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Pleistocene radiation of the serpentine-adapted genus *Hesperolinon* and other divergence times in Linaceae (Malpighiales)[1]

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PREMISE OF THE STUDY: *Hesperolinon* (western flax; Linaceae) is endemic to the western United States, where it is notable for its high and geographically concentrated species diversity on serpentine-derived soils and for its use as a model system in disease ecology. We used a phylogenetic framework to test a long-standing hypothesis that *Hesperolinon* is a neoendemic radiation.

METHODS: Five plastid and two ribosomal nuclear DNA gene regions were sampled from 105 populations of *Hesperolinon*, including all 13 recently recognized species across their known ranges. We used these data to generate population-level phylogenies of *Hesperolinon*. We also generated a robustly sampled chronogram of Linaceae using an eight-gene, 100-taxon supermatrix calibrated using fossil *Linum* pollen and a published chronogram of Malpighiales.

KEY RESULTS: Most diversification in *Hesperolinon* has taken place in the past 1–2 million yr, much more recently than previous estimates. Only the earliest-diverging species, *H. drymarioides*, was resolved as a clade. Denser taxon and gene sampling generally support previously proposed relationships within Linaceae, but with more recent diversification of key clades.

CONCLUSIONS: *Hesperolinon* is an excellent example of edaphic neoendemism, in support of Raven and Axelrod's hypothesis for the genus. Dense population-level sampling reveals a complex of incipient species, with clades poorly aligned with traditional morphological circumscriptions, likely due in part to continued gene flow. The diversification of Linaceae is more recent than previously estimated, and other recent radiations (e.g., *Hugonia*) warrant further study.

KEY WORDS California Floristic Province; chronogram; *Hesperolinon*; Linaceae; neoendemic; serpentine; supermatrix

Serpentine-derived soils are well known for extensive plant endemism by taxa capable of growing under conditions considered physically and chemically harsh, including low calcium-to-magnesium ratios, low concentrations of essential nutrients (N, P, K), and high levels of heavy metals (e.g., Mg, Fe, Ni) (Walker, 1954; Kruckeberg, 2002). In the highly diverse California flora, in which ~10% of endemic taxa are known only from serpentine (Kruckeberg, 1984), recent comparative phylogenetic studies have indicated that serpentine endemism commonly appears to be an evolutionary dead end, with a strong evolutionary trend toward edaphic specialization but low subsequent rates of diversification (Anacker et al., 2011). Important exceptions have, in part, been challenging to study using molecular systematic methods (e.g., *Streptanthus* Nutt.; Cacho et al., 2014). Improved understanding of the natural history, biogeography, and relationships of lineages that have diversified on serpentine soils has proved to be important in understanding fine-scale ecological and evolutionary phenomena. Recent examples include study of adaptations that limit apparency to herbivores on serpentine barrens (Strauss and Cacho, 2013) and the role of serpentine barrens themselves as refuges from pathogens (Springer, 2009). More generally, the recognition of the California Floristic Province as a global biodiversity hotspot (Mittermeier et al., 2011) gives strong impetus to efforts to understand the evolution and assembly of its flora.

To date, the time frame for plant diversification on Californian serpentines has been relatively poorly understood because of imprecise understanding of the timing of serpentine exposure, uncertainty

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about whether serpentine endemics were once more widely distributed (Stebbins, 1942), and lack of divergence time estimates for serpentine lineages—especially for lineages including a high diversity of serpentine-occurring taxa (but see, e.g., Baldwin, 2005). One such diverse example is the genus *Hesperolinon* (A. Gray) Small (Linaceae), a group of gracile annuals with highly localized distributions, which are commonly known as "western flaxes." *Hesperolinon* is ecologically and evolutionarily notable for exemplifying diversification on serpentine soils and for containing most of the diversity that renders *Linum* L. paraphyletic (Sharsmith, 1961; Raven and Axelrod, 1978; Anacker et al., 2011; McDill and Simpson, 2011). Every *Hesperolinon* species is able to persist on serpentine, and 69% (9 of 13) are serpentine endemics (Sharsmith, 1961; O'Donnell, 2006; Springer, 2009). Although other members of Linaceae can be found on serpentine (e.g., *Linum punctatum* C. Presl subsp. *pycnophyllum* (Boiss. & Heldr.) Gustavsson), only *Hesperolinon* shows evidence of such extreme diversification on serpentine, including several independent origins of serpentine endemism. This has made *Hesperolinon* a model group for testing hypotheses of ecological interactions in extreme environments such as the "pathogen refuge hypothesis" (Springer et al., 2007; Springer, 2009).

Geographically, *Hesperolinon* species have narrow, overlapping ranges. Twelve of the 13 species are present in an area smaller than the state of Delaware (Lake and Napa counties, California), and only the range of *H. micranthum* (A. Gray) Small extends outside of both the state of California and the California Floristic Province. At the population level, however, individuals of different *Hesperolinon* species only rarely intermingle. Instead, populations of two or more species are often found parapatrically along an ecological gradient (Sharsmith, 1961; A. C. Schneider et al. personal observation).

Biogeographic patterns in *Hesperolinon*, coupled with morphological similarities between species in the genus and close relatives, have largely been interpreted as evidence for a recent origin of this group. Raven and Axelrod (1978, p. 75) regarded *Hesperolinon* as "the most extreme of all serpentine genera" and a young, "rapidly evolving complex," in contrast to some putatively older, less diverse, woody serpentine lineages that have few close relatives and restricted, possibly contracted, ranges. A reliable estimate of the age of the *Hesperolinon* clade and knowledge of its closest relatives are necessary to fully test Raven and Axelrod's (1978) hypothesis that *Hesperolinon* represents a neoendemic radiation. Consequently, the present study builds on the foundational efforts of McDill et al. (2009) and McDill and Simpson (2011), with additional sampling of *Hesperolinon* and the benefit of recent evidence that Ixonanthaceae, rather than Irvingiaceae, is sister to Linaceae (Xi et al., 2012).

The close relationship between *Hesperolinon* and *Linum* is undisputed. Indeed, the first *Hesperolinon* species described were assigned to *Linum* (e.g., Bentham, 1839; Jepson, 1936; see Sharsmith, 1961). However, based on its distinctive yellow to orange or white to pink flowers, reduced number of carpels (two or three), 17 or 18 pairs of chromosomes, lack of staminodia, distinctive appendages at the base of each petal, and alternating attachment of petals and stamens to a staminal cup, *Hesperolinon* was soon described as a section of *Linum* (Gray, 1865), then elevated to generic rank (Small, 1907), as it has been treated subsequently (e.g., Sharsmith, 1961; Rogers et al., 1972; Rogers, 1975). Within *Hesperolinon*, flower color, inflorescence structure, carpel number, flower size, and vestiture have traditionally been used in delimiting taxa (Sharsmith, 1961; O'Donnell, 2010; McDill, 2012).

