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THE EVOLUTION OF UNISEXUALITY IN *CALLIGRAPHA* LEAF BEETLES: MOLECULAR AND ECOLOGICAL INSIGHTS ON MULTIPLE ORIGINS VIA INTERSPECIFIC HYBRIDIZATION

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Abstract.—Interspecific hybridization is a well-established cause of unisexual origins in vertebrates. This mechanism is also suspected in other apomictic taxa, but compelling evidence is rare. Here, we evaluate this mechanism and other hypotheses for the evolutionary origins of unisexuality through an investigation of *Calligrapha* leaf beetles. This group provides an intriguing subject for studies of unisexual evolution because it presents a rare insect example of multiple apomictic thelytokous species within a primarily bisexual genus. To investigate unisexual evolution, this study conducts the first molecular systematic analysis of *Calligrapha*. This involved the collection and analysis of about 3000 bp of DNA sequences—representing RNA and protein-coding loci from mitochondrial and nuclear genomes—from 54 specimens of 25 *Calligrapha* species, including four unisexual tetraploid taxa. Phylogenetic and molecular clock analyses indicated independent and single evolutionary origins of each of these unisexual species during the Pleistocene. Significant phylogenetic incongruence was detected between mitochondrial and nuclear datasets and found to be especially associated with the asexual taxa. This pattern is expected when unisexual lineages arise via interspecific hybridization and thus represent genetic mosaics that possess certain nuclear alleles from the paternal species lineage and mitochondrial DNA (mtDNA) alleles from the maternal parent. Analyzing the mtDNA and nuclear relatedness of unisexuals with corresponding haplotypes of bisexual *Calligrapha* species allowed the putative identification of these maternal and paternal species lineages for each unisexual species. Strong phenotypic similarities between unisexual taxa and their paternal parent species supported a model that involves both backcrosses of interspecific hybrids with a paternal parent and unreduced gametes. This model accounts for the origins of apomixis, polyploidy, and an overrepresentation of paternal nuclear alleles (and associated phenotypes) in unisexuals. This model is also consistent with the tetraploid karyotypes of unisexual *Calligrapha*, in which three sets of chromosomes (of presumed paternal ancestry) are quite morphologically homogeneous compared to the fourth. Especially intriguing was a consistent association of unisexual species with the host plant of the paternal parent but never with the maternal host. The statistical implausibility of these patterns occurring by chance further supports our inference of parental species. Moreover, it points to a potentially critical role for host-association in the formation and preservation of unisexual lineages. These findings suggest that ecological factors are critical for the diversification of unisexual as well as bisexual taxa and thus point out new research directions in the area of ecological speciation.

Key words.—Asexuality, Chrysomelidae, herbivorous insects, parthenogenesis, phylogenetic incongruence, polyploidy, speciation.

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Amphimictic bisexuality (i.e., sexual reproduction) represents the predominant reproductive mode in animals. By contrast, asexuality represents a comparatively rare and quite taxonomically scattered reproductive strategy that characterizes few higher taxa (bdelloid rotifers and darwinulid ostracods representing notable exceptions; Normark et al. 2003). The most common and taxonomically widespread form of animal asexuality is apomictic thelytokous parthenogenesis, a genetic system characterized by the complete absence of males and by female lineages that clonally produce a daughter-only progeny due to the complete suppression of meiosis (Maynard Smith 1978). The evolution of thelytoky is highly associated with that of polyploidy (White 1978). Evolutionary transitions involving thelytoky appear to be largely unidirectional: from an ancestral diplodiploid or haplodiploid sexual condition to a derived thelytokous strategy, sometimes via cyclical or facultative parthenogenetic phases (Normark 2003). Although reversals to sexuality are theoretically possible early in these transitions (Normark 2003), they are generally considered implausible, given the difficulty of ''reinventing'' maleness and restoring diploidy (Bull and Charnov 1985).

Why sexual reproduction should be so evolutionarily dominant represents one of the major unresolved questions in evolutionary biology (Maynard Smith 1978). Indeed, given the inherent twofold fitness disadvantage of sex compared to asex (sexual females pass on only half their alleles to each offspring whereas unisexuals clonally pass on their entire genome), the success of sexual reproductive modes represents something of a paradox (Williams 1975). Attempts to resolve this paradox typically involve potential long-term disadvantages to clonality (Stearns 1987). These disadvantages are sometimes invoked to argue that unisexual lineages must typically have brief evolutionary life spans and thus represent evolutionary dead ends (Maynard Smith 1978). The evaluation of the evolutionary persistence of unisexuals (Judson and Normark 1996) has thus become a major focus of interest.

Another reason for observed patterns might be that the

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unisexual state is difficult to evolve in the first place (Simon et al. 2003). On this view, understanding unisexual rarity demands investigations of the mechanisms and evolutionary frequency of unisexual origins. It has long been suspected that a common mechanism for the origin of apomictic thelytokous unisexuality is interspecific hybridization (White 1978, p. 291). This is certainly the case in the modest number of asexual vertebrates, in which the transition from bisexual to unisexual reproduction is believed to be nearly universally caused by this mechanism (Dawley and Bogart 1989; Avise et al. 1992). However, in other taxa, including insects, empirical evidence for this mechanism is rare. To date, introgressive hybridization has only been compellingly linked to unisexual origins in four insect genera: the stick insect *Bacillus* (Mantovani et al. 2001), the grasshopper *Warramaba* (Honeycutt and Wilkinson 1989), *Rhopalosiphum* aphids (Delmotte et al. 2003), and the weevil *Otiorhynchus* (Tomiuk and Loeschcke 1992; Stenberg et al. 2000). In some other organisms, such as *Aramigus* weevils (Normark and Lanteri 1998), this mechanism is suspected but not entirely confirmed. Whether this scarcity of evidence reflects the small evolutionary significance of this mechanism outside of the vertebrates or a lack of study is unknown. Thus, detailed investigations of this potential mechanism in additional groups of insects will be critical for an understanding of its broader evolutionary implications (Kearney 2005).

A recent renaissance in the study of ecological speciation has made clear that the investigation of pertinent ecological factors is critical to understanding mechanisms of species formation (Schluter 2001; Rundle and Nosil 2005). Such studies have so far focused on sexual species, but these issues may be no less pertinent to the study of unisexual origins. In the case of unisexual species that form via interspecific hybridization, such studies should evaluate the ecological factors that determine which potential parental species actually meet and mate and whether their unisexual offspring survive to establish new species. To date, however, the genetic factors underlying potential mechanisms of asexual origin (Simon et al. 2003) have received considerably more attention than potentially crucial ecological factors.

