branching order of the well-supported major lineages within each subgenus of *Tiquilia*. Even though the 5-marker, 16-taxon reduced data set indicated strong support for all nodes involving the major lineages of *Tiquilia*, a Shimodaira–Hasegawa (SH) test (Goldman et al., 2000; Shimodaira and Hasegawa, 1999) was therefore conducted to determine if any other topologies within *Tiquilia* were reasonable alternatives to the highest likelihood topology. The SH test is an appropriate test of topology if one of the topologies is selected on an *a posteriori* basis (Goldman et al., 2000). In order to ensure that the topology with the highest likelihood is always available when running the SH test, it is desirable to include every possible reasonable topology during the analysis (Goldman et al., 2000). To this end we exhaustively tested two sets of topologies: in one set of topologies, every possible branching order among the four well-supported major lineages of subg. *Eddya* (*T. canescens* + *T.greggii*, the *T. gossypina* clade, the *T. hispidissima* clade, and *T. purpusii*), while holding the topology of subg. *Tiquilia* constant, was included (15 possible topologies), along with a tree in which all four lineages formed a basal polytomy; while in the other set of topologies, we tested the two alternative branch orders of the three well-supported lineages of subg. *Tiquilia* (the *T. plicata* clade, the *T. palmeri* clade, and *T. nuttallii*) while holding subg. *Eddya* constant, again with a tree in which the three lineages formed a polytomy. This resulted in a total of 19 topologies that were included in the SH test, which employed the same ML settings used for estimating the ML topology of the 5-marker data set, with RELL optimization (1000 replicates; Goldman et al., 2000; Shimodaira and Hasegawa, 1999) as implemented in PAUP* 4.0b10.

2.6. Molecular-based dating

The tree topology with the highest likelihood score for the 41-taxon *ndhF* data set was used to infer divergence times. Because the favored likelihood topology did not recover *Vahlia* as sister to Boraginales (see Section 3) as has been found in some recent analyses (Bremer et al., 2002; Lundberg, 2001), divergence times were estimated using two different root positions on the likelihood tree: the root for one tree was unconstrained, while the root of the other tree was constrained to have *Vahlia* as sister to Boraginales (these topologies will be referred to as the unconstrained and *Vahlia*-constrained topologies, respectively). Similarly, because a different outgroup topology of ((*Ehretia*, *Bourreria*), *Tiquilia*) was recovered instead of (*Ehretia*, (*Bourreria*, *Tiquilia*)) in some of the individual marker preliminary analyses, this alternate topology was also included in the dating analyses. All topologies that could not be rejected by the SH test of the combined data set were also included in the dating analyses, to see what effect these alternative topologies had on divergence times. Branch lengths for all alternate topologies were estimated using the model selected by Modeltest for the *ndhF* data set.

We utilized the age inferred for the divergence of the crown group uniting *Borago* and *Nicotiana* (~106 Ma) in the chronogram of asterid ages in Bremer et al. (2004) as a fixed node age in both the unconstrained and *Vahlia*-constrained *ndhF* topologies. The ages of Bremer et al. are mean ages derived from analyses of six well-dated and well-placed fossil-based constraints, and represent the best current effort at dating divergence times in asterid angiosperms. For the tree in which *Vahlia* was constrained to be sister to Boraginales, we applied a second fixed divergence date of 104 Ma to the *Vahlia* + Boraginales node. The divergence date for this node was also derived from Bremer et al. (2004), and was selected because *Vahlia* is closest to Boraginales under this constraint. In addition, one possible alternate calibration point is available for Boraginaceae subfamily Ehretioideae in the form of a well-dated fossil fruit with clear affinities to *Ehretia* P. Browne that has been dated to the early Eocene (~50 Ma; Gottschling et al., 2002). This fossil has been utilized by Gottschling et al. (2004) to date divergence times in the woody Boraginales. However, we could not appropriately apply this date to *Ehretia* in the *ndhF* tree because the only node in the *ndhF* phylogeny for this genus occurs in a more derived position compared to the node to which the 50-Ma date was applied in Gottschling et al. (2004). Instead, we constrained subfamily Ehretioideae to have a minimum age of 50 Ma, as this is a logically appropriate, although less desirable, placement for such a constraint (Magallón, 2004).

Divergence times were estimated using the program r8s v. 1.70 (Sanderson, 2003). A likelihood ratio test of molecular clock-constrained and unconstrained ML trees indicated a significant level of rate heterogeneity ($p \ll 0.001$) in the *ndhF* data set, and for this reason we applied a rate smoothing approach using penalized likelihood (Sanderson, 2002) with the TN algorithm to estimate divergence times. The cross-validation procedure outlined in Sanderson (2002) was performed to determine the appropriate smoothing rate for penalized likelihood. The cross-validation analyses for the unconstrained *ndhF* topology indicated that a molecular clock approach was a more appropriate method for estimating divergence times (Sanderson, 2004) under this rooting scheme. We therefore implemented the Langley–Fitch (LF) method (Langley and Fitch, 1974) using the TN algorithm to derive dates for this topology. In all cases, the furthest outgroups were pruned from the analysis to eliminate the presence of arbitrary zero-length branches at the root of the tree, and all zero-length internal branches were collapsed. A total of five starts per optimization was conducted to check for multiple optima, and gradient checks of the correctness of each optimization solution (Sanderson, 2004) were performed.

Standard errors of divergence dates were estimated in two ways. The first method involved the nonparametric bootstrapping procedure outlined in Sanderson (2004), which measures the error that results from potential inaccuracy in tree reconstruction due to sampling a finite number of nucleotides. First, 100 bootstrapped data sets were created from the original data set with the program Mesquite v. 1.05 (Maddison and Maddison, 2004). Branch lengths were then reestimated for each bootstrapped data set in PAUP* using the original ML parameter estimates and both the unconstrained and *Vahlia*-constrained *ndhF* ML topologies. The resulting trees with branch lengths were then imported into r8s, and means and standard deviations were then estimated using the 'profile' command using both the 104 and 106 Ma calibration points. The second method for measuring standard error involved a parametric bootstrapping approach (Goldman et al., 2000; Huelsenbeck et al., 1996; Swofford et al., 1996) similar to that used by Davis et al. (2002), which measures the error generated by the process of molecular evolution. Using the *ndhF* ML tree topology, branch lengths, and parameter estimates, 100 data sets were simulated with Mesquite v. 1.05. Branch lengths were then reestimated for each simulated data set in PAUP* in the same manner as for the nonparametric bootstrap analysis, and means and standard deviations were again estimated using the 'profile' command in r8s using both calibration points.

3. Results

Preliminary phylogenetic analyses confirmed that *Tiquilia* is part of Boraginaceae subfamily Ehretioideae, and is sister to either *Bourreria* alone or to *Bourreria* + *Ehretia* (see Supplemental materials). These same analyses suggested the monophyly of both *Tiquilia* and its subgenera, and suggested the existence of seven well-supported major lineages. Subgenus *Eddya* contains four of these lineages: (1) *Tiquilia canescens* + *T. greggii*; (2) the *T. gossypina* clade; (3) the *T. hispidissima* clade; and (4) *T. purpusii*. Subgenus *Tiquilia* contains the remaining three major lineages: (5) the

Table 3
Summary of data set characteristics

Data set	Aligned length (bp)	G + C content (%)	# pars. inform. characters	# gap characters (# inform.)	# MPTs	RCI	RI	Model selected
5-marker combined (all markers)	6040	37.5	644	153 (59)	1	0.613	0.767	GTR+I+Γ
5-marker combined (*ndhF*)	2062	33.0	152	2 (1)	n/a	n/a	n/a	n/a
5-marker combined (*matK*)	1775	33.3	137	13 (6)	n/a	n/a	n/a	n/a
5-marker combined (*rps16*)	900	33.0	69	41 (15)	n/a	n/a	n/a	n/a
5-marker combined (ITS)	677	67.6	143	60 (27)	n/a	n/a	n/a	n/a
5-marker combined (*waxy*)	626	39.5	143	37 (10)	n/a	n/a	n/a	n/a
ndhF	2087	32.8	418	n/a (gaps not included)	10	0.481	0.754	TVM+I+Γ

MPTs, most parsimonious trees; RCI, rescaled consistency index; RI, retention index; I, proportion of invariant sites; and Γ, rate heterogeneity. References: GTR (Rodríguez et al., 1990), TVM (Posada and Crandall, 1998).

T. plicata clade; (6) the *T. palmeri* clade; and (7) *T. nuttallii*. This preliminary work was used to select both the 16 taxa to include in the 5-marker combined analyses and the 19 species of *Tiquilia* to include in the *ndhF* 41-taxon dating analyses.

The aligned lengths of the 5-marker (partitioned by marker) and the *ndhF* data sets are indicated in Table 3, along with general data set characteristics and the number of gap characters used in parsimony analyses. The 5.8S, *ndhF*, and *matK* genes had the least amount of variation, while the coding regions of *waxy* and the non-coding *trnK* and *rps16* introns had an intermediate amount of variation. The two introns of *waxy* and ITS were highly variable, with regions of ITS unalignable, even in some cases between major lineages of *Tiquilia*.

Waxy sequences of *Tiquilia* and its outgroups showed high similarity to the regions from exon 9 through exon 11 of *waxy* sequences of Solanaceae and Convolvulaceae in GenBank. Exon and intron boundaries were determined by sequence comparison to other GenBank *waxy* sequences and to 5′ and 3′ consensus sequences for intron splice sites in plants (Csank et al., 1990). *Waxy* sequences of *Tiquilia* and the three outgroups contained portions of exon 9 (ranging from 26 to 43 bp of the 3′-end) and exon 11 (ranging from 126 to 147 bp of the 5′-end), and all of intron 9 (ranging from 91 to 103 bp), exon 10 (all sequences contained 177 bp), and intron 10 (ranging from 105 to 120 bp). No indels were detected in any exon sequences.