Recent molecular phylogenetic evidence, especially from the work of McDill and colleagues, supports long-standing taxonomic and evolutionary hypotheses that *Hesperolinon* is nested within a paraphyletic *Linum* sect. *Linopsis* (Rchb.) Engelm. pro parte (Rogers, 1975; McDill and Simpson, 2011). Those higher-level studies included limited representation of *Hesperolinon* and yielded contrasting results as to whether the monotypic genus *Sclerolinon* C. M. Rogers is sister to *Hesperolinon* or more distantly related. That relationship, as well as the more general placement of *Hesperolinon* in Linaceae, is important to understanding both the timing of diversification and the biogeographic origin of *Hesperolinon*, which was not assigned to a source region other than the California Floristic Province by Raven and Axelrod (1978). Within *Hesperolinon*, Springer (2009) identified the morphologically disparate *H. drymarioides* (Curran) Small as sister to the rest of the genus using evidence from plastid DNA (cpDNA). He also found support for a clade consisting of *H. breweri* (A. Gray) Small, *H. californicum* (Benth.) Small, and *H. congestum* (A. Gray) Small, a group easily diagnosed by having alternate primary branching with congested, clustered cymes of relatively large flowers (Sharsmith, 1961). However, in addition to poor resolution at other key nodes, Springer's phylogeny was based exclusively on cpDNA and lacked geographic sampling outside of the San Francisco Bay Area and Inner North Coast Ranges, thereby excluding morphologically distinct and geographically disjunct populations of several species as treated by Sharsmith (1961).

We sought to address these gaps in knowledge of the western flaxes and relatives by estimating a geographically comprehensive molecular phylogeny of *Hesperolinon*, representing all currently accepted taxa across their respective ranges, and by pursuing a more informed divergence time estimate for *Hesperolinon* from a revised fossil-calibrated chronogram of Linaceae. Specifically, we had three objectives: (1) to reevaluate the divergence time of *Hesperolinon*, given the recent identification of Ixonanthaceae as the sister group of Linaceae, and in so doing reassess Raven and Axelrod's (1978) hypothesis that *Hesperolinon* represents a neoendemic Californian radiation; (2) to examine relationships of *Hesperolinon* to other taxa of Linaceae, in part to resolve its biogeographic origin; and (3) to assess the monophyly of *Hesperolinon* and species relationships within the genus.

MATERIALS AND METHODS

***Linaceae supermatrix assembly*—**SUMAC (Freyman, 2015) was used to download all Ixonanthaceae and Linaceae sequences from GenBank release 204 and assemble an eight-gene supermatrix (seven plastid regions plus ITS; Table 1) representing a total of 100 taxa (95 ingroup Linaceae plus five outgroup Ixonanthaceae), including complete generic and section-level sampling. The sequence matrix was constructed by clustering all sequences homologous to eight guide sequences obtained from GenBank of *Linum bienne* Mill. for the nuclear ribosomal (nrDNA) internal transcribed spacer region (ITS-1+5.8S gene+ITS-2; hereafter "ITS") and the following seven cpDNA regions: *rbcL*, *trnL–trnF* intergenic spacer, *trnK* 3′ intron, *ndhF*, *trnG*, *psbA–trnH* intergenic spacer, and *rpl16*. Sequences were aligned using MAFFT version 7.123b (Katoh and Standley, 2013). Default settings were used, except we used the direction adjustment option to ensure proper sequence polarity. Alignments were then concatenated according to species

TABLE 1. Regions of homologous sequences used in the Linaceae sequence matrix.

Region no.	Gene region	Number of OTUs [a]	Aligned length	Missing data (%)	Taxon coverage density
1	*rbcL*	85	1428	22.0	0.78
2	*trnL–trnF* spacer	65	1265	40.4	0.60
3	*trnK* 3′ intron	57	1842	47.7	0.52
4	*ndhF*	51	1006	53.2	0.47
5	ITS	51	857	53.2	0.47
6	*trnG*	16	744	85.3	0.15
7	*psbA–trnH* spacer	17	563	84.4	0.16
8	*rpl16* intron	30	1159	72.5	0.28
Total	–	100	8864	65	0.43

[a] Operational taxonomic units.

binomial, creating chimeric operational taxonomic units (OTUs) that do not necessarily represent a single individual. Five species from the Ixonanthaceae were included as the outgroup, based on a recent molecular phylogenetic study of Malpighiales that resolved Ixonanthaceae as sister to Linaceae (Xi et al., 2012). GenBank accession numbers for sequences used in constructing the Linaceae supermatrix are in Appendix S1 (see Supplemental Data with the online version of this article).

***Linaceae phylogenetic analysis*—**Divergence times and phylogeny were jointly estimated using RevBayes (Höhna et al., 2014) under an uncorrelated log-normal relaxed clock model. The GTR+Γ substitution model was used for each of the eight gene partitions. Rate variation across sites was modeled under a gamma distribution approximated by four discrete rate categories. The more general GTR model was used because many other nucleotide substitution models such as HKY85 and JC69 are nested within it, enabling the model to collapse down to a better-fitting model during Bayesian parameter estimation. The constant-rate birth–death sampling tree prior (Stadler, 2009) was used with the probability of sampling species at the present (ρ) set to 0.35; ρ was calculated by dividing the number of extant species sampled in the supermatrix (100) by the sum of the number of species recognized in Linaceae (~260) and in Ixonanthaceae (~30). Branch rates were drawn from a log-normal prior with the mean and standard deviation given exponentially distributed hyperpriors with rate parameters of 0.25.

Divergence times were estimated using two independent stochastic nodes to calibrate the tree age. For the root age, we used the crown age of the linoids reported by Xi et al. (2012) as part of a 16-fossil, 191-taxon study of Malpighiales (mean = 90.0 Ma [million years ago]; 95% highest probability density: 103.4–73.6 Ma). To model the uncertainty in root age, the root node was given a uniform calibration density with a range of 73.6–103.4 Ma. The fossil calibration used to date the crown node of *Linum* was based on *Linum* pollen grains from the Ebro Basin of northeastern Spain dated to 33.9–37.2 Ma (Cavagnetto and Anadón, 1996; McDill and Simpson, 2011). We fixed the fossil age to the mean of the age range (35.55 Ma) and modeled the crown age for *Linum* under a diffuse log-normal prior with a mean of 10 and standard deviation of 0.5. In other words, the sampled prior ages of the *Linum* crown node had a mean of 45.55 Ma and could not be more recent than 35.55 Ma. Four independent replicates of the Markov chain Monte Carlo (MCMC) were each run for 100,000 iterations. Each iteration consisted of 563 moves randomly scheduled from 236 different moves. The MCMC was sampled every 10 iterations, and the first 2500 samples from each replicate were discarded as burn-in. We used Tracer version 1.6 (Rambaut et al., 2014) to check for convergence. Results were considered reliable when the effective sampling size (ESS) for all parameters from each independent run exceeded 200, and when combined had ESS values >1000.

Taxon, population, and character sampling within* Hesperolinon*—Ingroup sampling consisted of 5–20 populations of each of the 13 recently recognized species in genus *Hesperolinon*. The only validly published taxon not included, *H. confertum* (A. Gray ex Trel.) Small, was not recognized by Sharsmith (1961) or subsequent authors, following doubts by Thomas (1955). It is poorly differentiated from *H. californicum*, diagnosed only as a "low form [of *H. californicum*], more densely leafy and with a contracted inflorescence, the median appendage of the petals obovate" (Trelease, 1887, p. 19). Ten outgroup individuals were also sampled. These included two exemplars of the putative sister genus *Sclerolinon*, the closely related *Linum neomexicanum* Greene, *L. rupestre* (A. Gray) Engelm. ex A. Gray, and *Cliococca selaginoides* (Lam.) C. M. Rogers & Mildner. The five remaining outgroup samples were selected from the more distantly related *Linum* sect. *Linum*: *L. bienne* (two exemplars), *L. lewisii* Pursh var. *lewisii*, *L. perenne* L., and *L. usitatissimum* L.