Based on the issues discussed above, our understanding of the evolution of sex versus asex would benefit from studies of unisexual origins in ecologically specialized invertebrate taxa. Such a system is offered by *Calligrapha* leaf beetles (Coleoptera: Chrysomelidae). Asexuality is very rare among beetles, with only about a hundred known unisexual taxa, mostly among the weevils (Curculionidae; e.g., Suomalainen et al. 1987). Among the eight leaf beetle genera in which asexuality has been suggested (Cox 1996), *Calligrapha* stands out because it includes seven distinct unisexual species and because female-only thelytoky and associated allotetraploidy has been well documented among them (Brown 1945; Robertson 1966; Gómez-Zurita et al. 2004). Each unisexual *Calligrapha* taxon is generally well differentiated from congeners, exhibiting diagnostic morphological characters that are shared by populations throughout their geographic range (Brown 1945; Gómez-Zurita et al. 2004). Among these species, *C. scalaris* is unique in having been described as facultatively parthenogenetic based on variably biased population sex ratios (Robertson 1966). However, the rarity of facultative thelytoky in general (Normark 2003) and the specific observation of diploid sexuals and tetraploid asexuals within this species (Robertson 1966) suggests that the local coexistence of obligately bisexual and unisexual lineages presents a more likely explanation for observed sex ratios in this species. Both bisexual and unisexual species of North American *Calligrapha* sensu stricto are monophagous on particular shrubs and trees (Brown 1945). They have ecologically intimate associations with these plants, which represent these beetles' native habitat throughout their life history. To wit, each species oviposits, undergoes larval development, pupates, feeds, and (in the case of bisexual species) mates as adults on their specific host plant.

The present study presents the first large-scale phylogenetic analysis of *Calligrapha* species (for the only related study see Gómez-Zurita et al. 2004). More importantly, by addressing a number of fundamental questions about patterns and mechanisms of unisexual origins, this paper introduces *Calligrapha* as a potential model system for investigations of unisexual evolution. Here, we evaluate the following issues: (1) Origins: how many times has unisexuality independently evolved, and how ancient are these origins? We address these questions using character reconstruction and the application of molecular clocks. (2) Mechanisms: have unisexual species been formed as a product of interspecific hybridization? We address this question by evaluating phylogenetic incongruence between mitochondrial and nuclear gene trees and the contributions of unisexual taxa to such disagreement. Hybrid unisexuals introduce such incongruence because their mitochondrial genes will reflect the maternal species lineage while some nuclear alleles will have originated in the paternal species, yielding character conflict (Crease et al. 1989; Simon et al. 2003). This observation is not expected to hold true under alternative mechanisms of unisexual origins such as spontaneous mutation in loci associated with sexual reproduction or infection with feminizing factors such as *Wolbachia* (Simon et al. 2003). (3) Parental species: if unisexuals are of hybrid origin, which extant bisexual species represent the maternal and paternal lineages involved in the original hybridization events? We address this question by evaluating patterns of relatedness between unisexual and bisexual mitochondrial and nuclear haplotypes, respectively (e.g., Birky 1996). Patterns of phenotypic similarity were evaluated as additional evidence on paternal species, which contribute the majority of nuclear alleles under the mechanism of hybrid origins favored here. (4) (Phylo)genetic factors: are certain clades particularly prone to produce unisexuals? Is a particular level of genetic divergence between potential parental species more likely to produce unisexual descendents? We addressed these questions by evaluating the random likelihood of the observed phylogenetic distribution of unisexuals and by examining the distribution of inferred parental divergences. (5) Ecological explanations: is the host-plant association of inferred maternal or paternal species predictive of that of their unisexual daughter species? What do such patterns suggest about the ecology of unisexual speciation? We addressed these issues by evaluating the probability of observed patterns and considering their implications in view of the underlying host-associated biology of these insects.

Materials and Methods

Taxon Sampling

This study treats all *Calligrapha* species available from field collections. Our analysis includes 54 specimens belonging to 25 (Table 1) of the approximately 100 New World *Calligrapha* species (Weise 1916). Most specimens could be unambiguously identified to species according to morphology. Because *Calligrapha* species are generally monophagous, identification was further corroborated by the plant taxon on which a specimen was collected. In only two cases were our identifications provisional and based primarily on the host record. First, we identified a *Calligrapha* larva collected on *Ulmus americana* as *C. scalaris*, the only *Calligrapha* species known to use this host plant. Because there are no comprehensive comparative descriptions of *Calligrapha* larvae, morphological identification of this specimen was not possible. Second, our *C. rhoda* specimen did not fully conform to its species description (or that of any other *Calligrapha* known to us), but was collected on *Corylus* species, which only *C. rhoda* is known to use (Brown 1945). We have mainly sampled North American species from the subgenus *Calligrapha* sensu stricto, to which all unisexual taxa belong. We have used species from two related *Calligrapha* subgenera as outgroups: *Bidensomela* (*B. bidenticola bidenticola*) and *Coreopsomela* (*C. californica coreopsivora*). Our sampling of parthenogens includes three of the five cytologically documented unisexual *Calligrapha* (*C. alnicola, C. apicalis, C. vicina*) and *C. suturella* (Gómez-Zurita et al. 2004), which is only known from female specimens. Our study also includes *C. scalaris*. However, because of the provisional identification of our *C. scalaris* specimen (see above) and a lack of information on its bisexual/unisexual status, we conservatively treat this species as a bisexual taxon in our analyses.

Gene Regions, DNA Extraction, Polymerase Chain Reaction Amplification, and DNA Sequencing

Our study treats sequences from both mitochondrial and nuclear genomes. Each of these included both protein-coding and ribosomal genes. The four sequenced gene regions were: (1) a fragment spanning the mitochondrial cytochrome oxidase 1 and 2 genes (cox1 = 737 bp, cox2 = 504 bp), along with the intervening leucine tRNA (tRNA-Leu = 64–67 bp); (2) a fragment of the large ribosomal RNA subunit of the mitochondrial genome (16S rRNA = 508–512 bp); (3) an intron-free fragment of the elongation factor 1-alpha gene (ef1-α = 471 bp); and (4) the complete second internal transcribed spacer of the nuclear ribosomal RNA cluster (ITS-2 = 647–740 bp). Together, these provided over 3000 bp per specimen. Whole genomic DNA was extracted nondestructively using the DNeasy kit (Qiagen, Valencia, CA) and each gene region was PCR amplified and sequenced as described in Gómez-Zurita et al. (2004). Primers are listed in Table 2. For certain specimens, we failed to produce sequences from particular gene regions: cox1 in *C. alnicola* 11; ef1-α in *C. alnicola* 10 and 11, *C. confluens* 2, *C. elegantula, C. pantherina* 2, and *C. suturella* 1; and ITS-2 in *C. alnicola* 10, *C. apicalis* 1, *C. ignota* 2, and *C. multipunctata* 2. Sequences can be found in GenBank under accession numbers AM160792–AM161000.

Sequence Editing, Alignment, and Homology Assessment

Nucleotide polymorphism was not observed in the mitochondrial sequences of individuals, but polymorphic sites were present in a few ef1-α sequences from bisexuals: two sites in *C. wickhami*, and one in each of *C. dislocata, C. ignota* 3 and *C. polyspila* 3. Variation at these sites was incorporated using the appropriate International Union of Pure and Applied Chemistry (IUPAC) symbol. Protein-coding sequences showed no length polymorphism and were readily aligned manually, then double checked by translation to amino acid sequences. 16S rRNA sequences presented some length variation that was unambiguously aligned using the default alignment parameters in ClustalX 1.8 (gap opening = 10.0, gap extension = 0.2; transition weight = 0.5; International Union of Biochemistry DNA weight matrix; Thompson et al. 1997).