Parsimony, ML, and Bayesian searches produced identical topologies for each data set and are therefore treated together. The replicate Bayesian analyses that were performed for both data sets were consistent with each other and indicated that the posterior distribution was properly sampled. Modeltest selected the GTR+I+Γ model as the model that best described the 5-marker combined data, while it selected the TVM (one transition and four transversion types; Posada and Crandall, 1998) +I+Γ model for the *ndhF* data. The ML tree for the 16-taxon, 5-marker data set is depicted in Fig. 1, with Bayesian posterior probabilities indicated (bootstrap values are omitted from the tree because they were identical to Bayesian posterior probabilities). The parsimony analysis of the 5-marker data set returned 10 most parsimonious trees that differed only in the branching order among the taxa of the *T. gossypina* clade. Biogeographic analysis supports a North American origin for *Tiquilia*, including both subgenera and all seven major lineages, with dispersals to South America and the Galápagos Islands (Fig. 1).

The unconstrained maximum likelihood topology for the *ndhF* data set is depicted in Fig. 2, with parsimony bootstrap percentages and Bayesian posterior probabilities also indicated. The root of the *Vahlia*-constrained topology is indicated by the arrow in Fig. 2; this topology is also depicted in Fig. S4 (Supplemental materials). The parsimony analysis

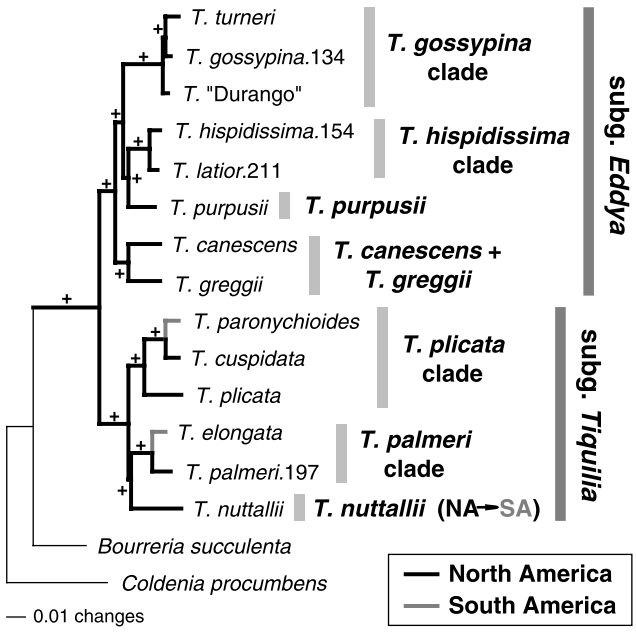

Fig. 1. Phylogram of the tree resulting from the maximum likelihood search of the 5-marker combined data set ($\ln L = -19287.127$), with a reconstruction of dispersal history mapped onto the tree. Plus signs (+) above or below the branches indicate Bayesian posterior probabilities of 1.0. The distributions of outgroup taxa are not shown because they have either Old World or pantropical distributions and because they do not influence the reconstruction of dispersal history in *Tiquilia*. The single dispersal event from North America to South America in *T. nuttallii* is not shown on the tree. The Galápagos taxa of subg. *Tiquilia* were not included in this analysis, but their absence does not affect the North American origin of all major lineages of *Tiquilia*.

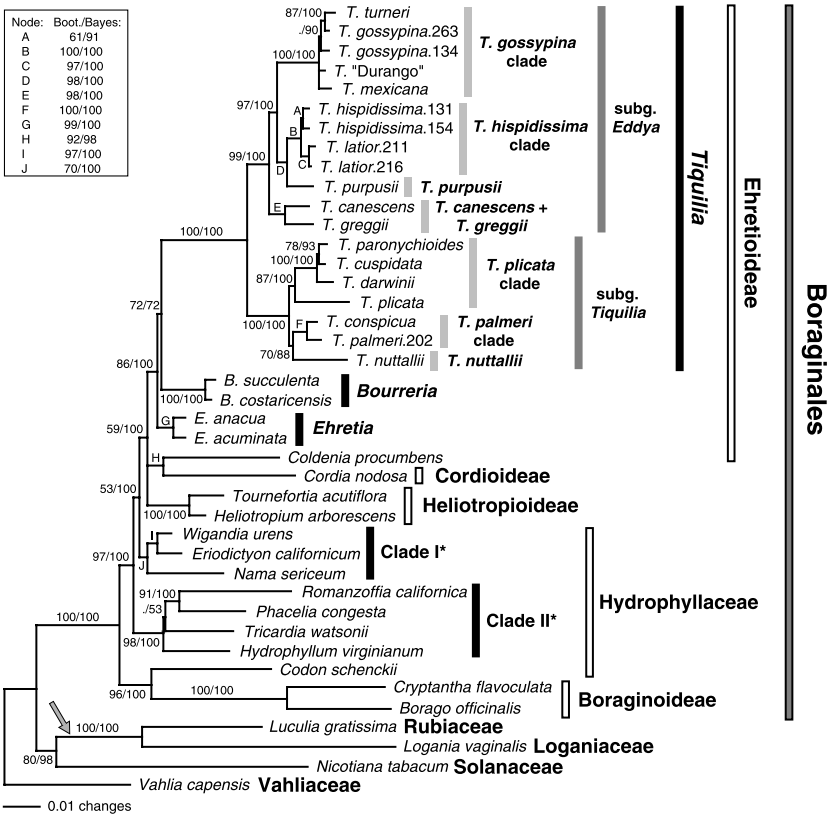

Fig. 2. Phylogram of the tree resulting from the maximum likelihood search of the *ndhF* data set, with unconstrained rooting, showing major lineages within *Tiquilia* and the Boraginales. Parsimony bootstrap proportions and Bayesian posterior probabilities above 50% respectively are indicated above or below the branches, or are indicated in the box at upper left. The arrow indicates the position of the root of the tree under the *Vahlia*-constrained *ndhF* topology (this topology is depicted in Fig. S4 in the Supplemental materials). *Sensu Ferguson (1999).

of the *ndhF* data set returned a single most parsimonious tree.

Seven of the 19 alternate combined data set topologies could not be rejected at the $p = 0.05$ level by the SH test. These seven topologies are depicted in a simplified form in Fig. 3.

Maximum likelihood analysis of the *ndhF* data set returned a tree with an unconstrained likelihood score of −11529.351 and a clock-constrained likelihood score of −11682.074. A χ^2 test of twice the difference in these likelihoods strongly rejected clock-like evolution in this data set ($2 \times \text{difference} = 305.446$; $df = 39$; $p \ll 0.001$).

The node ages derived from the r8s analyses of the *ndhF* data set using the 104 Ma calibration point on the *Vahlia*-constrained topology are depicted in the chronogram in Fig. 4. These values are also given in Table 4, along with the node ages derived from the 106 Ma calibration point using both the *Vahlia*-constrained and the unconstrained *ndhF* topology (the node ages derived for the unconstrained topology are also depicted in Fig. S5 in the supplemental materials). The divergence dates obtained using the *Vahlia*-constrained topology were consistently older than those derived from the unconstrained topology. The oldest divergence dates were obtained under the 104 Ma (*Vahlia* + Boraginales) calibration point. These dates were approximately 5–6% older than those obtained from the 106 Ma calibration point (*Nicotiana* + *Vahlia*/Boraginales) using the *Vahlia*-constrained *ndhF* topology, and varied considerably from 6 to 57% older than those obtained using the unconstrained topology (Table 4). The mean ages and standard deviations obtained for each node in the nonparametric and parametric bootstrap analyses were similar in all cases and are indicated in Table 4 and Table S1 (Supplemental materials), respectively. Cross-validation analyses suggested that a smoothing rate of 100 was the most appropriate value for the penalized likelihood analyses, and gradient checks of optimization solutions passed in all cases. Dates obtained from the 104 and 106 Ma calibration points did not conflict with the 50-Ma minimum age constraint for Boraginaceae subfam. Ehretioideae under the *Vahlia*-constrained topology; however, the 50 Ma constraint was enforced for Ehretioideae using the unconstrained topology. The analysis of the alternate topology of ((*Bourreria*, *Ehretia*), *Tiquilia*) produced divergence times that varied only slightly from the ML topology, and hence these times are not listed. Node ages derived from the analyses of the SH trees were essentially identical in most cases, with significant differences restricted to nodes that were not present in the original ML topology. These different node ages are depicted on the SH trees themselves in Fig. 3; other dates are omitted.

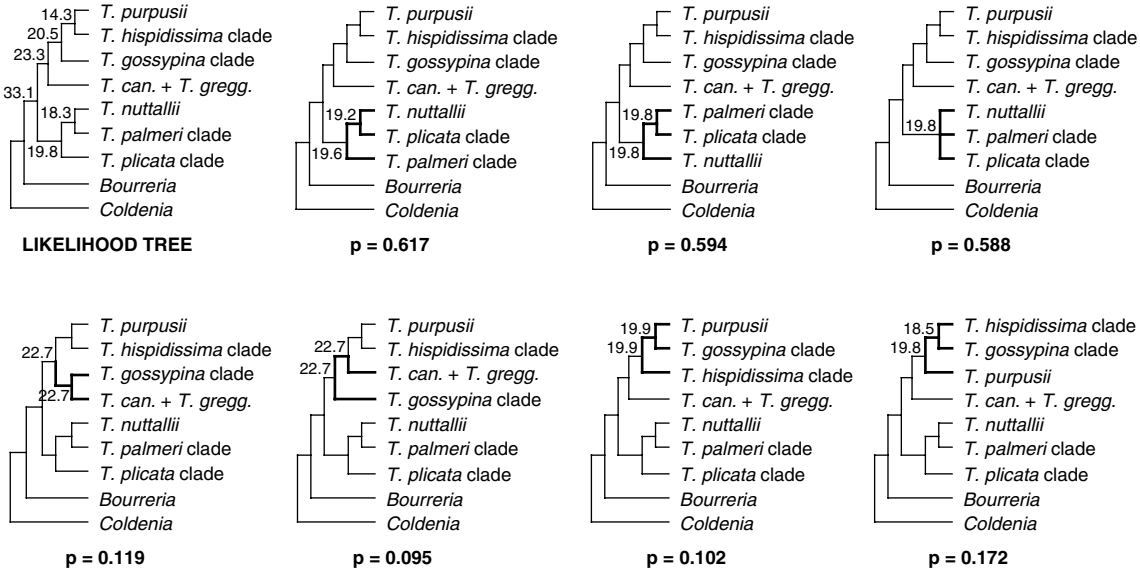

Fig. 3. Non-rejected SH test topologies, reduced to the seven major lineages of *Tiquilia* and two outgroups. The topology favored by the likelihood search of the 5-marker combined data set is shown at upper left, with crown ages of nodes derived from the r8s analysis (using the 104 Ma calibration point) given on the subtending stem branches. Branches in bold within the SH test trees represent differences in topology relative to the likelihood tree. Crown ages of these differing nodes are shown on the non-rejected trees; ages of other nodes did not vary appreciably from the node ages on the likelihood tree. Probability values derived from the SH test are given under the trees.