All sampled *Hesperolinon* populations are mapped in Fig. 1. Population selection and DNA extraction of dried aboveground tissue for 77 of the 105 sampled *Hesperolinon* populations and the outgroup *Linum neomexicanum* are described in Springer (2009). The remaining 28 *Hesperolinon* populations were selected to provide comprehensive geographic sampling of the known range of each species, as determined by a map of georeferenced herbarium specimens in the Consortium of California Herbaria (http://ucjeps.berkeley.edu/consortium/). For each of these additional populations and the nine remaining outgroup samples, leaf tissue was sampled from pressed and dried voucher collections at UC/JEPS, ground using 1.0 mm glass beads, and extracted using a DNeasy Plant Mini Kit (Qiagen, Valencia, California).

Four cpDNA regions were used to estimate relationships within *Hesperolinon*: *trnT–trnL* spacer, *trnL–trnF* spacer, *trnK* 3′ intron, and *rpl16* intron. They were selected because of their prior use in a phylogenetic study of *Hesperolinon* (Springer, 2009). Two transcribed spacer nrDNA regions were also used to provide data from an independently inherited genomic region. The ITS region was selected on the basis of its favorable properties for plant phylogenetics (Baldwin et al., 1995) and, in particular, its use in a recent analysis of the subfamily Linoideae, which included *Hesperolinon* (McDill et al., 2009). The external transcribed spacer (ETS) was selected to obtain additional rapidly evolving nrDNA characters for phylogenetic resolution, based in part on the availability of the

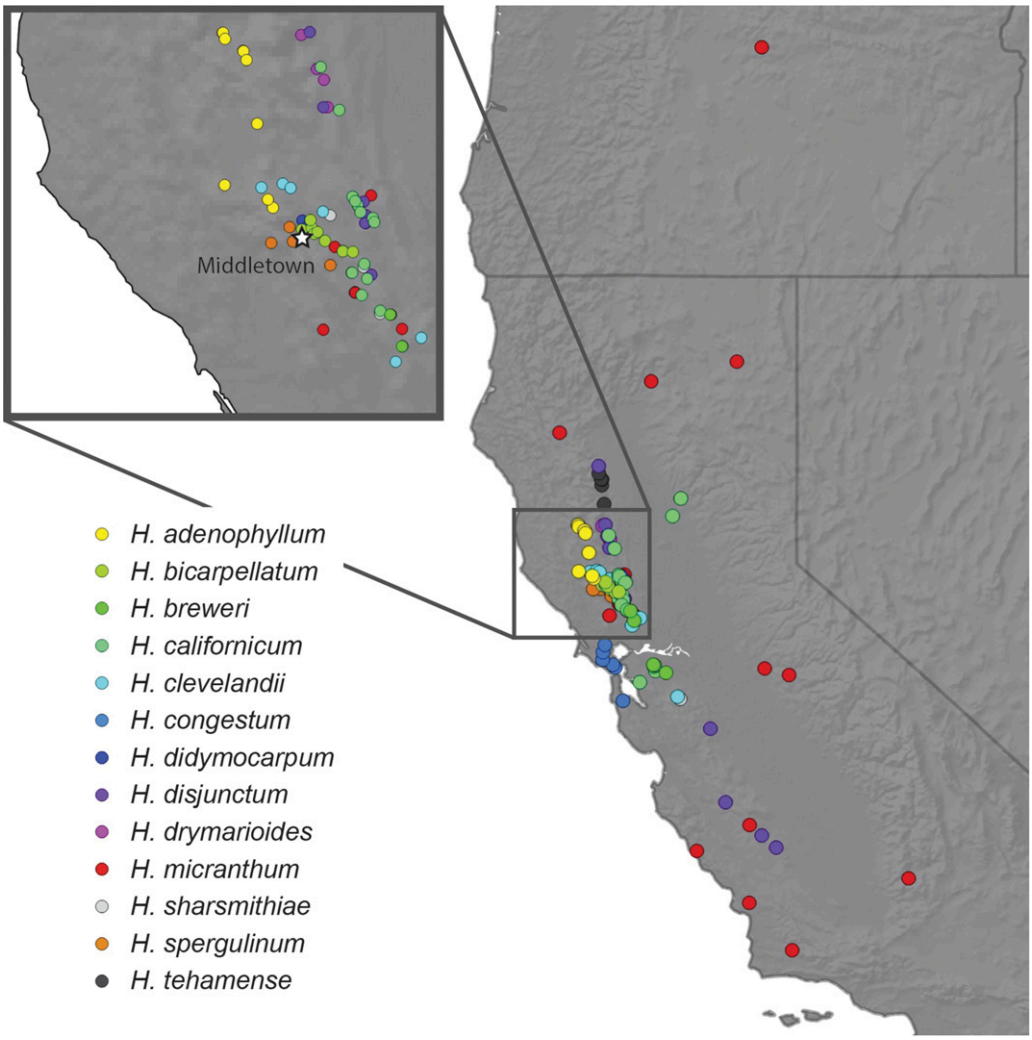

FIGURE 1 Locations of 105 sampled *Hesperolinon* populations in California and Oregon, USA.

Linum usitatissimum genome (Wang et al., 2012), which facilitated primer design.

Hesperolinon *DNA amplification, sequencing, and sequence alignment*—Polymerase chain reaction (PCR) amplifications of target gene regions were performed using AccuPower PCR PreMix kits (Bioneer, Alameda, California). Primer sequences and thermocycling parameters for plastid regions (*trnT–trnL* spacer, *trnL–trnF* spacer, *trnK* 3′ intron, *rpl16* intron) were obtained from Springer (2009) and references cited therein, with minor modifications (Table 2). No new sequences of the *trnK* 3′ intron were generated because of low sequence variability; only previously reported sequences by Springer (2009) were used. Primer sequences for ITS were obtained from White et al. (1990) and Urbatsch et al. (2000). ETS primers were designed using Primer3 (Untergasser et al., 2012) after sequencing the 18S-26S IGS region of several accessions following Baldwin and Markos (1998) and obtaining a published sequence from *Linum usitatissimum* (Wang et al., 2012). Identical primers were used for PCR and sequencing.

All PCR products were visualized by 1.0% agarose gel electrophoresis and purified using ExoSAP (USB Products, Cleveland, Ohio). Samples were sent to the UC Berkeley DNA Sequencing Facility (Berkeley, California) for sequencing of both DNA strands. Sequences were checked for base-calling errors using Geneious version 6.1.7 (Biomatters, Auckland, New Zealand), assembled into contigs using Geneious or custom Python scripts utilizing the Biopython package (Cock et al., 2009). Sequence alignments were generated using MUSCLE version 3.8.31 (Edgar, 2004) with default parameters, followed by inspection and manual adjustments in Geneious using Simmons's (2004) similarity criterion. The alignments are deposited in TreeBASE (http://treebase.org), study accession number 18150. Herbarium voucher and GenBank accession numbers for *Hesperolinon* sequences are in Appendix S2 (see Supplemental Data with the online version of this article).

Hesperolinon *phylogenetic analyses*—Maximum likelihood (ML) and Bayesian inference (BI) phylogenetic analyses were conducted on three datasets: the cpDNA matrix, consisting of concatenated *trnT–trnL* spacer, *trnL–trnF* spacer, *trnK* 3′ intron, and *rpl16* intron sequences; the nrDNA matrix consisting of concatenated ITS and ETS sequences; and a combined matrix with all sequences concatenated to evaluate support for clades weakly supported by both the cpDNA and nrDNA data (Kluge, 1989). The combined analyses used partitioned models that allowed the model parameters to vary between the nrDNA and cpDNA partitions. All analyses were conducted on the CIPRES Science Gateway (Miller et al., 2010).