ITS-2 variation was more complicated, exhibiting polymorphism in the form of two types of length variation. The first type was single repetitions of 2–4 bp motifs. In these instances, the shortest length variant was selected and aligned with a combination of ClustalX and manual methods. Because of the nonindependence of nucleotide characters within a given repetitive element, each repetition was re-coded as a single character—specifically, as the first nucleotide in the element—for phylogenetic analysis. For example, if a given length variant was represented by one, two, or three AGG repeats, these would be re-coded as: A--, AA-, and AAA, respectively. The second type of length variation was in the form of sequence segments that were highly dissimilar between diverged *Calligrapha* lineages, yet contained considerable phylogenetic information. Sequence homology for these segments was determined using the implied alignment (Wheeler 2003) provided by the one-step direct-optimization phylogenetic reconstruction implemented in POY 3.0.11 (Wheeler et al. 2002). This method determines homology among length-variable sequences using the principles of parsimony and maximum congruence among data partitions. For ITS-2, these partitions were 11 segments that initial manual alignments had identified as an alternating pattern of six conserved and five hypervariable segments (Table 3). Search conditions for direct optimization in POY were similar to those employed in Gómez-Zurita (2004), but included twice as many random sequence addition replicates and tree rearrangements, followed by the ratcheting procedure implemented in Giannini and Simmons (2003).

Alignment of the tRNA-Leu sequences was aided by secondary structure predictions for this gene from *Drosophila yakuba* (Clary and Wolstenholme 1985) and using the tRNA scan-SE Search Server (Lowe and Eddy 1997). Sequence length variation in this gene was limited to the TΨC-loop of the transcribed molecule's cloverleaf structure. Alignment of these regions was also obtained using POY 3.0.11, after partitioning the tRNA-Leu sequence into three presumably homologous segments: a 5′ segment, the TΨC-loop, and a 3′ segment.

Phylogenetic Analyses

Standard equally weighted parsimony analyses of six gene regions were conducted, both independently and in various combinations using PAUP* 4.0b10 (Swofford 2002). Heuristic tree searches employed 50 random sequence addition replicates and tree bisection-reconnection (TBR). To evaluate the effects of indels on phylogenetic hypotheses, analyses of indel-containing partitions were conducted twice: once including and once excluding indel-containing nucleotide positions. Node support was assessed in PAUP* using 500 pseudoreplicates of nonparametric bootstrapping (Felsenstein 1985) and by estimating decay indices (Bremer 1988) with the assistance of TreeRot 2 (Sorenson 1999). Bayesian inference coupled with Markov chain Monte Carlo techniques was implemented in MrBayes 3.0 (Huelsenbeck and Ronquist 2001) after selecting the best-fitting evolutionary models for our data using ModelTest 3.06 (Posada and Crandall 1998; Table 4). ModelTest uses two criteria to identify the most appropriate model: a hierarchical likelihood ratio test (hLRT) and the Akaike information criterion (AIC). When these criteria did not recommend the same model, we selected the model with fewer parameters, that is, the one less afflicted by sampling error and that allowed faster tree estimation. Searches included four chains running for 3×10^6 generations. Trees were sampled from the cold chain every 100 generations, and they and their branch lengths were saved. We determined that the likelihood of obtained trees had plateaued before 100,000 generations. Thus we discarded the first 1000 trees (burn-in) and used the remaining trees to estimate posterior probabilities for each model parameter and for tree topology and branch lengths.

Assessing Congruence among Gene Regions

Incongruence among the phylogenetic signals provided by different gene regions was assessed by statistically evaluating the incongruence length difference (ILD) index (Mickevich and Farris 1981; Farris et al. 1994) using the partition homogeneity test implemented in PAUP* 4.0b10 (Swofford 2002). This analysis treated 999 random data partitions. For each partition, one most parsimonious tree was retained from heuristic analyses that employed 20 random sequence addition replicates and excluded all invariant characters (Cunningham 1997).

We then evaluated the contribution of unisexual taxa to incongruence between mitochondrial and nuclear datasets as follows: First, we removed the four unisexual species from the datasets and determined the ILD value. Second, we iteratively removed 299 different and randomly selected sets of four bisexual species from the datasets and determined ILD values in each instance. Third, we evaluated the difference between the mean ILD values generated by the bisexual exclusions and the ILD value from the unisexual exclusion using a *t*-test (Sokal and Rohlf 1995). If unisexuals contribute more than bisexuals to dataset incongruence—as predicted by the interspecific hybridization hypothesis of unisexual origin—then mean ILD values after bisexual removal should be significantly higher.

Evaluating the Origins of Unisexuality

Given the characteristics of *Calligrapha* unisexuality (i.e., thelytokous and [hypothetically] allotetraploid), evolutionary reversals from unisexuality to the ancestral sexual condition should be biologically unlikely, as both maleness and diploidy would have to be reconstituted (e.g., Normark and Lanteri 1998). Therefore, most parsimonious character reconstructions of unisexual evolution were obtained using MacClade 4.0 (Maddison and Maddison 2000) after constraining transformations to unisexuality to be irreversible. Unisexuality was then optimized onto parsimony trees in which each species that proved monophyletic in the combined analyses (i.e., all species except *C. alni*) was represented by a single operational taxonomic unit (OTU).

Additionally, we employed the Shimodaira-Hasegawa test (SH; Shimodaira and Hasegawa 1999) to statistically evaluate the support of the data for the alternative hypotheses that unisexuality evolved four times (that is, separately for each analyzed unisexual species) versus fewer times with subsequent asexual speciation. To do so, we used PAUP* 4.0b10 to compare unconstrained maximum-likelihood tree topologies with topologies from analyses that constrained various subsets of unisexual taxa to be monophyletic. These analyses treated 1000 resampling estimated log-likelihood (RELL) bootstrap replicates, employed evolutionary models recommended by ModelTest, and were initiated using a neighbor-joining tree with subsequent rounds of TBR. Significant results from these tests would indicate that enforcing monophyly yields a poorer fit of the data to the tree, thus arguing against unisexual speciation and for independent origins of unisexuality from the bisexual condition. Likelihood scores were additionally used in subsequent likelihood ratio tests.

Phylogenetic relatedness of unisexual genotypes was further evaluated using phylogenetic trees and statistical parsimony networks for the different sets of markers. Statistical parsimony networks were constructed using the software TCS 1.18 (Clement et al. 2000). When phylogenetic resolution was not adequate for recognizing closely related lineages, these were tentatively established based on genetic distances as the pair of most similar sexual/asexual genotypes as estimated with PAUP* 4.0b10 (Swofford 2002).

Testing the Mitochondrial Clock

No reliable fossil or geological data are available for the calibration and dating of nodes in the phylogeny of *Calligrapha*. Instead, we estimated clade ages using an oft-employed arthropod mtDNA substitution rate of 2.3% per million years (Brower 1994) and a *Calligrapha* maximum-likelihood mtDNA topology. This topology was derived using a substitution model selected using ModelTest 3.6 under both constant (L_0) and nonconstant (L_1) rate assumptions. Likelihoods obtained from these alternative analyses were evaluated using a likelihood ratio test [$\delta = 2(\ln L_0 - \ln L_1)$] and comparing δ to a chi-square distribution with 52 df (= no. OTUs − 2) to determine significance.