4. Discussion

4.1. Phylogenetic relationships of Tiquilia

Tiquilia, its two subgenera, and all seven of the major lineages suggested by the preliminary data are strongly supported as monophyletic in both the *ndhF* and the 5-marker combined analyses (Figs. 1 and 3). The relationships within some of these seven lineages are sometimes difficult to recover using a larger sample of taxa, particularly within the *T. gossypina*, *T.hispidissima*, and *T. plicata* clades. Incongruence among one or more data partitions exists in all three of these lineages. Such incongruence is frequently encountered when reconstructing relationships among closely related species (McKinnon, 2005; Wendel and Doyle, 1998), and since it is of no consequence to the deeper-level relationships in the genus, it is not discussed further here.

Reduction of the combined data matrix to include two outgroups and 14 exemplar taxa within *Tiquilia* results in a non-significant ILD test result ($p = 0.15$) among all five data partitions, suggesting congruence among all markers in the relationships among the seven major monophyletic lineages of *Tiquilia*. This congruence is corroborated by the strong bootstrap and Bayesian support for the branching order in the 16-taxon combined data phylogeny (Fig. 1). However, the SH test of the branching order among these seven lineages fails to reject seven of 19 alternate topologies (Fig. 3), including all three alternate topologies in subg. *Tiquilia*. The remaining four alternate topologies are restricted to subg. *Eddya* and do not appear to follow an easibly discernible pattern. Therefore the SH test would seem to indicate a lack of confidence in the deeper-level branch order within *Tiquilia*. However, the topology in the combined data phylogeny makes sense morphologically (e.g., the *T. gossypina*, *T. hispidissima*, and *T. purpusii* clades form a natural group based on a similar fruit morphology; the same could be said about the *T. palmeri* clade and *T. nuttallii* based on foliar morphology), and it is possible, if not likely, that with even more sequence data the SH test would converge on the combined data topology. Because the combined data and *ndhF* topologies are identical, with very similar proportional branch lengths, and because a much larger sample of outgroups is available for *ndhF*, it seems reasonable to utilize the *ndhF* phylogeny for analyses of divergence times.

The *ndhF* data allow for a reasonably clear picture of the position of *Tiquilia* within the Boraginales (Fig. 2). The *ndhF* phylogeny is largely congruent with the ITS phylogeny of Boraginales in Gottschling et al. (2001) with respect to the major lineages of Boraginales, and supports the position of *Tiquilia* within Ehretioideae, with *Tiquilia* sister to the American/African tropical sister genera *Bourreria* and *Hilsenbergia* (represented by *Bourreria* only in the *ndhF* phylogeny; Gottschling and Hilger, 2001; Miller, 2003). However, there is only moderate support for this relationship (72% bootstrap proportion; Bayesian posterior probability = 0.72). In some of the preliminary individual marker analyses that include both *Ehretia* and *Bourreria*, the two instead form a sister group to *Tiquilia*. The *ndhF* phylogeny also strongly supports the monophyly of Ehretioideae (excluding *Coldenia*, which in our analysis is sister to *Cordia*) and of Boraginales. The other subfamilial taxa of Boraginaceae [including subfamilies Boraginoideae,

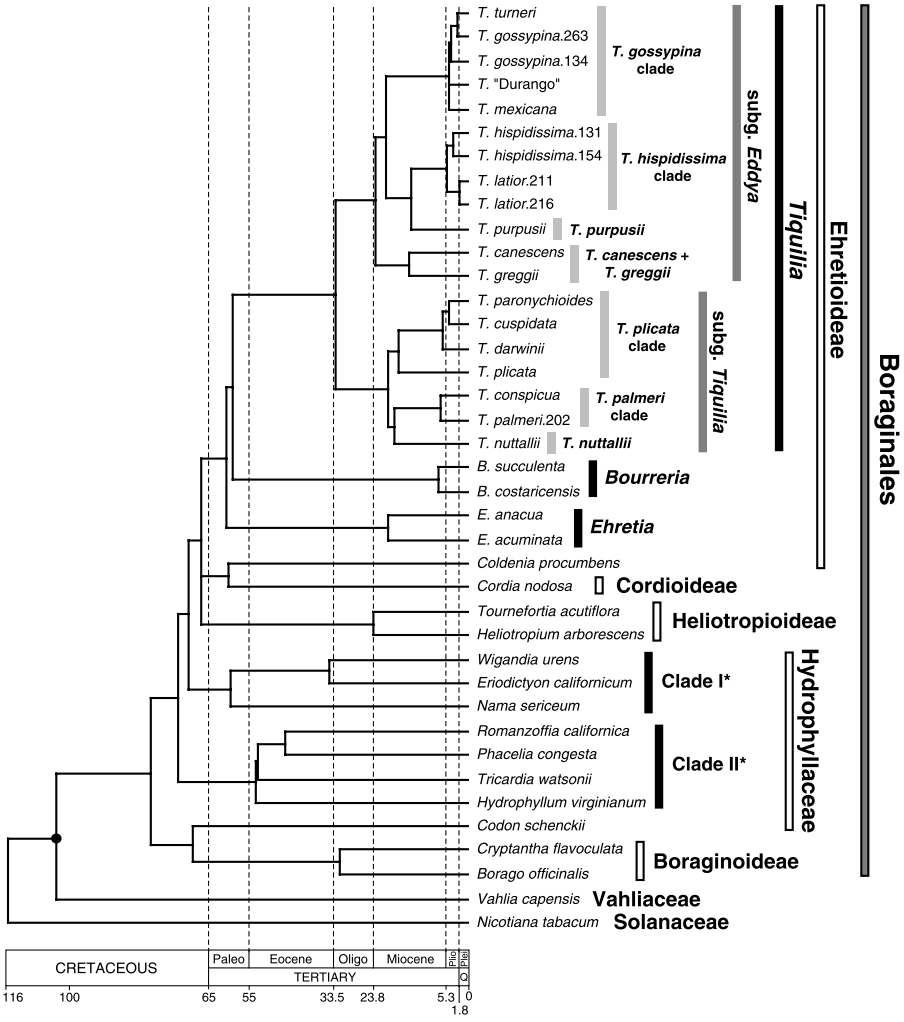

Fig. 4. Chronogram of the *ndhF* lnL tree derived from the penalized likelihood analysis of the *Vahlia*-constrained topology, showing major lineages within *Tiquilia* and the Boraginales. The black circle indicates the node whose age was fixed to 104 Ma in the analyses. A Cenozoic and partial Cretaceous timescale are shown at bottom; units are Ma (million years ago). *Abbreviations:* Paleo, Paleocene; Oligo, Oligocene; Plio, Pliocene; Plei, Pleistocene; and Q, Quaternary. Dates follow Berggren et al. (1995). *Sensu Ferguson (1999).

Cordioideae (including *Coldenia*), and Heliotropioideae] and Hydrophyllaceae (including clades I and II of Ferguson, 1999) are also recovered as monophyletic lineages, with reasonably strong support. The branching order among these lineages is well-supported by *ndhF*, although the branch lengths are relatively short.

4.2. Molecular dating in the Boraginales

The differing topologies that result from the alternate placement of the root on the *ndhF* ML tree produce varying divergence time estimates across the phylogeny, but the most significant age discrepancies occur near the base of the Boraginales. Both the unconstrained and *Vahlia*-constrained topologies suggest that extant Boraginales first diversified in the late Cretaceous (79.7–74.5 Ma; Table 4), and both agree on a relatively rapid diversification of the major lineages of Boraginales, including *Codon*, the Boraginoideae, Hydrophyllaceae clades I and II (sensu Ferguson, 1999), and the Heliotropioideae, Cordioideae (including *Coldenia*), and Ehretioideae. However, the *Vahlia*-constrained ML topology favors a late Cretaceous to early Tertiary diversification of these lineages, while the unconstrained topology suggests that these events occurred anywhere from 5 to 15 Ma later (Table 4; see also Fig. S5 in the Supplemental material). Within the Ehretioideae, an initial rapid radiation into the stem lineages of *Ehretia*, *Bourreria*, and *Tiquilia* is implied in the Paleocene (61.0–55.6 Ma) by the *Vahlia*-constrained analyses or in the Eocene (50.0–47.8 Ma) by the unconstrained analyses (Table 4).