Maximum likelihood analyses were performed in RAxML-HPC2 version 8.0.24 (Stamatakis, 2014) using the GTRCAT model with 25 rate categories and 100 rapid bootstrap (BS) replicates. For the optimal tree a complex process of iterative searches was used (Stamatakis et al., 2008); however, for each bootstrap replicate a single search was performed. Rapid bootstrapping with the GTRCAT model has been criticized for generating inflated bootstrap values, particularly when few bootstrap replicates are sampled (Simmons and Norton, 2014). Therefore, we performed much more thorough Bayesian analyses using MrBayes version 3.2.2 (Yang and Rannala, 1997; Ronquist et al., 2012). Uncertainty about the correct substitution model was integrated out by sampling across all possible submodels of the general time reversible (GTR) model space using reversible jump MCMC (rjMCMC; Green, 1995; Huelsenbeck et al., 2004). The distribution of rates across sites was modeled using a gamma distribution approximated by four discrete rate categories. Uniform priors were used for the tree topology and

TABLE 2. Molecular regions used in the phylogenetic analyses of *Hesperolinon*, approximate lengths of complete ingroup sequences, PCR primers (5′–3′), and thermocycler parameters. ETS primer pair ETS_seq/ETS_680 was used for all *Hesperolinon* accessions but would not amplify outgroup ETS. For outgroup ETS amplification, primer pair ETS_seq/ETS_620 was used, yielding a slightly shorter amplicon (or ETS_seq/ETS_480, for *Linum bienne* and *L. lewisii*).

Gene region	Amplicon length	Primer sequences	Thermocycling parameters
ITS	635 bp	ITS4: TCC TCC GCT TAT TGA TAT GC ITS-I: GTC CAC TGA ACC TTA TCA TTT AG	96°C, 1min; 40× (96°C, 10s; 48°C, 30s; 72°C, 20s + 4s/cycle); 72°C, 7min
ETS	600 bp	ETS_seq: TGG CAG GAT CAA CCA GGT A ETS_680: TTT GGC TGA ATG GTG GAT TT ETS_620: AAG TTG GTT GCA TGT TTT CG ETS_480: GTG ATG CRC ATG AST GGT AT	96°C, 2min; 35× (94°C, 30s; 56°C, 30s; 72°C, 1min); 72°C, 3min
trnT–trnL spacer	480–500 bp	trnT 'a': CAT TAC AAA TGC GAT GCT C trnL 'b': TCT ACC GAT TTC GCC ATA TC	94°C, 4min; 40× (92°C, 45s; 53°C, 50s; 72°C, 1min); 72°C, 10min
trnL–trnF spacer	890 bp (*H. drymarioides*) 975 bp (others)	trnL 'c': CGA AAT CGG TAG ACG CTA CG trnF 'f': ATT TGA ACT GGT GAC ACG AG	94°C, 5min; 40× (92°C, 1min; 53°C, 1min; 72°C, 1min 20s); 72°C, 10min
trnK 3′ intron	480 bp	matK '8F': CTT CGA CTT TCT TGT GCT trnK '2R': AAC TAG TCG GAT GGA GTA	94°C, 5min; 35× (92°C, 1min; 50°C, 1min; 72°C, 1min 30s); 72°C, 10min
rpl16 intron	1020–1060 bp	rpl16 'F71': GCT ATG CTT AGT GTG TGA CTC GTT G rpl16 'R1516': CCC TTC ATT CTT CCT CTA TGT TG	95°C, 5min; 40× (95°C, 1min; 50°C, 1min; +15°C 0.2°C/s; 65°C, 4min); 72°C, 10min

the alpha shape parameter of the gamma distribution of rate variation. The substitution rates and stationary nucleotide frequencies used flat Dirichlet priors. The branch length prior used the exponential distribution with the lambda rate parameter set to the default 10.0. Two independent runs of four chains each (three heated and one cold) were sampled every 1000 generations until the average standard deviation of split frequencies was <0.01. The first 25% of samples were discarded as burn-in. Convergence was further assessed by ensuring that the potential scale reduction factor (PSRF) value was close to 1.0 for all parameters, and by using Tracer to confirm that the ESS of parameter values was >200.

RESULTS

Divergence times and relationships within Linaceae—A chronogram (maximum a posteriori topology) is presented in Fig. 2. The most recent common ancestor of *Hesperolinon* was found to have a Pliocene origin, diverging from its common ancestor with *Sclerolinon* ~6 Ma. However, most diversification of extant lineages occurred during the Pleistocene, within the past 2 million yr (Table 3). *Hesperolinon* was strongly supported as monophyletic and sister to the monotypic *Sclerolinon*. Together, these two genera were resolved as sister to the yellow-flowered *Linum* sect. *Linopsis* pro parte plus the monotypic genus *Cliococca* Bab. All other sections of *Linum* sensu McDill and Simpson (2011) were found to be monophyletic with strong support. *Linum* sect. *Linopsis* was found to be paraphyletic, with *Linum* sect. *Syllinum* Griseb. and the genera *Cliococca*, *Sclerolinon*, and *Hesperolinon* nested within it.

More broadly, Linaceae was resolved as monophyletic with high support (posterior probability [PP] = 1.0), as were the two subfamilies of Linaceae: Linoideae and Hugonioideae. Linaceae and its sister family Ixonanthaceae likely diverged from their most recent common ancestor in the late Cretaceous. Diversification of extant lineages in Linaceae did not begin until the Eocene, during which the Linoideae and Hugonioideae split and *Linum* began to diversify (Fig. 2 and Table 3). Most extant diversity in these clades has origins in the Miocene or later, including the divergence and diversification of most major sections of *Linum*, southern Asian genera of the Linaceae, and the Hugonioideae.

Within Hugonioideae, the Old World genera *Hebepetalum* Benth. and *Roucheria* Planch. were each resolved as monophyletic with moderate support (PP > 0.8). These genera form a poorly supported grade subtended by New World Hugonioideae. *Hugonia* L. was found to be nonmonophyletic, with *Hugonia* sect. *Durandea* Planch. sister to two Southeast Asian genera. Within Linoideae, the small southern Asian genera *Anisadenia* Wall. ex Meisn. and *Tirpitzia* Hallier f. are each strongly supported as monophyletic and, together with the monotypic *Reinwardtia* Dumort., constitute a robust clade with *Linum* and its segregate genera (PP = 1.0). The blue-flowered species in *Linum* (sect. *Linum* and sect. *Dasylinum* (Planch.) Juz.) were found to be sister to the yellow-flowered species in *Linum* and segregate genera *Cliococca*, *Hesperolinon*, *Radiola* Hill, and *Sclerolinon*. These clades diverged in the late Eocene shortly after diverging from the Southeast Asian lineage of *Anisadenia+Tirpitzia+Reinwardtia*, but did not substantially diversify until the Neogene (Fig. 2).