Next, a linearized mtDNA tree for *Calligrapha* was obtained using the semiparametric penalized likelihood method (PL) of Sanderson (2002) as implemented in r8s version 1.7

TABLE 1. Specimens treated in this study. B, bisexual reproduction; U, obligate parthenogenetic unisexuality; FP, putatively facultative parthenogenesis. NA, information not available.

Taxon	Ind. no.	Collection locality	Collectors	Host plant collected from	Reproductive mode
Ingroup					
Calligrapha (sensu stricto) Chevrolat					
C. alni Schaeffer	1	USA: WV, Tucker Co.	J. Gómez-Zurita and D. P. Duran	*Alnus* spp.	B
C. alni Schaeffer	2	Canada: QUE, Chicoutimi	D. J. Funk	*Alnus incana/rugosa*	B
C. alnicola Brown	1	Canada: QUE, Ste. Marie	D. J. Funk	*Alnus incana/rugosa*	U
C. alnicola Brown	2	USA: VT, Orleans Co.	D. J. Funk	*Alnus incana/rugosa*	U
C. alnicola Brown	3	Canada: QUE, Vallee-Jonction	D. J. Funk	*Alnus incana/rugosa*	U
C. alnicola Brown	4	Canada: QUE, St. Theophile	D. J. Funk	*Alnus incana/rugosa*	U
C. alnicola Brown	5	USA: ME, Somerset Co.	D. J. Funk	*Alnus incana/rugosa*	U
C. alnicola Brown	6	Canada: QUE, Quyon	J. Gómez-Zurita and A. Cardoso	*Alnus incana/rugosa*	U
C. alnicola Brown	7	Canada: QUE, Quyon	J. Gómez-Zurita and A. Cardoso	*Alnus incana/rugosa*	U
C. alnicola Brown	8	Canada: QUE, Quyon	J. Gómez-Zurita and A. Cardoso	*Alnus incana/rugosa*	U
C. alnicola Brown	9	Canada: QUE, Cowansville	D. J. Funk	*Alnus incana/rugosa*	U
C. alnicola Brown	10	Canada: QUE, Cowansville	D. J. Funk	*Alnus incana/rugosa*	U
C. alnicola Brown	11	USA: MN, Cook Co.	D. J. Funk	*Alnus incana/rugosa*	U
C. alnicola Brown	12	USA: MN, Cook Co.	D. J. Funk	*Alnus incana/rugosa*	U
C. amator Brown		Canada: ON, Selkirk	J. Gómez-Zurita and A. Cardoso	*Tilia americana*	B
C. apicalis Notman	1	Canada: QUE, Cowansville	D. J. Funk	*Alnus incana/rugosa*	U
C. apicalis Notman	2	Canada: QUE, Cowansville	D. J. Funk	*Alnus incana/rugosa*	U
C. confluens Schaeffer	1	Canada: QUE, East Angus	D. J. Funk	*Alnus incana/rugosa*	B
C. confluens Schaeffer	2	USA: VT, Caledonia Co.	D. J. Funk	*Alnus incana/rugosa*	B
C. dislocata (Rogers)		USA: NM, Torrance Co.	D. P. Duran	NA	B
C. elegantula Jacoby		Costa Rica: Zarcero, Alfaro Ruiz	A. Solís	NA	B
C. felina Stål		Mexico: Morelos, Cuernavaca	C. N. Duckett	*Sida* sp.	B
C. fulvipes Stål		Mexico: Puebla	S. J. Kim	NA	B
C. ignota Brown	1	USA: MN, Anoka Co.	D. J. Funk	*Betula papyrifera*	B
C. ignota Brown	2	USA: MN, Anoka Co.	D. J. Funk	*Betula papyrifera*	B
C. ignota Brown	3	USA: MN, Anoka Co.	D. J. Funk	*Ostrya virginiana*[3]	B
C. knabi Brown	1	USA: MD, Prince George's Co.	C. and S. Staines	*Cornus* spp.	B
C. knabi Brown	2	USA: MD, Prince George's Co.	C. and S. Staines	*Cornus* spp.	B
C. multipunctata (Say)[1]	1	Canada: QUE, Aylmer	J. Gómez-Zurita and D. P. Duran	*Salix bebbiana*	B
C. multipunctata (Say)[2]	2	USA: UT, Cache Co.	T. H. Hsiao	*Salix* sp.	B
C. pantherina Stål	1	Mexico: Oaxaca	S. J. Kim	NA	B
C. pantherina Stål	2	Mexico: Oaxaca, Miahuatlan	C. N. Duckett	NA	B
C. philadelphica (Linnaeus)	1	USA: OH, Pike Co.	J. Gómez-Zurita and D. P. Duran	*Cornus sericea*	B
C. philadelphica (Linnaeus)	2	USA: MI, Schoolcraft Co.	D. J. Funk	*Cornus* sp.	B
C. polyspila (Germar)	1	Uruguay: Rocha	E. Petitpierre	*Sida rhombifolia*	B
C. polyspila (Germar)	2	Brazil: PR, Areia Branca	C. N. Duckett	NA	B
C. polyspila (Germar)	3	Brazil: PR, Betania	C. N. Duckett	NA	B
C. ramulifera Stål		Costa Rica: Santa Elena	A. Solís	*Guazuma ulmifolia*	B

TABLE 1. Continued.

Taxon	Ind. no.	Collection locality	Collectors	Host plant collected from	Reproductive mode
C. rhoda? Knab		USA: NY, Saratoga Co.	D. J. Funk	*Corylus americana*	B
C. rowena Knab	1	Canada: ON, Ottawa	J. Gómez-Zurita and A. Cardoso	*Cornus* spp.	B
C. rowena Knab	2	USA: MN, Anoka Co.	D. J. Funk	*Cornus alba*	B
C. scalaris (Le Conte)		USA: ND, Pembina Co.	D. J. Funk	*Ulmus* spp.	FP
C. spiraea (Say)		USA: WV, Greenbrier Co.	J. Gómez-Zurita and D. P. Duran	*Physocarpus opulifolia*	B
C. suturella Schaeffer	1	Canada: QUE, Weedon Ctre.	D. J. Funk	*Salix bebbiana*	U
C. suturella Schaeffer	2	Canada: QUE, Stoneham	D. J. Funk	*Salix bebbiana*	U
C. suturella Schaeffer	3	USA: ME, Somerset Co.	D. J. Funk	*Salix bebbiana*	U
C. suturella Schaeffer	4	USA: ME, Kennebec Co.	D. J. Funk	*Salix bebbiana*	U
C. suturella Schaeffer	5	USA: MI, Grand Traverse Co.	D. J. Funk	*Salix bebbiana*	U
C. suturella Schaeffer	6	USA: ME, Kennebec Co.	D. J. Funk	*Salix bebbiana*	U
C. suturella Schaeffer	7	Canada: Manitoba	D. J. Funk	*Salix bebbiana*	U
C. vicina Schaeffer		Canada: ON, Ottawa	J. Gómez-Zurita and A. Cardoso	*Cornus sericea*	U
C. wickhami Bowditch		USA: TX, Culberson Co.	D. P. Duran	NA	B
Outgroup					
Bidensomela Monrós					
B. bidenticola bidenticola Brown		USA: WV, Pendleton Co.	J. Gómez-Zurita and D. P. Duran	*Bidens* sp.	B
Coreopsomela Monrós					
C. californica coreopsivora Brown		Canada: ON, Lanark Co.	J. Gómez-Zurita and A. Cardoso	*Coreopsis* sp.	B
Leptinotarsa Stål					
L. cacica Stål		Mexico: Veracruz, N of Chocamar	C. N. Duckett		

[1] *C. m. bigsbyana* form.
[2] *C. m. multipunctata* form.
[3] Specimen collected (beaten from) atypical host plant.