Although we cannot be certain whether the node ages implied by the *Vahlia*-constrained or unconstrained analyses are more likely to be correct, the former dates seem more realistic given the presence of the ~50 Ma fossil *Ehretia* fruit described above, and are at least possible given the phylogenetically uncertain position of the Boraginales. While a sister relationship between Boraginales and Vahliaceae is not directly supported by the *ndhF* data, other analyses have recovered this relationship using a combination of

Table 4
Crown ages of nodes derived from the r8s molecular dating analyses, along with standard deviations of node ages derived from nonparametric bootstrap analyses

Clade name	*Vahlia*-constrained		Unconstrained	*Vahlia*-constrained		Unconstrained
	Crown age of clade—104 Ma cal. point (Ma)	Crown age of clade—106 Ma cal. point (Ma)	Crown age of clade—106 Ma cal. point (Ma)	Mean crown age ± SD, from nonparametric bootstrap (Ma)	Mean crown age ± SD, from nonparametric bootstrap (Ma)	Mean crown age ± SD, from nonparametric bootstrap (Ma)
Root	115.8	**106.0**	**106.0**	115.9 ± 5.6	**106.0**	**106.0**
Vahlia + Boraginales	**104.0**	99.1	—	**104.0**	99.4 ± 3.0	—
Nicotiana + *Logania*/*Luculia*	—	—	96.8	—	—	99.7 ± 2.9
Logania + *Luculia*	—	—	68.3	—	—	70.6 ± 4.5
Boraginales	79.7	75.6	74.5	80.7 ± 3.9	76.7 ± 4.3	74.1 ± 4.3
Codon + Boraginoideae	69.1	65.6	64.3	69.5 ± 4.3	66.2 ± 4.6	64.9 ± 4.5
Boraginoideae	31.9	30.4	31.9	31.8 ± 3.5	30.4 ± 3.4	31.3 ± 3.5
Hydr. Clade II + Clade I/ woody Borag.	73.0	69.0	59.7	73.9 ± 4.2	70.0 ± 4.7	59.2 ± 2.7
Hydrophyllace Clade II	53.6	50.6	42.0	54.2 ± 5.1	51.3 ± 5.1	41.3 ± 3.4
Tricardia + *Romanzoffia*/*Phacelia*	52.6	49.7	41.3	52.6 ± 5.2	49.8 ± 5.2	40.2 ± 3.4
Romanzoffia + *Phacelia*	46.1	43.6	35.6	46.8 ± 4.6	44.2 ± 4.4	35.7 ± 3.1
Hydroph. Clade I + woody Boraginales	70.3	66.2	55.5	70.7 ± 4.3	66.9 ± 4.8	55.3 ± 2.2
Hydrophyllaceae Clade I	59.5	55.7	37.3	60.6 ± 6.1	56.9 ± 6.7	35.4 ± 4.6
Wigandia + *Eriodictyon*	34.8	31.6	14.8	36.8 ± 10.1	33.6 ± 9.4	14.2 ± 3.1
Woody Boraginales	67.1	63.3	52.9	67.1 ± 4.2	63.5 ± 4.7	52.8 ± 1.9
Heliotropioideae	23.4	21.9	15.5	24.0 ± 4.9	22.4 ± 4.7	15.3 ± 3.0
Coldenia + *Cordia*	60.4	57.0	45.8	59.9 ± 5.0	56.7 ± 5.3	47.2 ± 2.7
Ehretioideae	61.1	57.5	**50.0**	61.2 ± 4.4	57.8 ± 4.5	**50.0**
Ehretia	19.8	17.9	10.3	21.9 ± 8.8	19.7 ± 7.7	9.4 ± 2.5
Bourreria + *Tiquilia*	59.0	55.6	47.8	58.8 ± 4.5	55.4 ± 4.5	48.0 ± 1.7
Bourreria	7.3	6.9	5.0	7.3 ± 2.3	6.8 ± 2.1	4.7 ± 1.5
Tiquilia	33.1	31.4	28.8	33.6 ± 3.4	31.9 ± 3.3	28.3 ± 2.1
T. subg. *Tiquilia*	19.8	18.8	18.1	20.1 ± 2.2	19.2 ± 2.2	17.5 ± 1.8
T. nuttallii + *T. palmeri* clade	18.3	17.4	16.7	18.3 ± 2.3	17.5 ± 2.2	15.9 ± 1.7
T. palmeri clade	6.8	6.5	6.2	6.6 ± 1.9	6.3 ± 1.8	5.7 ± 1.5
T. plicata clade	17.4	16.6	16.0	17.6 ± 2.0	16.8 ± 1.9	15.4 ± 1.7
T. darwinii + *T. paron.*/*T. cusp.*	6.0	5.8	5.7	6.4 ± 1.7	6.1 ± 1.7	5.6 ± 1.5
T. paronychioides + *T. cuspidata*	4.7	4.5	4.4	4.7 ± 1.4	4.5 ± 1.4	4.1 ± 1.2
T. subg. *Eddya*	23.3	22.2	20.6	23.5 ± 3.1	22.4 ± 3.0	19.8 ± 2.0
T. goss. clade + *T. hisp.* clade/*T. purp.*	20.5	19.5	18.2	20.5 ± 2.8	19.5 ± 2.7	17.3 ± 2.0
T. hispidissima clade + *T. purpusii*	14.3	13.6	12.8	14.9 ± 2.7	14.1 ± 2.6	12.4 ± 1.9
T. hispidissima clade	4.9	4.6	4.4	5.1 ± 1.6	4.8 ± 1.5	4.2 ± 1.3
T. latior	2.0	1.9	1.8	2.2 ± 1.0	2.1 ± 0.9	1.8 ± 0.8
T. hispidissima	3.4	3.2	3.0	3.1 ± 1.3	3.0 ± 1.2	2.6 ± 1.0
T. gossypina clade	4.4	4.2	4.1	4.5 ± 1.0	4.3 ± 1.0	3.9 ± 0.8
T. gossypina + *T. turneri*	3.8	3.6	3.6	4.0 ± 0.7	3.8 ± 0.7	3.5 ± 0.6
T. gossypina.263 + *T.turneri*	2.7	2.6	2.5	2.7 ± 0.9	2.6 ± 0.9	2.4 ± 0.7
T. canescens + *T. greggii*	14.6	13.9	12.9	15.0 ± 2.6	14.3 ± 2.6	12.5 ± 2.0

Clade names refer only to the taxa in Figs. 2 and 4. The calibration point ages and minimum age constraint are depicted in bold.

other markers (Bremer et al., 2002; Lundberg, 2001). Furthermore, the older ages derived from the *Vahlia*-constrained analyses do not conflict with the 50 Ma minimum age constraint imposed on the Ehretioideae (node ages of 61.0 and 57.5 Ma, respectively), whereas the unconstrained analyses are affected by the Ehretioideae time constraint (Table 4). Gottschling et al. (2002) report that the ~50 Ma fossil *Ehretia* fruit shows clear affinities with an extant subclade of the genus, thereby suggesting that *Ehretia* may have existed for some time previously. Thus, the 50 Ma minimum age constraint on Ehretioideae used in the *ndhF* analyses may be overly conservative, in which case the Paleocene initial diversification of Ehretioideae implied by the *Vahlia*-constrained analyses is a more attractive scenario. We have therefore chosen to emphasize the *Vahlia*-constrained chronogram shown in Fig. 4.

It should be noted that our dates for the initial diversification and radiation of the major clades of Boraginales are considerably younger than the dates implied in Gottschling et al. (2004), who based their analysis largely on the ~50 Ma *Ehretia* fossil fruit described above. They interpret their analysis as suggesting a mid-Cretaceous origin for the primarily woody Boraginales (a group including Boraginaceae subfamilies Cordioideae, Heliotropioideae, and Ehretioideae), whereas the same node in our *Vahlia*-constrained analysis suggests an origin for these subfamilies near the Cretaceous/Tertiary boundary (67.1–63.3 Ma; Table 4). Constraining this node to have a mid-Cretaceous date (90 Ma) in the *ndhF* tree results in a highly improbable mid-Jurassic age for the divergence of Vahliaceae and Boraginales. While we admit that our analysis does not rely on ingroup fossil data (and likely never will), we feel that the use of inferred divergence dates from the work of Bremer et al. (2004), which utilizes multiple well-placed and well-dated fossils from throughout the asterids, represents a more conservative approach.

We must also emphasize that the calibration points used for the molecular dating analyses in *Tiquilia* are secondary in nature (i.e., they are not independent fossil-based dates) and represent mean dates based on six fossil analyses (Bremer et al., 2004). The divergence dates derived from these calibration points must therefore be treated cautiously (Graur and Martin, 2004). Nevertheless, as is summarized below, the general timeline of evolution inferred for *Tiquilia* by the molecular dating analyses makes sense in light of the morphological diversity within the genus and the climate history of southwestern North America.

4.3. The role of aridity in the evolutionary history of Tiquilia

We can now examine whether *Tiquilia* conforms to the general outline of desert plant evolution implied by Axelrod (1950, 1979a,b) by revisiting the three questions asked in the introduction:

(1) Is *Tiquilia* a relatively old arid-adapted lineage that evolved in North America?

Even in the absence of molecular data, there are a number of reasons to suspect that *Tiquilia* is a relatively old North American group. Most of the extant diversity in the genus is found in North America, including all of subg. *Eddya*, which is suggestive of an origin on that continent. That this origin may be relatively ancient is suggested by the morphological divergence of *Tiquilia* from its relatives, and by the sheer diversity of extant *Tiquilia*. *Tiquilia* possesses a number of unique traits compared to the rest of Ehretioideae, including a low-growing habit, distinctive leaf venation, nutlets instead of drupes, dichotomous branching, and a number of other characters indicative of adaptation to aridity (including a number of leaf characters; Richardson, 1977). In fact, *Tiquilia* is the only genus in Ehretioideae that is restricted to arid and semi-arid habitats (Miller, 2003). A potentially long history for the genus is also suggested by its great diversity, which is best illustrated by contrasting the two subgenera, *Tiquilia* and *Eddya*. Morphologically, these two monophyletic groups differ substantially in their leaf venation, branching pattern, and fruit morphology (Richardson, 1977). They also differ in base chromosome number (subg. *Eddya*, $x=9$; subg. *Tiquilia*, $x=8$) and habitat preferences (subg. *Eddya*, calcareous substrates; subg. *Tiquilia*, loose sand; Richardson, 1977). The major lineages within each subgenus are themselves quite distinct from each other morphologically, and frequently occur sympatrically (in the case of subg. *Eddya*, with up to five species growing together) with no signs of hybridization. This tremendous diversity within *Tiquilia* is therefore likely to be the result of relatively ancient speciation events, and not the product of a recent, rapid radiation.