Hesperolinon *relationships inferred from cpDNA*—The majority-rule consensus tree of 5332 trees sampled from the posterior distribution of the BI analysis of *Hesperolinon* cpDNA (Fig. 3A) did not substantially differ from the ML topology (data not shown). *Hesperolinon* was found to be monophyletic (PP = 0.67; BS = 95%) and to constitute a clade with *Sclerolinon*, albeit with negligible support (PP = 0.57; BS = 63%). *Hesperolinon drymarioides* was found to a clade with high support (PP = 1.0; BS = 100%); the rest of the genus constituted a weakly supported clade (PP = 0.52; BS = 98%). None of the remaining taxonomic species within *Hesperolinon* was resolved as monophyletic, although several deeper clades showed moderate to strong support (PP > 0.8; BS > 68%), united in a backbone polytomy. These clades included two that represent all sampled populations of *H. adenophyllum* (A. Gray) Small (cp1; Fig. 3A) united in a polytomy with all samples of *H. didymocarpum* H. Sharsm. and populations of *H. bicarpellatum* (H. Sharsm.) H. Sharsm. found in the hills 5-6 km northeast of Middletown, California, but not >8 km southeast along Butts Canyon. Clade cp3 represents all sampled populations of *H. spergulinum* (A. Gray) Small plus several accessions of

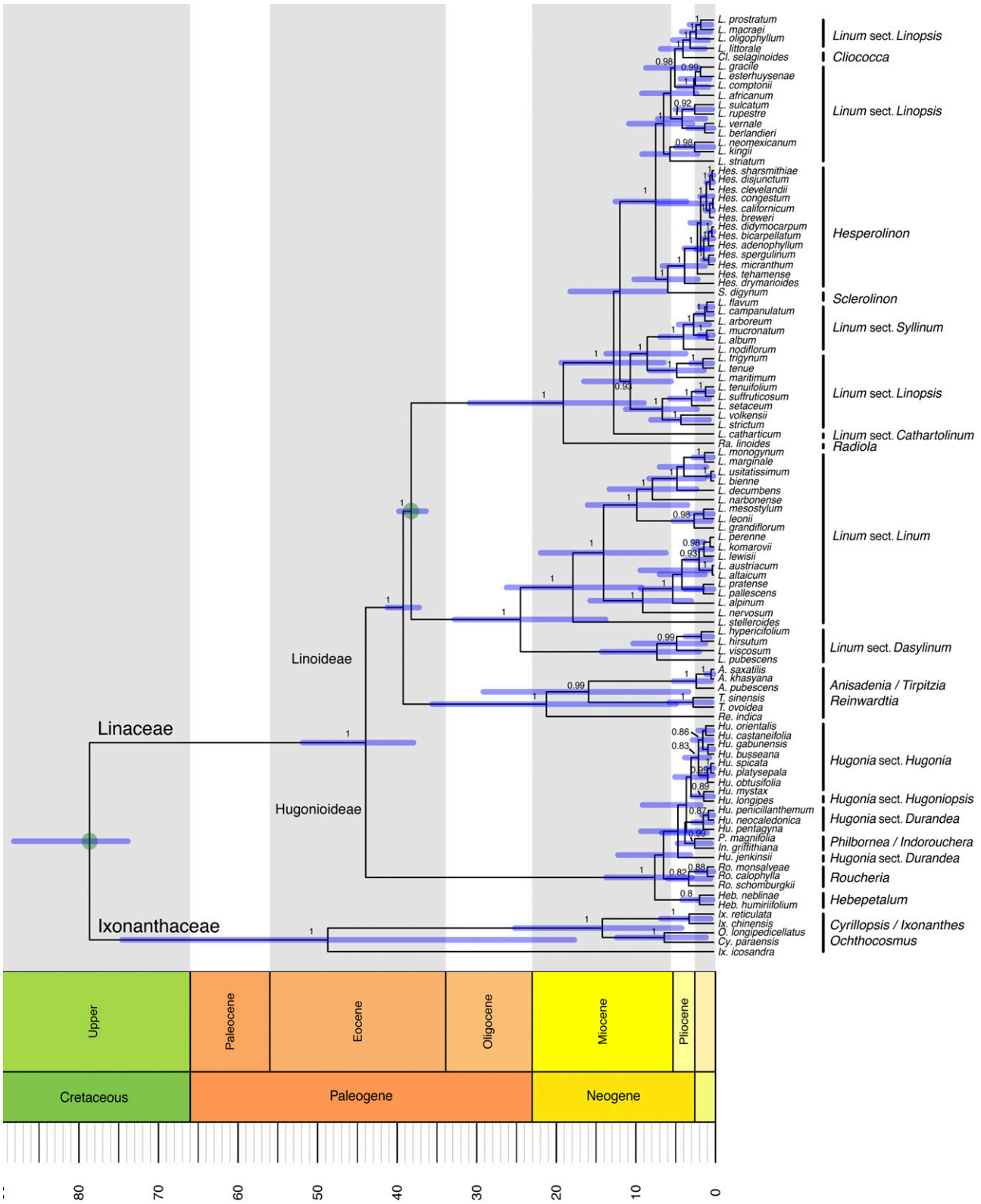

FIGURE 2 Bayesian inference chronogram (maximum a posteriori topology) of 95 Linaceae and 5 Ixonanthaceae taxa. Approximate positions of fossil calibration points are shown as green circles; blue bars represent 95% highest probability density [HPD] intervals. Only posterior probabilities >0.8 are shown. A summary of the divergence time estimates and 95% HPD intervals is in Table 3.

TABLE 3. Divergence times (Ma) and credible intervals (95% highest probability density [HPD]) of key well-supported clades within the linoids (PP = 1.0). Blue-flowered *Linum* are defined as sections *Linum* and *Dasylinum*; yellow-flowered *Linum* include sections *Linopsis*, *Syllinum*, and *Carthartolinum*, plus segregate genera *Hesperolinon*, *Sclerolinon*, and *Cliococca*.

	Crown age	
Clade	**Mean**	**95% HPD**
Linaceae + Ixonanthaceae (linoids)	78.6	87.9–73.6
Ixonanthaceae	48.7	74.3–17.5
Linaceae	43.9	51.6–37.7
Linoideae	39.2	41.0–37.0
Linum (including segregate genera)	38.2	39.5–36.1
Blue-flowered *Linum*	24.4	32.6–13.6
Yellow-flowered *Linum* + *Radiola*	19.1	30.7–8.7
Yellow-flowered *Linum*	12.7	19.2–6.3
Hugonioideae	7.6	13.5–2.9
Hesperolinon	3.8	6.4–1.1

H. micranthum. Clade cp4 represents mostly *H. micranthum* from across its range, but also two yellow-flowered specimens from the Diablo Range of central California, collected ~2.5 miles apart, that most closely resemble *H. clevelandii* (Greene) Small and *H. sharsmithiae* R. O'Donnell. Clade cp5 contains all but the southernmost sampled population of *H. tehamense* H. Sharsm. as well as a nearby population of *H. disjunctum* H. Sharsm. Clade cp6 is a polytomy of all other sampled *H. clevelandii* populations along with a subclade including *H. bicarpellatum* from Butts Canyon, the three-carpellate populations from Lake and Napa counties assigned to *H. sharsmithiae*, and nearby *H. disjunctum* populations.