TABLE 2. Primers used to amplify and sequence gene regions analyzed in this study. NA, information not available.

Marker	Primer	Sequence	Reference
cox1	C1-J-2183	5′-CAA CAT TTA TTT TGA TTT TTT GG-3′	Simon et al. 1994
	TL2-N-3014	5′-TCC AAT GCA CTA ATC TGC CAT ATT A-3′	Simon et al. 1994
cox2	C1-J-2797	5′-ATA CCT CGA CGT TAT TCA GA-3′	Simon et al. 1994
	C2-N-3661	5′-GCT CCA CAA ATT TCT GAG CA-3′	Simon et al. 1994
16S rRNA	LR-N-13398	5′-CGC CTG TTT ATC AAA AAC AT-3′	Simon et al. 1994
	LR-J-12887	5′-CTC CGG TTT GAA CTC AGA TCA-3′	Simon et al. 1994
ef1-α	efs149	5′-ATC GAG AAG TTC GAG AAR GAR GC-3′	Normark et al. 1999
	NA	5′-CCA YCC CTT RAA CCA NGG CAT-3′	NA
ITS-2	ITS-3	5′-GCA TCG ATG AAG AAC GCA GC-3′	White et al. 1990
	ITS-4	5′-TCC TCC GCT TAT TGA TAT GC-3′	White et al. 1990

(http://ginger.ucdavis.edu/r8s/). The r8s analysis involved two steps. First, we estimated the optimal smoothing parameter. This balances the contributions to tree linearization of the tree likelihood and a roughness penalty that penalizes lineage heterogeneities in substitution rates. This was accomplished by cross-validation using the Bayesian tree reconstruction and estimated branch lengths as the starting topology, and the truncated Newton algorithm, which evaluated 30 smoothing values at 0.1 increments starting from 0.0. The most basal node was arbitrarily fixed at age 100.0 (r8s manual; Sanderson 2002). Rooting the topology for this analysis required an outgroup more distant than those used for our phylogenetic analyses in order to scale the most basal divergences in our tree, which otherwise would be decomposed arbitrarily. For this purpose, we used sequences from *Leptinotarsa cacica*, a member of the same subtribe as *Calligrapha* that clearly represents a divergent lineage on morphological and genetic grounds (as confirmed in this study). Second, we conducted iterative PL searches that fixed the previously obtained smoothing parameter (= 2.35 = the average of two equally optimal values: 2.3 and 2.4) and assigned a range of fixed arbitrary ages (from 1 to 100) to the root. This strategy (Leys et al. 2003) was used to determine the root age that yielded average substitution rates that were most similar to Brower's (1994) estimate.

Clade ages were thereafter estimated from the linearized tree after calibration using this root age. Ages of each unisexual species were calculated as the age of the node uniting the clade of haplotypes from each species with its bisexual sister lineage. This approach yields age estimates that are conservative with respect to the hypothesis that unisexual species are of recent origin.

TABLE 3. Hypervariable ITS-2 rRNA segments.

Position	Motif[1]	Taxa[2]
204–217	YATGTGTGW(GT)$_{0-1}$ATG	all other
	YATRTGTACGTGK	*dis*, *ele*
	TATGTGTTGTACG	*ram*
	TATA	*fel*, *ful*, *pan*, *pol*, *wic*
	TATATA	*bid*, *cor*
231–246	AC(GCC/GAC/– – –)GCCGYCGC	all other
	ACGCCGTCGCCGC	*dis*
	ACACGGCCKC	*ele*, *ram*
	C	*fel*, *ful*, *pan*, *pol*, *wic*
	GTAT	*bid*, *cor*
299–327	CGATTCWCACWMTW[TA][3]CAAYRTGTGAGAA	all other
	YGATTCR(CA)$_{1-2}$CAST[ACACA][4][ACATGT][5](GA)$_{1-2}$GAA	*dis*, *ele*, *ram*
	CGATTCACGAAAGTGAG	*fel*, *ful*, *pan*, *pol*
	GTG	*wic*
	TGA	*bid*, *cor*
349–387	ATCTCCTATTAT[CGTCATCGATAATAGCGTGTGTAC][6]	all other
	ACCTCCTATTATCATCATCGATAATAATAGCGTGTAC	*dis*
	GCGTGTGC	*ele*, *ram*
	GRCCTATTCTCAAAAATAGAAC	*fel*, *ful*, *pan*, *pol*
	ATCCAATGGAC	*wic*
	ATTCTGTAT	*bid*, *cor*
665–674	[CGTAGGCGWT][7]	all other
	A	*dis*, *ele*, *ram*, *fel*, *ful*, *pan*, *pol*, *wic*, *bid*, *cor*

[1] IUPAC ambiguity symbols are used for polymorphic positions.
[2] The first three letters of the specific epithet identify taxa sharing a particular motif.
[3] In *C. amator*.
[4] In *C. ramulifera*.
[5] In *C. ramulifera* and *C. dislocata*.
[6] Missing in *C. philadelphica* and *C. vicina*.
[7] Missing in *C. knabi*, *C. philadelphica*, and *C. vicina*.

TABLE 4. Optimal evolutionary models as selected using ModelTest 3.6. Abbreviations for models and parameters: TVM (transversion model; Rodríguez et al. 1990), HKY (Hasegawa et al. 1985), K81 (Kimura 1981), TrN (Tamura and Nei 1993), K80 (Kimura 1980), GTR (general time-reversible model; Rodríguez et al. 1990); *ef* and *uf*: equal and unequal nucleotide frequencies, respectively; Γ: heterogeneity in substitution rates (α: shape of the gamma distribution); I: invariable sites (i: proportion of invariable sites).

		Parameters										
Marker	Model	A	C	G	ti/tv ratio	A↔C	A↔G	A↔T	C↔G	C↔T	α	i
cox1[1]	TVM + Γ + I	0.33	0.14	0.12		2.31	21.13	2.73	1.29	21.13	0.92	0.54
tRNA-Leu[1]	HKY	0.37	0.15	0.15	2.33	—	—	—	—	—	—	—
cox2[1]	TVM + Γ	0.38	0.14	0.08	—	5.51	36.35	4.22	2.09	36.35	0.17	—
16S rRNA[1]	K81*uf* + Γ + I	0.37	0.08	0.15	—	1.00	6.46	2.12	2.12	6.46	0.75	0.68
ef1-α[1,2]	TrN*ef* + Γ	—	—	—	—	1.00	3.36	1.00	1.00	6.96	0.27	—
ITS-2[1]	K80 + Γ	—	—	—	1.00	—	—	—	—	—	0.18	—
mtDNA[1]	GTR + Γ + I	0.35	0.12	0.13		3.68	19.08	3.51	1.24	30.67	0.87	0.60
Nuclear DNA[1,2]	TrN*ef* + Γ + I	—	—	—	—	1.00	2.65	1.00	1.00	4.36	1.24	0.67
All data[1,2]	GTR + Γ + I	0.31	0.17	0.18		1.83	7.71	2.51	1.63	12.14	0.76	0.58

[1] As selected by the hLRT.
[2] As selected by the Akaike information criterion.