The *ndhF* and combined analyses strongly suggest that *Tiquilia* is in fact an old North American lineage. Biogeographic reconstruction confirms a North American origin for *Tiquilia* and all of its major lineages (Fig. 1), and molecular-based dating of the *ndhF* phylogeny indicates that the stem lineage of *Tiquilia* may have diverged from its nearest extant relatives as early as the Paleocene (Fig. 4). Furthermore, the long branch separating *Tiquilia* from its nearest relatives suggests a potentially long period of isolation (on the order of 20–25 million years) for the stem lineage of *Tiquilia*. Such isolation would help to explain the great morphological divergence between *Tiquilia* and the rest of the Ehretioideae. This long branch is probably a real phenomenon, and not an artifact of undersampling of potential sister groups in the Ehretioideae. Various molecular-based phylogenies of Boraginales (Gottschling et al., 2001; Gottschling, 2003; Gottschling and Hilger, 2004) that include broad sampling of the genera within Ehretioideae (including *Bourreria*, *Carmona* Cav., *Cortesia* Cav., *Ehretia*, *Halgania* Gaudich., *Hilsenbergia*, *Lepidocordia* Ducke, *Rochefortia* Sw., *Rotula* Lour., and *Tiquilia*) suggest that no other genera within the subfamily are likely closer to *Tiquilia* than the sister genera *Bourreria* and *Hilsenbergia*. According to the molecular dating analyses, the divergence of the two subgenera of *Tiquilia* occurred in the early to mid-Oligocene (33.1–28.8 Ma), with all of the major

lineages diverging in the early to mid-Miocene (23.3–12.8 Ma; Table 4, Fig. 4). Such relatively ancient divergence times would account for the considerable morphological diversity of *Tiquilia* and for the reproductive isolation suggested by the morphological integrity of the sympatric species within each subgenus.

The early divergence dates suggested by the *ndhF* analyses for the stem and crown lineages of *Tiquilia* have important implications for the origin of adaptation to aridity in the genus. Because all extant species of *Tiquilia* occupy arid habitats, it is very likely that the most recent common ancestor of *Tiquilia* was also arid-adapted. The *ndhF* r8s analysis indicates that this ancestor lived no later than the late Eocene/early Oligocene, judging from the age of the divergence implied for the two subgenera (Fig. 4). However, *Tiquilia* in the morphological sense (that is, an arid-adapted subshrub) may have existed many millions of years prior to this in the middle Eocene, in local xeric sites among the subtropical dry forest and savanna that existed in western North America at the time (Axelrod, 1950, 1979a; Graham, 1999). This drier vegetation type probably first spread significantly in the middle to late Eocene as a response to the gradual cooling and drying that had been occurring since the Eocene peak of warmth around 55–50 Ma (Graham, 1999). Based on the current knowledge of the phylogeny, distribution, and ecology of the taxa of Ehretioideae, it is possible that the earliest Ehretioids were members of a seasonally dry tropical forest or scrub community (many extant species of Ehretioideae grow in such environments at present; Leon and Alain, 1957; Miller, 1989, 2002; Standley, 1924), and it is likely that at least part of this ancient range included North America. These putative North American ancestral Ehretioids could have inhabited the middle Eocene subtropical/tropical dry scrub or sclerophyll woodland communities of western North America, eventually giving rise to *Tiquilia* in areas of local aridity or semi-aridity in the middle to late Eocene. Such a scenario, whereby a tropical plant group becomes adapted to aridity during the Eocene in time to benefit from the increasingly dry climates that followed, is exactly in line with the hypotheses of Axelrod (1950, 1979a) concerning the evolution of the current American desert flora. However, it should be emphasized that the lack of fossil information for early *Tiquilia* and Ehretioids in general precludes the confirmation of such a hypothesis, in spite of the suggestiveness of the *ndhF* phylogeny and r8s analyses.

Tiquilia may also conform to two of Axelrod's corollary arguments concerning the origin of arid-adapted plant lineages. First, Axelrod argued that local islands of edaphic aridity promoted the evolution of an autochthonous, arid-adapted flora (Axelrod, 1967, 1972, 1979a). Both subgenera of *Tiquilia* prefer their own, often arid, substrates: the species of subg. *Tiquilia* are restricted to loose sand, while the species of subg. *Eddya* are restricted to barren calcareous substrates (Richardson, 1977). Perhaps more importantly, however, species in both subgenera nearly always establish in substrate lacking plant cover, and consequently are frequently encountered in recently disturbed substrate. Such bare sites are characterized by high insolation of the soil, resulting in relatively high soil temperatures and low soil moisture near the surface, particularly in arid or semi-arid regions (Brady and Weil, 2002; MacMahon, 1999). It is likely that ancestral *Tiquilia* evolved as a specialist of these dry, open habitats, which were probably more restricted in extent prior to the advent of regional deserts. Evolution of new species or ecotypes can proceed rapidly when populations occupy extreme environments such as these [the catastrophic selection of Lewis (1962); see also Axelrod, 1967; Levin, 2005; Stebbins, 1952], and it is possible that such was the case for ancestral *Tiquilia*.

Second, Axelrod (1979a) pointed out that essentially all of the modern plants thought of as desert-adapted either occur outside of the deserts or have close relatives that do, and thus the ancestors of these plants could have evolved and/or survived in local patches of semi-arid woodland or tropic scrub, even in the complete absence of truly arid sites. *Tiquilia* follows just such a pattern, particularly with respect to the members of subg. *Eddya*. Although they are more common in arid sites, all of the species of subg. *Eddya* also occur outside of the deserts in semi-arid regions of desert grassland or oak-juniper-piñon savanna. In these semi-arid environments individuals grow either in recently disturbed substrate or on arid, calcareous slopes with low plant cover. Although we cannot determine the exact place and substrate of origin for ancestral *Tiquilia*, such dry microhabitats would have existed in the Eocene prior to the advent of regional deserts, just as they exist today. It is certainly possible that ancestral *Tiquilia* evolved in such arid microhabitats during the Eocene, and that the genus was able to survive in these habitats, albeit with a restricted range, throughout much of its evolutionary history.

(2) When did the majority of diversification within the genus occur, and is any of this diversification correlated with episodes of aridification during the Cenozoic?

There appear to have been two episodes of deeper-level diversification within *Tiquilia*. The divergence of the modern subgenera of *Tiquilia*, which the molecular dating analyses imply occurred soon after the end of the Eocene ~33.5 Ma (Fig. 4), constitutes the first episode. If correctly dated, this divergence would coincide with the end of one of the greatest episodes of Cenozoic aridification. This significant cooling of the Earth began near the end of the Eocene, and resulted in a terrestrial average temperature drop of 6–8 °C in the middle latitudes of North America (Wolfe, 1992, 1997). The mean annual range of temperature also increased significantly near the Eocene/Oligocene boundary, perhaps as much as 8–10 °C (Wolfe, 1992). These temperature shifts were accompanied by decreases in rainfall over the interior of North America (Graham, 1999), causing the tropical forests that dominated large swaths of southern North America during the middle Eocene to

retreat southward (Wolfe, 1992, 1997). Also by the early Oligocene, oak-pine savanna occupied at least portions of the western United States (Graham, 1999), and drier chaparral vegetation may have also spread over portions of this same area (Graham, 1999). The drier, cooler climates of the Oligocene also favored the beginning of the rise of mostly herbaceous angiosperm plant families (such as Asteraceae; Wolfe, 1997). The relatively sudden increase in aridity near the Eocene/Oligocene boundary likely would have provided new opportunities for the geographic and possibly ecological expansion of *Tiquilia*, which hitherto was probably restricted to very local dry areas. However, it is difficult to prove that this great aridification event directly encouraged the divergence of the two subgenera.

The second episode of diversification seems to have been confined to the early to mid-Miocene, and involved the radiation of the subgenera into all of the major lineages of extant *Tiquilia* (Fig. 4). It is more difficult to correlate any of these earlier divergence events, which the r8s analysis suggests occurred over ~9 million years, with a particular episode of aridification. The Oligocene and early Miocene were climatically similar times, but were followed by a mid-Miocene warming trend that ended around 15 Ma (Graham, 1999; Wolfe, 1997). After 15 Ma, cooling and drying resumed, resulting in the first appearance of true grasslands, the expansion of piñon-juniper woodland, and the possible appearance of some semi-desert vegetation in the modern Sonoran Desert region (Axelrod, 1979a; Graham, 1999). According to the molecular dating analyses, the divergence of *T. canescens* and *T. greggii* dates to this time (~14–13 Ma; Fig. 4, Table 4), as does the divergence of *T. purpusii* and *T. hispidissima*/*T. latior*, and it is possible that these diversifications are somehow linked to the aridifying conditions of the time. Other drought-adapted plant lineages are also thought to have diversified after the mid-Miocene cooling event, including C_4 grasses (Sage, 2004) and North American tarweeds (Asteraceae; Baldwin and Sanderson, 1998). Finally, it should be noted that alternative reconstructions of the branching order within the major lineages of *Tiquilia*, as suggested by the SH test, result in age differences for several nodes (Fig. 3). However, in all cases these differing ages are confined to the early to mid-Miocene, and therefore they do not interfere with any of the potential conclusions of this study.

Although widespread desert habitat was probably nonexistent prior to the late Miocene (Graham, 1999; Wolfe, 1985), the fact that the modern subgenera of *Tiquilia* likely began to diversify ~20 Ma indicates that suitable habitat for *Tiquilia* must have existed. Axelrod (1979a) predicts that local semi-arid or arid sites may have been present at this time across southwestern North America, and it is probable that *Tiquilia* was restricted to these limited areas. Such a fragmented distribution may have promoted allopatric speciation in *Tiquilia*. If isolated long enough, localized species such as these would evolve postzygotic reproductive barriers, thereby prohibiting subsequent hybridization (as opposed to closely related species, which are often isolated by geography or by prezygotic barriers; Levin, 2004). This type of scenario would account for the current sympatric ranges of many of the major lineages within each subgenus, which is otherwise difficult to explain due to the seemingly similar habitat requirements and pollination syndromes of the lineages involved.

(3) Is there any evidence of increased species- or population-level diversity in *Tiquilia* with the onset of semi-arid and arid conditions since the late Miocene?