Hesperolinon *relationships inferred from nrDNA*—The majority-rule consensus tree of 3852 trees sampled from the posterior distribution of the BI analysis of *Hesperolinon* nrDNA (ITS+ETS; Fig. 3B) did not substantially differ from the ML topology (data not shown). *Hesperolinon* was found to be monophyletic (PP = 0.98; BS = 84%) and nested within *Linum* (i.e., in a clade with a subset of *Linum* taxa; PP = 1.0; BS = 100%) with strong support. *Hesperolinon drymarioides* was resolved as monophyletic with strong support (PP = 1.0; BS = 100%), as was a clade containing all other samples from the genus (PP = 0.95; BS = 82%). Of the remaining species, two well-supported subclades of *H. adenophyllum* (nr1, PP = 0.96 and 1.0; BS = 64% and 100%; Fig. 3B) form a polytomy with a clade of *H. spergulinum* plus some populations of *H. micranthum* (nr2, PP = 1.0; BS = 84%). This clade (nr1+nr2, PP = 1.0; BS = 95%) is sister to another well-supported clade (PP = 1.0; BS = 88%) that is made up of the rest of the genus (minus *H. drymarioides*). Another well-supported clade in the genus unites several populations of *H. micranthum* (nr5) with all but one population of *H. clevelandii* (nr6) and all populations of *H. bicarpellatum*, *H. sharsmithiae*, and *H. didymocarpum* (nr7) as well as northern populations of *H. disjunctum* (except for the anomalous UC 1212496; PP = 1; BS = 87%). At finer phylogenetic scales, no taxonomic species was resolved as strictly monophyletic aside from *H. drymarioides*. In several cases, a single population of one taxonomic species grouped elsewhere, as in *H. clevelandii*, or a single population of a taxonomic species was nested within a clade otherwise representing all sequences of a different taxonomic species, such as one sequence attributed to *H. disjunctum* (UC1212496) nested within a clade otherwise including all sequences of *H. tehamense* (nr3). In other instances, strongly supported infraspecific clades were found in separate parts of the tree, such as separate clades for northern and southern populations of *H. disjunctum*, or some clades of *H. micranthum* (e.g., nr2 and nr5).

Hesperolinon *relationships inferred from a combined analysis*—Results of the BI analysis of the combined data matrix are summarized in Appendix S3 (see Supplemental Data with the online version of this article). *Hesperolinon* was again found to be monophyletic, with *H. drymarioides* sister to the rest of the genus. *Hesperolinon adenophyllum* also was supported as monophyletic (PP = 0.8; BS = 59%) and sister to a clade of *H. spergulinum* and *H. micranthum* pro parte (clade cp/nr1, PP = 0.96; BS = 91%). *Hesperolinon didymocarpum* populations were also found to form a monophyletic group (PP = 1.0; BS = 100%), nested within a grade of *H. bicarpellatum* from the hills north of Middletown (cp/nr2, PP = 1; BS = 96%). Most of the remaining sampled populations are resolved in several strongly supported clades, but with weak backbone support (PP < 0.7). Several *H. micranthum* populations collected between central Oregon and the edge of the Mojave Desert form a clade (cp/nr3; PP = 1.0; BS = 93%), as well as all sampled populations of *H. tehamense* plus a nearby collection of *H. disjunctum* (Sharsmith 4024, cp/nr4; PP = 0.94; BS = 98%). All but one population of *H. clevelandii* are strongly supported as a clade (PP = 0.98; BS = 94%) and sister to a mix of populations of *H. sharsmithiae*, remaining *H. disjunctum* from northern California, and *H. bicarpellatum* from Butts Canyon (cp/nr5, PP = 1.0; BS = 99%). A moderately supported clade includes all *H. breweri*, *H. californicum*, *H. congestum*, and southern *H. disjunctum* populations (cp/nr6; PP = 0.74; BS < 70%). Some substructure within this clade is better supported, although only occasionally corresponding to current taxonomic concepts. Given conflict between cpDNA and nrDNA and a high likelihood of introgression in some nested clades, we urge caution in interpreting results of the combined data analyses.

DISCUSSION

***Origin and recent diversification of* Hesperolinon—**Our Linaceae and *Hesperolinon* analyses show a close relationship between *Hesperolinon* and the other, yellow-flowered North American Linaceae. *Hesperolinon* and *Sclerolinon*, both treated in *Linum* by some early botanists (e.g., Trelease, 1887), can be distinguished from other members of that genus by the reduction of carpel number from five to two or three and by their biogeographic affinities to the western United States. Raven and Axelrod (1978) regarded them separately as genera of uncertain affinity, but primarily associated with the California Floristic Province. Our results show that *Hesperolinon* and *Sclerolinon* represent a single, larger clade (Figs. 2 and 3A). This pattern of endemic taxa of uncertain relationship being found to constitute a clade has been repeatedly discovered for other western North American taxa with extensive representation in the California flora (Baldwin, 2014). Raven and Axelrod's (1978) suggestion that *Hesperolinon* was ultimately of southwestern North American ("Madrean") origin remains a viable hypothesis for the larger clade including both *Hesperolinon* and *Sclerolinon*. This is consistent with conclusions by McDill et al. (2009) and warrants further investigation in light of the placement of North American members of *Linum* sect. *Linopsis* as the sister group of *Hesperolinon* plus *Sclerolinon* (Fig. 2).