RESULTS

Phylogenetic Findings

With respect to individual loci (Fig. 1, Table 5), strict consensus trees for the mitochondrial genes presented very similar topologies, each of which recovered the four clades and one paraphyletic group highlighted in Figure 2. The tRNA-Leu tree (not shown) resolved six nodes and is compatible with the other mtDNA trees. The combined mtDNA analysis provided high resolution and considerable statistical and character support for most clades. Trees from the two nuclear loci each agree with the mitochondrial loci with respect to the basal placement of the species in clade A (Fig. 2). The rest of the ef1-α-based tree is poorly resolved, and most of the additional multispecies clades recovered in this tree conflict with those supported by mtDNA. Including indels greatly increased the number of informative characters in the ITS-2 analysis (Table 5), yielding a rather highly resolved ITS-2 tree. Nonetheless, this tree fails to recover any clades from the mtDNA tree outside of clade A, and often places particular species in very different parts of the tree. The analysis of combined nuclear partitions (not shown) resolved only six of the more basal nodes (Fig. 2). All Bayesian trees were compatible with parsimony strict consensus topologies and further resolved some of their polytomies, although often with low posterior probabilities (<0.60). The only notable disagreement between parsimony and Bayesian trees were the different branching orders of *C. ramulifera*, *C. elegantula*, and *C. dislocata*.

All loci and partitions agreed that the four unisexual *Calligrapha* taxa do not collectively form a monophyletic group. Indeed, in no instance are any pair of these four species supported as sister taxa. In the mtDNA tree, all three unisexual species represented by multiple samples were monophyletic. By contrast, in ITS-2 one *C. suturella* was placed divergently from the others, and *C. alnicola* was also nonmonophyletic (but see resolution of networks below). Bootstrap support for these relationships was not strong, however.

Further insights on genealogical relationships among nuclear genotypes from the North American clade were provided by genotype networks (Fig. 3). Shared alleles between bisexual and unisexual species were found for *C. multipunctata* with *C. suturella* (ITS-2), *C. confluens* with *C. apicalis* (ef1-α) and *C. alnicola* (ITS-2), *C. alni* with *C. alnicola* (ef1-α and ITS-2), and *C. knabi* with *C. apicalis* (ef1-α). In two cases, *C. apicalis* and *C. alnicola*, asexual taxa share genotypes with at least two bisexual species. Close relationships between species pairs could also be established for *C. multipunctata* and *C. suturella* (ITS-2) and *C. philadelphica* and *C. vicina* (ef1-α and ITS-2).

Incongruence among Gene Regions

The ILD was consistently nonsignificant for all the comparisons of mitochondrial markers and consistently highly significant for all intergenomic comparisons and for the two nuclear genes (Table 6). These results held regardless of whether gapped positions were included in the analyses. The ILD values based on the combined mtDNA versus nuclear DNA datasets were also significant (ILD including gaps = 0.119; ILD excluding gaps = 0.088; $P < 0.05$ for both).

Our analyses of the effects of unisexual versus bisexual species exclusion on ILD values strongly supported the hypothesis that the unisexual taxa contribute disproportionately to observed disagreement between the mitochondrial and nuclear data. The great majority of replicates with bisexual removal (263/299 = 87%) yielded a higher ILD value than was observed upon unisexual removal (ILD = 0.043; a value approximately one-third as large as that obtained before unisexual exclusion). Furthermore, the mean of these bisexual-removal ILDs was highly significantly greater than the unisexual-removal ILD ($P \ll 0.001$).

Both of the nuclear genes exhibited some intraindividual polymorphisms. In principle, these polymorphisms could have compromised our phylogenetic reconstructions and estimates of dataset incongruity. This is because our use of IUPAC ambiguity codes may have yielded sequences representing inappropriate ''averages'' of two phylogenetically divergent alleles. However, such heterozygosity was rare, being found at only five nucleotide sites and in only four sequences. When different possible resolutions of each poly-