The *ndhF* analyses suggest that diversification within each of the major lineages of *Tiquilia* dates to the late Miocene (~6.5 Ma) or later (Table 4; Fig. 4), and is likely correlated with the expansion of semi-arid and then arid habitats during the Pliocene. From the late Miocene, mountain ranges and plateaus in Asia and western North American were uplifted (Graham, 1999). The changes in global air circulation that followed increased the extent of polar ice and eventually initiated the glacial/interglacial cycles of late Pliocene and Pleistocene times. Until the late Pliocene, it is likely that deserts were at best a local phenomenon (Axelrod, 1979a); nevertheless, semi-arid habitats expanded, allowing for the continued spread of arid-adapted plants such as *Tiquilia* (Graham, 1999). The *ndhF* analysis indicates that *Tiquilia* underwent a new round of diversification within several of the major lineages during the early Pliocene (e.g., in the *T. gossypina* and the *T. hispidissima* clades; Fig. 4), resulting in the origin of a number of new species. However, even though *Tiquilia* likely spread significantly during the late Miocene and early Pliocene, it probably still had a patchier distribution than today. It was only during the late Pliocene that climate conditions aridified enough to favor the spread of regional deserts. Because *Tiquilia* was preadapted to the newly arid conditions, it would have expanded significantly and potentially rapidly into its new desert surroundings. This expansion may have brought into contact a number of hitherto isolated species or populations of *Tiquilia*, and it is possible that the widespread sympatry of the major lineages of the genus may initially date from this time. The glacial/interglacial cycles of the Pleistocene created a concomitant cycle of contraction (during glacial periods) and expansion (during interglacial periods) of the desert biome in southwestern North America (Axelrod, 1979a; Graham, 1999; Lowenstein et al., 1999), which could be expected to cause simultaneous range contractions and expansions in populations of *Tiquilia*. The *T. gossypina* clade exhibits a somewhat confusing pattern of molecular variation across its range that may relate to the climate upheavals of the Pleistocene. The closely related species of this lineage show evidence of probable hybridization among formerly isolated populations (unpublished data) that could have resulted from repeated isolation and contact among these populations during the Pleistocene. Finally, the spread of *Tiquilia* in North America also increased the likelihood of amphitropical dispersal to the newly arid regions of the Pacific coast of South America and the Galápagos Islands. The *ndhF* phylogeny suggests that the *T. palmeri* and

T. plicata clades did not disperse to these regions prior to the late Miocene, approximately 6–7 Ma (Table 4 and Fig. 4), which correlates well with the initial advent of aridity in coastal South America at about the same time (Hartley and Chong, 2002).

5. Conclusions

All of the evidence derived from the molecular dating and phylogenetic analyses of *Tiquilia* supports Axelrod's hypotheses of the evolution of the modern North American desert flora. Although its exact place of origin within North America cannot be determined, it is possible that the Ehretioid ancestors of *Tiquilia* inhabited dry tropic scrub in southwestern North America during the Paleocene. *Tiquilia* likely first appeared in the middle to late Eocene as a specialist of open, edaphically dry sites, and probably remained restricted to locally semi-arid sites through much of its early history. The divergence of the two modern subgenera of *Tiquilia* probably occurred shortly after the great aridification event of the late Eocene/early Oligocene, ~33 Ma. The current major lineages of *Tiquilia* all likely arose in the early to mid-Miocene, perhaps evolving allopatrically in locally arid or semi-arid pockets. The drying and cooling of the late Miocene and early Pliocene prompted further expansion and diversification within the major lineages of *Tiquilia* (from ~7 to 4 Ma), followed by a potentially rapid range expansion with the onset of widespread aridity over southwestern North America in the late Pliocene and Pleistocene interglacial periods.

Although this sequence of events corresponds well with Axelrod's ideas of North American desert flora assembly, we must qualify the ages derived from the molecular dating analyses because they rely on secondary calibration points far removed from the ingroup. However, there are a number of independent reasons to accept the ancient divergence dates implied within *Tiquilia* by the molecular analyses: the calibration points used in this study are based on a thorough and well-calibrated analysis; the phylogeny of *Tiquilia* and its outgroups is well-supported, allowing a good degree of confidence in our interpretation of the sequence of evolutionary events; and the morphological and ecological divergence of *Tiquilia* from its nearest relatives, combined with the great morphological diversity within *Tiquilia*, independently suggests an ancient age for the genus and its main lineages. In arid-adapted plant groups such as *Tiquilia* that lack a fossil record, there is no choice but to seek alternative methods for calibrating divergence times. This study demonstrates that the utilization of a reasonably dated calibration point located far from the ingroup, while less desirable, can still yield useful information about evolutionary diversification in fossil-poor groups.

Acknowledgments

The authors wish to thank Alan Tye and the Charles Darwin Research Station, Asunción Cano, Fátima Cáceres, Mónica Arakaki, Blanca León, Martín Timaná, and the Plant Resources Center at the University of Texas for their help with collecting; and Marc Gottschling and Richard Olmstead for providing general help and outgroup DNA. We also want to thank Randy Linder, Alan Lloyd, Ulrich Mueller, Beryl Simpson, and the two anonymous reviewers for their comments on this manuscript. We gratefully acknowledge the following funding sources: the National Science Foundation [DEB 0206440 (a Doctoral Dissertation Improvement Grant to M.J.M. and R.K.J. and DEB 9982091 to R.K.J.)], the American Society of Plant Taxonomists, the Botanical Society of America, Sigma Xi, the Plant Biology Program and the Sidney F. and Doris Blake Centennial Professorship in Systematic Botany at the University of Texas at Austin.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.01.020.

References

Al-Shehbaz, I.A., 1991. The genera of Boraginaceae in the southeastern United States. J. Arnold Arbor. Suppl. Ser. 1, 1–169.

Álvarez, I., Wendel, J.F., 2003. Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phylogenet. Evol. 29, 417–434.

Axelrod, D.I., 1950. Evolution of desert vegetation in western North America. In: Axelrod, D.I. (Ed.), Studies in Late Tertiary Paleobotany, vol. 590. Carnegie Institution of Washington Publication, Washington DC USA, pp. 215–323.

Axelrod, D.I., 1958. Evolution of the Madro-Tertiary Geoflora. Bot. Rev. 24 (7), 433–509.

Axelrod, D.I., 1967. Drought, diastrophism, and quantum evolution. Evolution 21 (2), 201–209.

Axelrod, D.I., 1972. Edaphic aridity as a factor in angiosperm evolution. Am. Nat. 106, 311–320.

Axelrod, D.I., 1979a. Age and origin of Sonoran Desert vegetation. Occas. Paper. Calif. Acad. Sci. 132.

Axelrod, D.I., 1979b. Desert vegetation, its age and origin. In: Goodin, J.R., Northington, D.K. (Eds.), Arid Land Plant Resources, Proceedings of the International Arid Lands Conference on Plant Resources, Texas Tech University. International Center for Arid and Semi-Arid Land Studies, Texas Tech University, Lubbock, TX, USA, pp. 1–72.

Backlund, M., Oxelman, B., Bremer, B., 2000. Phylogenetic relationships within the Gentianales based on *ndhF* and *rbcL* sequences, with particular reference to the Loganiaceae. Am. J. Bot. 87 (7), 1029–1043.

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S., Donoghue, M.J., 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. Mo. Bot. Gard. 82 (2), 247–277.

Baldwin, B.G., Sanderson, M.J., 1998. Age and rate of diversification of the Hawaiian silversword alliance (Compositae). Proc. Natl. Acad. Sci. USA 95, 9402–9406.

Barkman, T.J., Lim, S.-H., Salleh, K.M., Nais, J., 2004. Mitochondrial DNA sequences reveal the photosynthetic relatives of *Rafflesia*, the world's largest flower. Proc. Natl. Acad. Sci. USA 101 (3), 787–792.

Berggren, W.A., Kent, D.V., Swisher, C.C. III, Aubry, M.-P., 1995. A revised Cenozoic geochronology and chronostratigraphy. In: Berggren, W.A., Kent, D.V., Aubry, M.-P., Hardenbol, J. (Eds.), Geochronology, Time Scales, and Global Stratigraphic Correlation. Special Publication No. 54, Society for Sedimentary Geology, Tulsa, OK, USA, pp. 129–212.

Betancourt, J.L., Van Devender, T.R., Martin, P.S. (Eds.), 1990. Packrat Middens: The Last 40,000 Years of Biotic Change. University of Arizona Press, Tucson, AZ, USA.

Bogler, D.J., Simpson, B.B., 1995. A chloroplast DNA study of the Agavaceae. Syst. Bot. 20 (2), 191–205.

Bogler, D.J., Simpson, B.B., 1996. Phylogeny of Agavaceae based on ITS rDNA sequence variation. Am. J. Bot. 83 (9), 1225–1235.

Brady, N.C., Weil, R.R., 2002. The Nature and Properties of Soils, 13 ed. Prentice Hall, Upper Saddle River, NJ, USA.

Bremer, B., Bremer, K., Heidari, N., Erixon, P., Olmstead, R.G., Anderberg, A.A., Källersjö, M., Barkhordarian, E., 2002. Phylogenetics of asterids based on 3 coding and 3 non-coding chloroplast DNA markers and the utility of non-coding DNA at higher taxonomic levels. Mol. Phylogenet. Evol. 24, 274–301.

Bremer, K., Friis, E.M., Bremer, B., 2004. Molecular phylogenetic dating of asterid flowering plants shows early Cretaceous diversification. Syst. Biol. 53 (3), 496–505.

Butterworth, C.A., Cota-Sanchez, J.H., Wallace, R.S.., 2002. Molecular systematics of tribe Cacteae (Cactaceae: Cactoideae): a phylogeny based on *rpl16* intron sequence variation. Syst. Bot. 27 (2), 257–270.

Caujapé-Castells, J., Jansen, R.K., Membrives, N., Pedrola-Monfort, J., Montserrat, J.M., Ardanuy, A., 2001. Historical biogeography of *Androcymbium* Willd. (Colchicaceae) in Africa: evidence from cpDNA RFLPs. Bot. J. Linn. Soc. 136, 379–392.

Crisp, M., Cook, L., Steane, D., 2004. Radiation of the Australian flora: What can comparisons of molecular phylogenies across multiple taxa tell us about the evolution of diversity in present-day communities? Philos. Trans. R. Soc. Lond. B Biol. Sci. 359, 1551–1571.