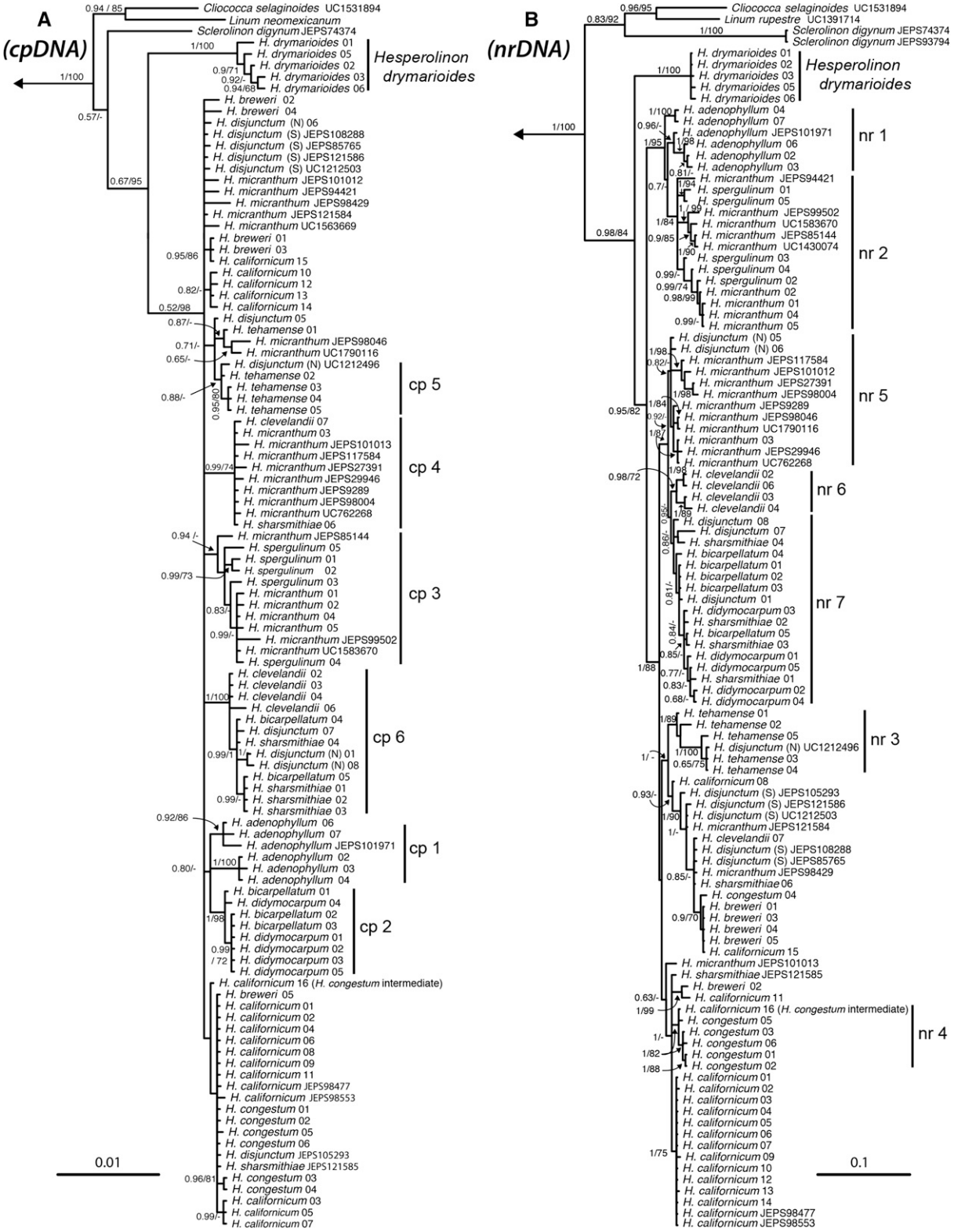

FIGURE 3 Bayesian inference majority-rule consensus tree of 104 *Hesperolinon* populations and three outgroup taxa inferred from (**A**) cpDNA and (**B**) nrDNA (ITS+ETS). The posterior probabilities and maximum likelihood bootstrap values (%) for nodes with PP > 0.6 and BS > 70% are given along the branches. Dash (–) denotes BS < 70%. Ingroup tip labels represent the most recent taxonomic determination followed by the herbarium accession number or the two-digit population code assigned by Springer (2009). *Hesperolinon disjunctum*, populations north (N) and south (S) of the Sacramento Delta are indicated. Species of *Hesperolinon* are abbreviated with the letter H. Several more distantly related outgroup samples from *Linum* sect. *Linum* are not shown. Numbered clades are discussed in the text.

Our results also confirm Raven and Axelrod's (1978) hypothesis that *Hesperolinon* is a recently diversifying lineage strongly associated with serpentine. We estimate that diversification of extant members of *Hesperolinon* from their most recent common ancestor took place since the late Miocene, likely in the Pliocene (~3.8 Ma), which is several million years later than previously thought (McDill and Simpson, 2011). Moreover, the diversification of most extant lineages within the genus has taken place only in the past 1–2 million yr, well after the divergence of the monotypic sister genus *Sclerolinon* and of *H. drymarioides* from the rest of *Hesperolinon* (Fig. 2).

Multiple considerations lead us to propose that *Hesperolinon* diversified in situ in the southern Inner North Coast Ranges, in the vicinity of Lake and Napa counties, with later range expansion of nested groups (e.g., *H. californica* + *H. congestum* and the *H. micranthum* complex). First, the southern Inner North Coast Ranges have long been recognized as having a high diversity of endemic species (Stebbins and Major, 1965; Thorne et al., 2009). Second, the closest extant relatives to this clade, including *H. drymarioidies* and *Sclerolinon*, share similar if not overlapping ranges. Third, the timing of diversification is coincident with the opening of available habitat in central California. Minimum age estimates of serpentine exposures in the North Coast Ranges and northern San Francisco Bay Area vary between 1 and 5 Ma (Harrison et al., 2004). In the past 3.5 Ma the Mendocino Triple Junction, formed by the convergence of the Pacific, Gorda, and North American tectonic plates, has migrated northwestward, leading to the uplift of the Coast Ranges and westward recession of the coastline (Montgomery, 1993; Lock et al., 2006).

In addition to uplift and subsequent weathering exposing new habitat and serpentine outcroppings, periodic volcanism from the late Miocene to as recently as ~10,000 yr ago created lava and ash deposits over the southern Inner North Coast Ranges (Donnelly-Nolan et al., 1981). Colonization of bare habitats like these has recently been suggested as an intermediary step to edaphic (particularly serpentine) specialization (Cacho and Strauss, 2014). This dynamic landscape also likely led to periodic isolation of small populations of *Hesperolinon*, perhaps followed by secondary contact or extinction. Thus, expanding habitat with the formation of the Coast Ranges and additional exposure of serpentine, coupled with dynamic volcanism and a strong Mediterranean-type climate in the past several million years, may help to explain the recent diversification of *Hesperolinon* even though individual serpentine outcrops in northern California may date back to the Eocene (Nilsen and McKee, 1979; Harrison et al., 2004). Our ability to generalize these findings to other western North American serpentine radiations such as *Allium* (Alliaceae), *Navarretia* (Polemoniaceae), or the *Streptanthus–Caulanthus* complex (Brassicaceae) is limited by the lack of fossil-calibrated chronograms for these and other comparable groups.

***Phylogenetic incongruence and taxonomy of* Hesperolinon**—Within *Hesperolinon*, DNA sequences representing different populations of the same taxonomic species sensu Sharsmith (1961) do not form clades. Only *H. drymarioides*, based on nuclear and plastid data, and *H. adenophyllum*, based on the combined data analysis, were resolved as monophyletic. With the exception of single populations, *H. clevelandii*, *H. congestum*, and *H. tehamense* form clades in the nrDNA analyses. Taxonomy notwithstanding, independent analyses of both the cpDNA data and the nrDNA data did resolve a number of congruent clades. For example, *H. spergulinum* and *H. micranthum* pro parte form a clade of white flowered, tricarpellate, mostly long-pedicelled plants (cp3 and nr2; Fig. 3). Congruent placements were also recovered for a number of populations resolved in unexpected clades, such as a population of *H. disjunctum* (Sharsmith 4024) in an otherwise monophyletic clade of *H. tehamense* (cp5, nr3).

Geographic proximity of taxonomically distinct populations resolved in the same clades (Fig. 2) suggests that hybridization and subsequent introgression could be a possible source of incongruence between gene trees and also may explain the congruent placement of certain populations like UC1212496. Although parapatric populations of *Hesperolinon* are generally well marked morphologically and separated spatially along ecological gradients, Sharsmith (1961) reported several populations that were intermediate between *H. disjunctum* and *H. tehamense*, *H. californicum* and *H. micranthum*, and *H. californicum and H. didymocarpum*, and even speculated that *H didymocarpum* was of hybrid origin. Forms intermediate between the two-carpellate *H. bicarpellatum* and the recently described tricarpellate segregate *H. sharsmithiae* have led some to question whether *H. bicarpellatum* and *H. sharsmithiae* are distinct species (McDill, 2012). Conflict between cpDNA and nrDNA trees may be due to hybridization involving *H. bicarpellatum*. In the nuclear tree, all sampled populations of *H. bicarpellatum* are in a clade with other taxonomic species diagnosable by a strong diachasial cymose inflorescence (i.e., *H. didymocarpum* and *H. sharsmithiae*; nr7; Fig. 3B), in contrast to the monochasial cymes found in the rest of the genus. In comparison with the nrDNA tree, cpDNA clades more strongly reflect geographic proximity of populations rather than inflorescence architecture. Populations of *H. bicarpellatum* found within 3–4 miles of Middletown form a strongly supported clade with *H. didymocarpum*, but populations >5 miles away along Butts Canyon are in a different strongly supported clade with populations of several other taxa, including *H. sharsmithiae*. Chloroplasts are generally inherited uniparentally and are subject to introgressive "chloroplast capture," which may well explain some fine-scale discordance between the nuclear and plastid trees (see Liston et al., 2007). However, the exact plastid inheritance mechanism in *Hesperolinon* remains unknown, and two closely related species (*Linum usitatissium* and *L. stelleroides* Planch.) both show biparental inheritance (Zhang et al., 2003; but see Corriveau and Coleman, 1988).