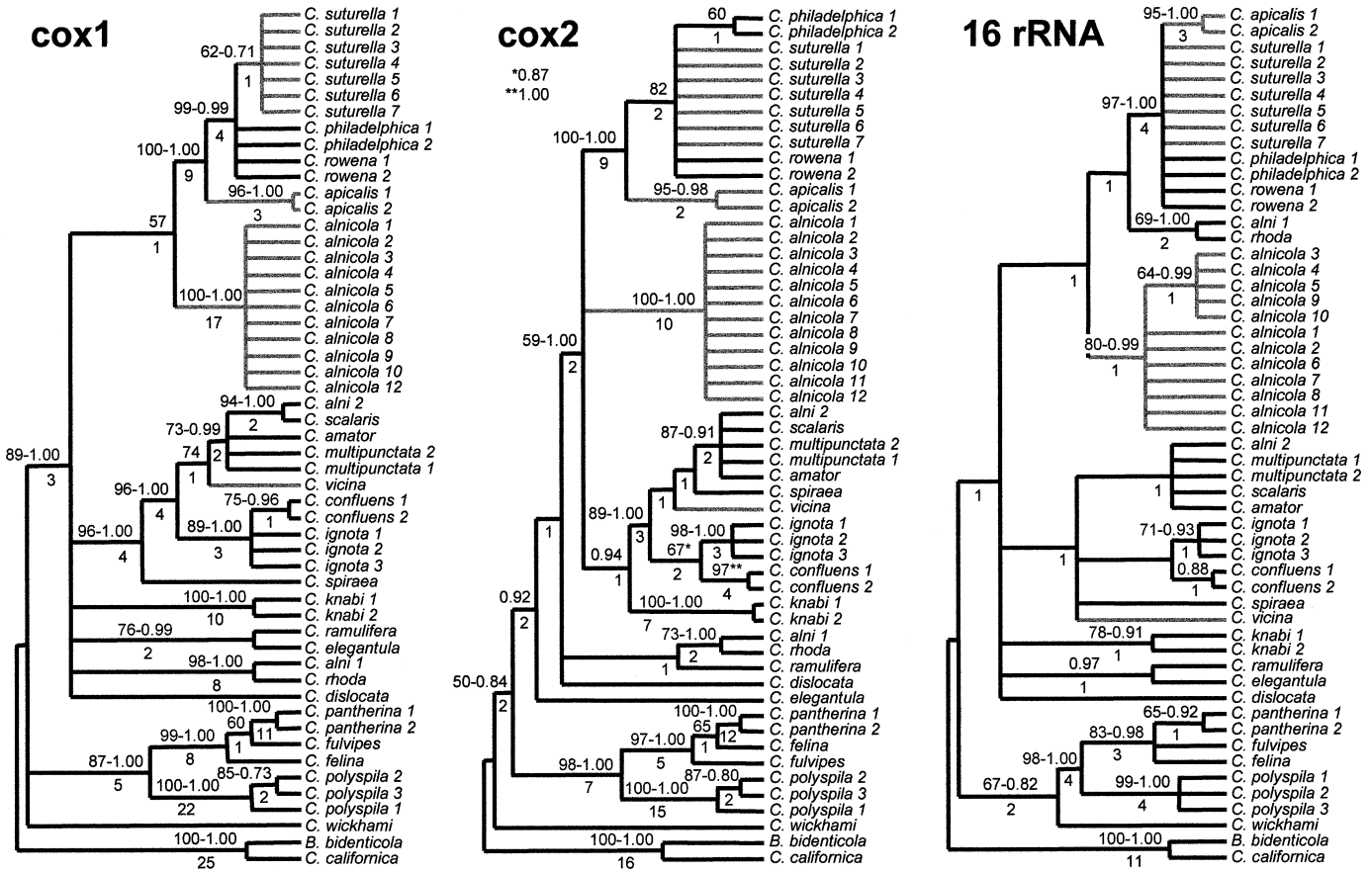

FIG. 1. Strict consensus trees from parsimony analyses of particular gene regions. The mtDNA tree represents a combined analysis of all mitochondrial regions. Bootstrap proportions (>50%) and Bayesian posterior probabilities (>0.50) are presented above branches and decay indices below branches as estimates of nodal support. Due to space constraints, see Figure 4 for Bayesian posterior probabilities for the mtDNA tree. Samples corresponding to parthenogenetic taxa are indicated with gray-shaded branches.

morphic site were considered in the construction of possible alternative ''alleles,'' the alleles from a given individual inevitably formed a monophyletic group. Thus, coexisting alleles were not phylogenetically divergent and so should not compromise our phylogenetic reconstructions. Furthermore, none of the individuals with polymorphic sites was unisexual. Thus, polymorphism cannot explain the special contribution of unisexuals to dataset incongruence.

Applying a Mitochondrial Clock

Maximum-likelihood analyses failed to reject the null hypothesis of constancy in substitution rate across lineages under the optimal GTR + Γ + I evolutionary model (χ^2 = 42.01, df = 52; P = 0.83). Our inferred root age of 10.6 million years ago (Mya) indicated a mean substitution rate of 0.0115 substitutions/per million years (My; Fig. 4). Using this calibration, the origin of *Calligrapha* was estimated to be 9.3 ± 0.22 Mya, and that of the ''North American clade'' to be 4.1 ± 0.10 Mya. Maximum ages (± SE) for the mitochondrial lineages of unisexual taxa were estimated to be 0.3 ± 0.01 My for *C. suturella*, 0.8 ± 0.02 My for *C. apicalis*, 0.9 ± 0.02 My for *C. vicina*, and 3.1 ± 0.07 My for *C. alnicola* (Fig. 4).

Origins of Unisexuality

At least as many unisexual origins as unisexual species included in the analysis were observed for reconstructions employing each of our separate- and combined-dataset tree topologies. This support for multiple independent unisexual origins received strong and consistent statistical corroboration from the SH and likelihood ratio tests (Table 7). These rejected multispecies unisexual clades for all but one possible combination of unisexual species. All phylogenetic analyses placed all unisexual sequences within the North American clade (Fig. 2).

In principle, the above-mentioned nucleotide polymorphisms could be informative of the mechanism of unisexual origins. If chromatographs consistently showed that the two peaks at polymorphic sites exhibited a 3:1 size ratio, this would be consistent with the particular cytogenetic model of hybrid origins posited below. In addition, if there were many polymorphic sites, cloning could be used to identify the divergent alleles yielding this heterozygosity, thereby providing evidence on the particular *Calligrapha* lineages that represent the maternal and paternal ancestors of a unisexual species. However, very few sites were polymorphic in ef1-α and

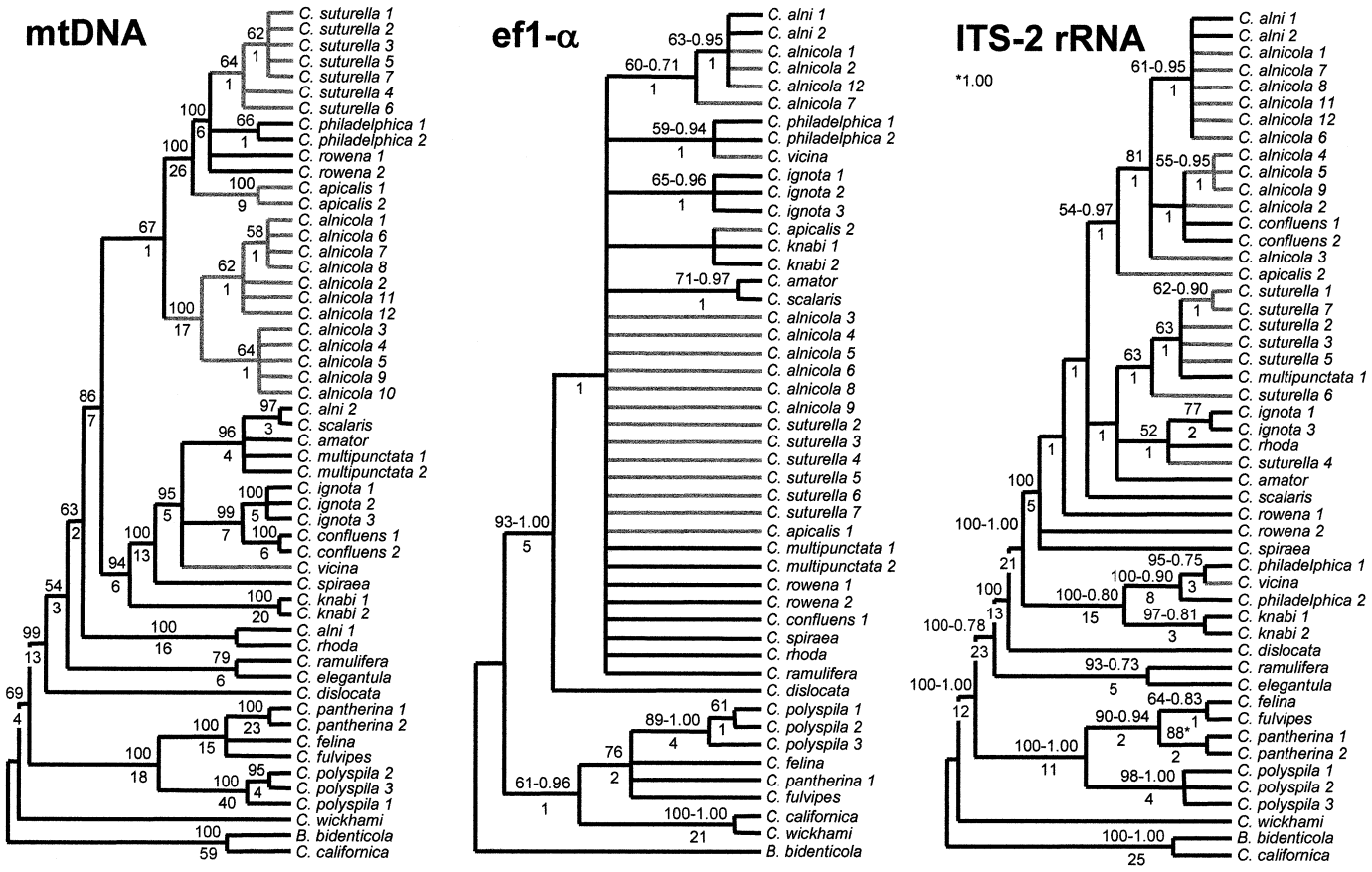

FIG. 1. Continued.