Csank, C., Taylor, F.M., Martindale, D.W., 1990. Nuclear pre-mRNA introns: analysis and comparison of intron sequences from *Tetrahymena thermophila* and other eukaryotes. Nucleic Acids Res. 18 (17), 5133–5141.

Cuénoud, P., Savolainen, V., Chatrou, L.W., Powell, M., Grayer, R.J., Chase, M.W., 2002. Molecular phylogenetics of Caryophyllales based on nuclear 18S rDNA and plastid *rbcL*, *atpB*, and *matK* DNA sequences. Am. J. Bot. 89 (1), 132–144.

Davis, C.C., Bell, C.D., Mathews, S., Donoghue, M.J., 2002. Laurasian migration explains Gondwanan disjunctions: evidence from Malpighiaceae. Proc. Natl. Acad. Sci. USA 99 (10), 6833–6837.

Davis, C.C., Fritsch, P.W., Bell, C.D., Mathews, S., 2004. High-latitude Tertiary migrations of an exclusively tropical clade: evidence from Malpighiaceae. Int. J. Plant Sci. 165 (Suppl. 4), S107–S121.

Donoghue, M.J., Smith, S.A., 2004. Patterns in the assembly of temperate forests around the Northern Hemisphere. Philos. Trans. R. Soc. Lond. B Biol. Sci. 59, 1633–1644.

Downie, S.R., Katz-Downie, D.S., 1996. A molecular phylogeny of Apiaceae subfamily Apioideae: evidence from nuclear ribosomal DNA internal transcribed spacer sequences. Am. J. Bot. 83 (2), 234–251.

Evans, R.C., Alice, L.A., Campbell, C.S., Kellogg, E.A., Dickinson, T.A., 2000. The granule-bound starch synthase (GBSSI) gene in the Rosaceae: multiple loci and phylogenetic utility. Mol. Phylogenet. Evol. 17 (3), 388–400.

Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C., 1994. Testing significance of incongruence. Cladistics 10 (3), 315–319.

Ferguson, D.M., 1999. Phylogenetic analysis and relationships in Hydrophyllaceae based on *ndhF* sequence data. Syst. Bot. 23 (3), 253–268.

Gilbert, D.G., 1992. SeqApp, v. 1.9a169. <http://iubio.bio.indiana.edu/soft/molbio/seqapp/>.

Goldblatt, P., Savolainen, V., Porteous, O., Sostaric, I., Powell, M., Reeves, G., Manning, J.C., Barraclough, T.G., Chase, M.W., 2002. Radiation in the Cape flora and the phylogeny of peacock irises *Moraea* (Iridaceae) based on four plastid DNA regions. Mol. Phylogenet. Evol. 25, 341–360.

Goldman, N., Anderson, J.P., Rodrigo, A.G., 2000. Likelihood-based tests of topologies in phylogenetics. Syst. Biol. 49 (4), 652–670.

Gottschling, M., Hilger, H.H., 2001. Phylogenetic analysis and character evolution of *Ehretia* and *Bourreria* (Ehretiaceae, Boraginales) and their allies based on ITS1 sequences. Bot. Jahrb. Syst. Pflanzengesch. Pflanzengeogr. 123 (2), 249–268.

Gottschling, M., Hilger, H.H., Wolf, M., Diane, N., 2001. Secondary structure of the ITS1 transcript and its application in a reconstruction of the phylogeny of Boraginales. Plant Biol. 3, 629–636.

Gottschling, M., Mai, D.H., Hilger, H.H., 2002. The systematic position of *Ehretia* fossils (Ehretiaceae, Boraginales) from the European Tertiary and implications for character evolution. Rev. Palaeobot. Palynol. 121, 149–156.

Gottschling, M., 2003. Phylogenetic Analysis of Selected Boraginales. Ph.D. thesis, Freien Universität Berlin. <http://www.diss.fu-berlin.de/2003/30/>.

Gottschling, M., Diane, N., Hilger, H.H., Weigend, M., 2004. Testing hypotheses on disjunctions in the primarily woody Boraginales: Ehretiaceae, Cordiaceae, and Heliotropiaceae, inferred from ITS1 sequence data. Int. J. Plant Sci. 165 (Suppl. 4), S123–S135.

Gottschling, M., Hilger, H.H., 2004. The systematic position of *Ehretia cortesia* nom. nov. (*Cortesia cuneifolia*: Ehretiaceae, Boraginales) inferred from molecular and morphological data. Taxon 53 (4), 919–923.

Gradstein, F.M., Agterberg, F.P., Ogg, J.G., Hardenbol, J., Van Veen, P., Thierry, J., Huang, Z., 1995. A Triassic, Jurassic, and Cretaceous time scale. In: Berggren, W.A., Kent, D.V., Aubry, M.-P., Hardenbol, J. (Eds.), Geochronology, Time Scales, and Global Stratigraphic Correlation. Special Publication No. 54, Society for Sedimentary Geology, Tulsa, OK, USA, pp. 95–128.

Graham, A., 1999. Late Cretaceous and Cenozoic History of North American Vegetation. Oxford University Press, New York, NY, USA.

Graur, D., Martin, W., 2004. Reading the entrails of chickens: molecular timescales of evolution and the illusion of precision. Trends Genet. 20 (2), 80–86.

Hall, J.C., Iltis, H.H., Sytsma, K.J., 2004. Molecular phylogenetics of core Brassicales, placement of orphan genera *Emblingia, Forchhameria, Tirania*, and character evolution. Syst. Bot. 29 (3), 654–669.

Hartley, A.J., Chong, G., 2002. Late Pliocene age for the Atacama Desert: implications for the desertification of western South America. Geology 30 (1), 43–46.

Huelsenbeck, J.P., Hillis, D.M., Jones, R., 1996. Parametric bootstrapping in molecular phylogenies: application and performance. In: Ferraris, J.D., Palumbi, S.R. (Eds.), Molecular Zoology: Advances, Strategies, and Protocols. Wiley Publishers, New York, NY, USA, pp. 19–45.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17 (8), 754–755.

Ingram, A.L., Doyle, J.J., 2004. Is *Eragrostis* (Poaceae) monophyletic? Insights from nuclear and plastid sequence data. Syst. Bot. 29 (3), 545–552.

Jacobs, B.F., Kingston, J.D., Jacobs, L.L., 1999. The origin of grass-dominated ecosystems. Ann. Mo. Bot. Gard. 86 (2), 590–643.

Johnson, L.A., Soltis, D.E., 1994. *mat*K DNA sequences and phylogenetic reconstruction in Saxifragaceae s. str. Syst. Bot. 19 (1), 143–156.

Kadereit, G., Borsch, T., Weising, K., Freitag, H., 2003. Phylogeny of Amaranthaceae and Chenopodiaceae and the evolution of C_4 photosynthesis. Int. J. Plant Sci. 164 (6), 959–986.

Kelchner, S.A., 2002. Group II introns as phylogenetic tools: structure, function, and evolutionary constraints. Am. J. Bot. 89 (10), 1651–1669.

Klak, C., Reeves, G., Hedderson, T., 2004. Unmatched tempo of evolution in southern African semi-desert ice plants. Nature 427 (1), 63–65.

Langley, C.H., Fitch, W., 1974. An estimation of the constancy of the rate of molecular evolution. J. Mol. Evol. 3, 161–177.

Lavin, M., Wojciechowski, M.F., Gasson, P., Hughes, C., Wheeler, E., 2003. Phylogeny of robinioid legumes (Fabaceae) revisited: *Coursetia* and *Gliricidia* recircumscribed, and a biogeographical appraisal of the Caribbean endemics. Syst. Bot. 28 (2), 387–409.

Leon, H., Alain, H., 1957. Flora de Cuba, vol. IV. Contribuciones ocasionales del museo de historia natural de La Salle 16.

Leopold, E.B., Liu, G., Clay-Poole, S., 1992. Low-biomass vegetation in the Oligocene? In: Prothero, D.R., Berggren, W.A. (Eds.), Eocene-Oligocene Climatic and Biotic Evolution. Princeton University Press, Princeton, NJ, USA, pp. 399–420.

Levin, D.A., 2004. Ecological speciation: crossing the divide. Syst. Bot. 29 (4), 807–816.

Levin, D.A., 2005. Niche shifts: the primary driver of novelty in angiosperm genera. Syst. Bot. 30 (1), 9–15.

Lewis, H., 1962. Catastrophic selection as a factor in speciation. Evolution 16 (3), 257–271.

Linder, H.P., 2003. The radiation of the Cape flora, southern Africa. Biol. Rev. 78, 597–638.

Linder, H.P., Hardy, C.R., 2004. Evolution of the species-rich Cape flora. Philos. Trans. R. Soc. Lond. B Biol. Sci. 359, 1623–1632.

Lowenstein, T.K., Li, J., Brown, C., Roberts, S.M., Ku, T.-L., Luo, S., Yang, W., 1999. 200 ky paleoclimate record from Death Valley salt core. Geology 27 (1), 3–6.

Lundberg, J., 2001. A well resolved and supported phylogeny of Euasterids II based on a Bayesian inference, with special emphasis on Escalloniaceae and other incertae sedis. In: Lundberg, J. (Ed.), Phylogenetic Studies in the Euasterids II with Particular Reference to Asterales and Escalloniaceae. Acta Universitatis Upsaliensis, Uppsala, Sweden. <http://www.diva-portal.org/diva/getDocument?urn_nbn_se_uu_diva-1597-1__fulltext.pdf>.

MacMahon, J.A., 1999. Disturbance in deserts. In: Walker, L.R. (Ed.), Ecosystems of the World 16: Ecosystems of Disturbed Ground. Elsevier, Amsterdam, pp. 307–330.

Maddison, W.P., Maddison, D.R., 2003. MacClade, v. 4.0 (release version 4.06). Sinauer Associates, Sunderland, MA, USA.

Maddison, W.P., Maddison, D.R., 2004. Mesquite, v. 1.05. <http://mesquiteproject.org/mesquite/mesquite.html>.