At least two other factors in addition to gene flow across divergent lineages may contribute to the lack of congruence among cpDNA clades, nrDNA clades, and past circumscriptions of *Hesperolinon* taxa. First, incomplete lineage sorting may contribute to the discordance, especially given the recent diversification in *Hesperolinon*. Second, species may not be well circumscribed; that is, morphological characters used to recognize species may be unreliable for diagnosing clades, in spite of previous taxonomic attention to the group. In Sharsmith's (1961) monograph, which remains the primary systematic and taxonomic resource on the genus, the author noted the difficulty in determining species boundaries due to intermediate populations, going so far as to say "the combination of characters found in any homogeneous, isolated colony of *Hesperolinon*, regardless of the species, is not duplicated by the combination of characters found in any other homogenous and isolated colony of the same species" (p. 244). Contributing to this pattern may be the island-like distribution of serpentine outcrops throughout California, which could promote a mosaic of small diverging

populations (Harrison et al., 2004), with putative self-compatibility in *Hesperolinon* also leading to higher levels of inbreeding punctuated by occasional outcrossing or hybridization (Sharsmith, 1961). In any event, interspecific gene flow likely has been more prevalent than suspected by Sharsmith, at least on evolutionary time scales.

***Putative cryptic diversity in* Hesperolinon**—In contrast to evidence for interspecific gene flow among recognized taxonomic species, we found evidence of at least two instances of cryptic diversity supported by both nuclear and plastid data. Several *H. micranthum* populations from across the geographic range of the species are resolved in a strongly supported clade with all samples of *H. spergulinum* (cp2, nr3; Fig. 3). A concatenated dataset resolves this clade as sister to *H. adenophyllum* (cp/nr1; Appendix S3). Remaining populations of *H. micranthum* are resolved elsewhere in the tree, grouping similarly in both the plastid and nuclear analyses. Flowers of *H. micranthum* are the smallest and most inconspicuous in the genus, but close inspection reveals significant variation among populations. Sharsmith (1961) described the morphology of one *H. micranthum* population as "on the periphery of the arbitrary limits set for this species" (p. 246). More generally, she declared that "morphologically distinct, essentially homogeneous, geographically isolated populations are to be found within most or perhaps all species of *Hesperolinon*" (p. 245), further speculating that some of those isolates may be "genetically distinct units, perhaps nascent species" (p. 246). In this case, the phylogenetic distance between clades of *H. micranthum* and a likely ancestral state of larger yellow petals probably indicate evolutionary convergence toward small white flowers (i.e., polyphyly of *H. micranthum*) rather than nascent speciation. Convergent evolution of small-flowered selfing annuals has been inferred in other Californian genera, such as *Leptosiphon* (Goodwillie, 1999).

Second, we found evidence from the nrDNA tree that the populations of *H. disjunctum* north and south of the Sacramento Delta are not closely related (though plastid evidence is inconclusive because of low resolution). All southern populations are resolved with several populations of other taxa in a moderately supported clade related to *H. tehamense* (Fig. 3B). With the exception of the single specimen that forms a clade with *H. tehamense* (Sharsmith 4024), all northern populations are found in a large, distantly related clade with poorly supported substructure (nr5+nr6+nr7). Sharsmith (1961) also noted variability in *H. disjunctum* and suggested that the northern and southern populations may be distinct, an idea also considered and extended by Raven and Axelrod (1978). Counterintuitively, although the southern populations of *H. disjunctum* were resolved as more closely related to *H. tehamense* than were the northern populations (except Sharsmith 4024), the northern populations of *H. disjunctum* are geographically closer to *H. tehamense* and most closely resemble that species, which is nearly indistinguishable aside from flower color (Sharsmith, 1961).

Divergence times and higher-level relationships in Linaceae—In contrast to complicated infrageneric patterns in *Hesperolinon*, we found strong support for broader-scale relationships within Linaceae. Greater taxon sampling, particularly within *Linum*, and additional genetic data generally have reinforced previous conclusions about relationships in Linaceae, such as paraphyly of the yellow-flowered clade of *Linum* (including *Cliococca*, *Hesperolinon*, *Sclerolinon*, *Radiola*, and *Linum* sections *Linopsis*, *Syllinum*, and *Cathartolinum* (Rchb.) Griseb.) and monophyly of most sections of *Linum* (McDill et al., 2009; McDill and Simpson, 2011). In contrast to earlier studies, we found the monotypic southeastern Asian genus *Reinwardtia* to belong to a well-supported clade with *Anisadenia* + *Tirpitzia*. A complete discussion of relationships and taxonomy in Linaceae is found in McDill et al. (2009) and McDill and Simpson (2011).

Our estimated divergence times in the Hugonioideae generally fall within the recent end of the ranges estimated by McDill and Simpson (2011), although some clades like *Hesperolinon* show much more recent origins. This is in contrast to other studies in which incomplete taxon sampling and high rate variation among lineages resulted in an underestimate of node ages (Xiang et al., 2011; Schulte, 2013; Soares and Schrago, 2015). Our results support the late Eocene diversification for the Linoideae clade hypothesized by McDill and Simpson (2011), coincident with the expansion of temperate grassland and herb-dominated habitats in which these species are generally found today. However, two other groups for which we estimated substantially more recent node ages include the yellow-flowered clade of *Linum* and the Hugonioideae, particularly the Old World subclade. Additional taxon and gene sampling suggests that the yellow-flowered clade of *Linum* diverged from *Radiola* in the early Miocene, but did not begin diversifying until the mid-Miocene Climatic Optimum (Zachos et al., 2008). *Hugonia*, which represents much of the diversity in its subfamily, appears to have undergone extensive diversification during the past 5 million yr throughout the Paleotropics. This is likely too late for the expansion of global rainforests in the early to mid-Miocene to have played much of a direct role in diversification, as previously suggested for tropical Linaceae (McDill and Simpson, 2011), although it could have allowed for the range expansion of a common ancestor of extant Hugonioideae (Zachos et al., 2008). The late Neogene was a period of global cooling and drying but also of intensifying summer monsoons in Southeast Asia, where many *Hugonia* species are currently found (Ravelo et al., 2004; Nie et al., 2014). Such a radiation, comparable in magnitude to *Hesperolinon*, warrants more detailed phylogenetic and evolutionary study, particularly given limited taxon sampling to date.

CONCLUSIONS

We have provided fossil-calibrated phylogenetic evidence supporting several long-standing hypotheses about the genus *Hesperolinon*. These include the recent radiation of *Hesperolinon* onto serpentine soils over the past several million years, a close phylogenetic relationship of *Hesperolinon* to *Linum* section *Linopsis* pro parte, and a sister-group relationship between *Hesperolinon* and *Sclerolinon*. The result that taxonomic species previously recognized within *Hesperolinon* are generally not resolved as clades is likely due to multiple causes, potentially including ongoing gene flow within this incipient species complex, incomplete lineage sorting associated with rapid diversification, overreliance on plesiomorphic or homoplastic characteristics in species delimitation, and probably some instances of undescribed diversity, such as within *H. micranthum* and *H. disjunctum*. A full taxonomic revision, although beyond the scope of this paper, is sorely needed in light of our results. To meet that goal, additional study of morphology and leveraging of additional genomic data are needed to understand the morphological variation in *Hesperolinon* in relation to phylogenetic relationships

and to better characterize why morphological and molecular interpretations of *Hesperolinon* often conflict. More broadly, our additional taxon sampling and revised chronogram could be used to test many of the biogeographical hypotheses generated by McDill et al. (2009) to explain the cosmopolitan distribution of Linaceae. These results could also inform a taxonomic revision of the family, which will be necessary to address paraphyly in the two largest genera, *Linum* and *Hugonia*.

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