Magallón, S.A., 2004. Dating lineages: molecular and paleontological approaches to the temporal framework of clades. Int. J. Plant Sci. 165 (4 Suppl.), S7–S21.

Mason-Gamer, R.J., Weil, C.F., Kellogg, E.A., 1998. Granule-bound starch synthase: structure, function, and phylogenetic utility. Mol. Biol. Evol. 15 (12), 1658–1673.

Mathews, S., Spangler, R.E., Mason-Gamer, R.J., Kellogg, E.A., 2002. Phylogeny of Andropogoneae inferred from phytochrome B, GBSSI, and *ndhF*. Int. J. Plant Sci. 163 (3), 441–450.

McKinnon, G., 2005. Reticulate evolution in higher plants. In: Henry, R.J. (Ed.), Plant Diversity and Evolution: Genotypic and Phenotypic Variation in Higher Plants. CABI Publishing, Cambridge, MA, USA, pp. 81–96.

Miller, J.S., 1989. A revision of the New World species of *Ehretia* (Boraginaceae). Ann. Mo. Bot. Gard. 76 (4), 1050–1076.

Miller, J.S., 2002. A revision of *Ehretia* (Boraginaceae) for Madagascar and the Comoro Islands. Adansonia 24 (2), 137–157.

Miller, J.S., 2003. Classification of Boraginaceae subfam. Ehretioideae: resurrection of the genus *Hilsenbergia* Tausch ex Meisn. Adansonia 25 (2), 151–189.

Miller, R.E., Rausher, M.D., Manos, P.S., 1999. Phylogenetic systematics of *Ipomoea* (Convolvulaceae) based on ITS and *waxy* sequences. Syst. Bot. 24 (2), 209–227.

Nickrent, D.L., Duff, R.J., Colwell, A.E., Wolfe, A.D., Young, N.D., Steiner, K.E., de Pamphilis, C.W., 1998. Molecular phylogenetic and evolutionary studies of parasitic plants. In: Soltis, D.E., Soltis, P.S., Doyle, J.J. (Eds.), Molecular Systematics of Plants II: DNA Sequencing. Kluwer Academic Publishers, Boston, MA, USA, pp. 211–241.

Nyffeler, R., 2002. Phylogenetic relationships in the cactus family (Cactaceae) based on evidence from *trnK/matK* and *trnL–trnF* sequences. Am. J. Bot. 89 (2), 312–326.

Olmstead, R.G., Ferguson, D., 2001. A Molecular Phylogeny of the Boraginaceae/Hydrophyllaceae. P. 131, in Botany 2001: Plants and People, Abstracts, meeting of the Botanical Society of America, Albuquerque, NM, USA.

Olmstead, R.G., Reeves, P.A., 1995. Evidence for the polyphyly of the Scrophulariaceae based on chloroplast *rbcL* and *ndhF* sequences. Ann. Mo. Bot. Gard. 82 (2), 176–193.

Olmstead, R.G., Sweere, J.A., 1994. Combining data in phylogenetic systematics: an empirical approach using three molecular data sets in the Solanaceae. Syst. Biol. 43 (4), 467–481.

Oxelman, B., Liden, M., Berglund, D., 1997. Chloroplast *rps16* intron phylogeny of the tribe Sileneae (Caryophyllaceae). Plant Syst. Evol. 206, 393–410.

Oxelman, B., Backlund, M., Bremer, B., 1999. Relationships of the Buddlejaceae s.l. investigated using branch support analysis of chloroplast *ndhF* and *rbcL* sequence data. Syst. Bot. 24, 164–182.

Peralta, I.E., Spooner, D.M., 2001. Granule-bound starch synthase (GBSSI) gene phylogeny of wild tomatoes (*Solanum* L. section *Lycopersicon* [Mill.] Wettst. subsection *Lycopersicon*). Am. J. Bot. 88 (10), 1888–1902.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14 (9), 817–818.

Posada, D., Buckley, T.R., 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Syst. Biol. 53 (5), 793–808.

Retallack, G.J., 1997. Neogene expansion of the North American Prairie. Palaios 12, 380–390.

Richardson, A.T., 1977. Monograph of the genus *Tiquilia* (*Coldenia*, sensu lato), Boraginaceae: Ehretioideae. Rhodora 79, 467–572.

Rodríguez, F., Oliver, J.L., Marin, A., Medina, J.R., 1990. The general stochastic model of nucleotide substitution. J. Theor. Biol. 142, 485–501.

Sage, R.F., 2004. The evolution of C4 photosynthesis. New Phytologist 161, 341–370.

Sanderson, M.J., 2002. Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. Mol. Biol. Evol. 19 (1), 101–109.

Sanderson, M.J., 2003. r8s: inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock. Bioinformatics 19 (2), 301–302.

Sanderson, M.J., 2004. r8s, version 1.70 User's Manual. <http://ginger.ucdavis.edu/r8s/r8s1.7.manual.pdf>.

Sanderson, M.J., Thorne, J.L., Wikstrom, N., Bremer, K., 2004. Molecular evidence on plant divergence times. Am. J. Bot. 91 (10), 1656–1665.

Sang, T., Crawford, D.J., Stuessy, T.F., 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). Am. J. Bot. 84 (9), 1120–1136.

Schultheis, L.M., Baldwin, B.G., 1999. Molecular phylogenetics of Fouquieriaceae: evidence from nuclear rDNA ITS studies. Am. J. Bot. 86 (4), 578–589.

Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16 (8), 1114–1116.

Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torazawa, K., Meng, B.Y., Sugita, M., Deno, H., Kamogashira, T., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Tohdoh, N., Shimada, H., Sugiura, M., 1986. The complete nucleotide sequence of tobacco chloroplast genome: its gene organization and expression. EMBO J. 5, 2043–2049.

Simmons, M.P., Ochoterena, H., 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. 49 (2), 369–381.

Smith, R.A., 1998. Phylogenetic placement of the holoparasitic family Lennoaceae: preliminary molecular evidence. Am. J. Bot. 85 (6), 157.

Smith, R.A., Ferguson, D.M., Barkman, T.J., dePamphilis, C.W., 2000. Molecular phylogenetic evidence for the origin of Lennoaceae: a case of adelphoparasitism in the angiosperms? Am. J. Bot. S87, 158.

Standley, P.C., 1924. Trees and Shrubs of Mexico (Passifloraceae-Scrophulariaceae). Contributions from the United States National Herbarium, vol. 23, Part 4.

Stebbins, G.L., 1952. Aridity as a stimulus to plant evolution. Am. Nat. 86, 33–44.

Steele, K.P., Vilgalys, R., 1994. Phylogenetic analyses of Polemoniaceae using nucleotide sequences of the plastid gene *mat*K. Syst. Bot. 19 (1), 126–142.

Stefanovic, S., Olmstead, R.G., 2004. Testing the phylogenetic position of a parasitic plant (*Cuscuta*, Convolvulaceae, Asteridae): Bayesian inference and the parametric bootstrap on data drawn from three genomes. Syst. Biol. 53 (3), 384–399.

Swofford, D.L., 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods), v. 4.0b10, for Macintosh. Sinauer Associates, Sunderland, MA, USA.

Swofford, D.L., Olsen, G.J., Waddell, P.J., Hillis, D.M., 1996. Phylogenetic inference. In: Hillis, D.M., Moritz, C., Mable, B.K. (Eds.), Molecular

Systematics, second ed. Sinauer Associates, Sunderland, MA, USA, pp. 407–514.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The Clustal_X windows interface: flexible strategies for multiple alignment aided by quality analysis tools. Nucleic Acids Res. 25 (24), 4876–4882.

Thompson, R.S., Anderson, K.H., 2000. Biomes of western North America at 18,000, 6000 and 0 ^{14}C yr BP reconstructed from pollen and packrat midden data. J. Biogeogr. 27, 555–584.

Walsh, B.M., Hoot, S.B., 2001. Phylogenetic relationships of *Capsicum* (Solanaceae) using DNA sequences from two noncoding regions: the chloroplast *atpB–rbcL* spacer region and nuclear *waxy* introns. Int. J. Plant Sci. 162 (6), 1409–1418.

Weeks, A., Daly, D.C., Simpson, B.B., 2005. The phylogenetic history and biogeography of the frankincense and myrrh family (Burseraceae) based on nuclear and chloroplast sequence data. Mol. Phylogenet. Evol. 35 (1), 85–101.

Wells, P.V., 1966. Late Pleistocene vegetation and degree of pluvial climatic change in the Chihuahuan Desert. Science 153, 970–975.

Wen, J., 1999. Evolution of eastern Asian and eastern North American disjunct distributions in flowering plants. Annu. Rev. Ecol. Syst. 30, 421–455.

Wendel, J.F., Doyle, J.J., 1998. Phylogenetic incongruence: window into genome history and molecular evolution. In: Soltis, D.E., Soltis, P.S., Doyle, J.J. (Eds.), Molecular Systematics of Plants II: DNA Sequencing. Kluwer Academic Publishers, Boston, MA, USA, pp. 265–296.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA, USA, pp. 315–322.

Winkworth, R.C., Donoghue, M.J., 2004. *Viburnum* phylogeny: evidence from the duplicated nuclear gene GBSSI. Mol. Phylogenet. Evol. 33 (1), 109–126.

Wolfe, J.A., 1985. Distribution of major vegetational types during the tertiary. In: Sundquist, E.T., Broecker, W.S. (Eds.), The Carbon Cycle and Atmospheric CO_2: Natural Variations Archean to Present, vol. 32. Geophysical Monograph, American Geophysical Union, Washington, DC, USA, pp. 357–375.

Wolfe, J.A., 1992. Climatic, floristic, and vegetational changes near the Eocene/Oligocene boundary in North America. In: Prothero, D.R., Berggren, W.A. (Eds.), Eocene–Oligocene Climatic and Biotic Evolution. Princeton University Press, Princeton, NJ, USA, pp. 421–436.

Wolfe, J.A., 1997. Relations of environmental change to angiosperm evolution during the late Cretaceous and Tertiary. In: Iwatsuki, K., Raven, P.H. (Eds.), Evolution and Diversification of Land Plants. Springer, Tokyo, Japan, pp. 269–290.