TABLE 5. Characteristics of analyzed gene regions and parsimony analyses for separate and combined datasets and alternative coding of indels. *D*, the maximum interspecific uncorrected p-distance.

	Length	AT%	ti/tv ratio	*D*	No. trees	Tree length	No. variable	No. informative	CI	RI
Gaps (5th state)										
Mitochondrial					65	957	467	360	0.6186	0.8615
cox1	737	72.1	2.027	0.127	22	486	227	177	0.6091	0.8513
tRNA-Leu	64–67	71.7	1.014	0.116	24	23	14	13	0.7826	0.9524
cox2	504	75.7	1.931	0.125	3	303	145	112	0.5974	0.8598
16S rRNA	508–512	76.8	1.228	0.058	75	133	81	58	0.7293	0.9058
Nuclear					$>10^6$	595	368	258	0.7395	0.9149
Nuclear[1]					$>10^5$	357	251	153	0.7983	0.9211
ef1-α	471	53.5	3.196	0.110	1205	177	123	60	0.7966	0.8475
ITS-2	647–740	50.5	1.074	0.065	9	379	245	198	0.7889	0.9495
ITS-2[1]					696	159	128	93	0.9057	0.9778
All data					239	1675	835	618	0.6161	0.8557
All data[1]					384	1408	718	513	0.6229	0.8503
Gaps (excluded)										
Mitochondrial					64	925	451	348	0.6173	0.8605
tRNA-Leu					1	8	7	6	0.8750	0.9722
16S rRNA					72	117	72	53	0.7436	0.9150
Nuclear					$>10^6$	280	194	106	0.7893	0.8793
Nuclear[1]					$>10^6$	271	189	102	0.7934	0.8796
ITS-2[2]					$>10^6$	86	71	46	0.9302	0.9763
ITS-2[1,2]					$>10^6$	79	66	42	0.9367	0.9782
All data					922	1254	645	454	0.6316	0.8473
All data[1]					51,920	1244	640	450	0.6318	0.8474

[1] ITS-2 rRNA data was alternatively analyzed excluding the hypervariable regions.
[2] The number of TBR rearrangements per replicate was limited to 10^8 due to the extremely high number of equally parsimonious trees found during the search.

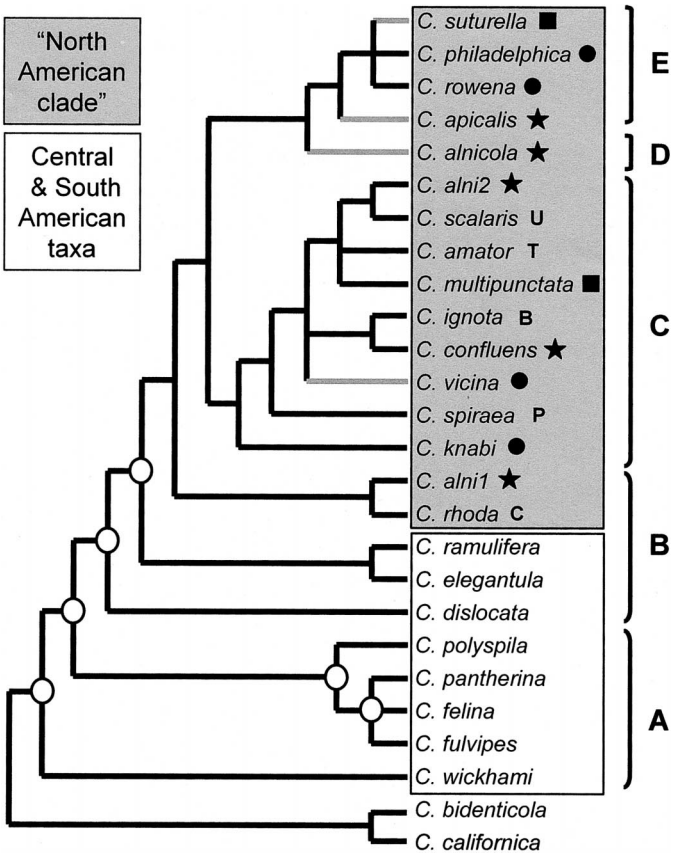

FIG. 2. Simplified mtDNA tree, illustrating major phylogenetic findings. This topology includes the two divergent haplotypes of *Calligrapha alni*, but otherwise depicts a single sequence for each study species. Choice of sequences does not affect this topology as no other *Calligrapha* species were found to be mitochondrially polyphyletic (see Fig. 1). Circles indicate those clades also recovered in a combined analysis of the two nuclear genes and thus highlight relationships that are congruent across genomes. Phylogenetic elements A–E are also discussed in the context of agreement among data partitions. Gray branches indicate the most parsimonious reconstruction of unisexual origins on the assumption that reversals to the bisexual condition are not possible. All species in the ''North American clade'' have distributions that include northern regions of this continent. The remaining species have distributions centered in Latin America. Different symbols are used to indicate the host-plant associations of species in the North American clade: square, *Salix*; circle, *Cornus*; star, *Alnus*; U, *Ulmus*; T, *Tilia*; B, *Betula*; P, *Physocarpus*; C, *Corylus*.

polymorphic sites did not exhibit the predicted peak size ratios, so no such inferences were possible.

DISCUSSION

The Evolutionary History of Unisexual Origins in Calligrapha

Our phylogenetic analyses of *Calligrapha* indicate that thelytokous parthenogenetic unisexuality is evolutionarily derived from an ancestral bisexual condition for each of the four unisexual species studied here. This finding presents no evidence that unisexual *Calligrapha* lineages have diversified through subsequent speciation, consistent with the hypothesis that unisexuality represents an evolutionary dead end (Maynard Smith 1978). Networks of nuclear markers were not very informative about the occurrence of multiple independent origins of asexuality, since most alleles harbored by different unisexual species show on average little divergence and appear clustered within the network core (see Fig. 3). However, monophyly tests are unequivocal in this respect (Table 7). Furthermore, lower molecular evolutionary rates at nuclear compared to mtDNA loci and considerably longer coalescent times for the nuclear markers (increased in polyploid organisms) may account for high similarity and low polymorphism among nuclear haplotypes from different species. Thus, low mutation rates may account for a pattern that might otherwise be interpreted as being inconsistent with independent origins.

Our findings are more equivocal on whether each of these unisexual taxa is itself the product of single or multiple (polyphyletic) origins, the latter resulting from separate matings involving different and possibly genetically varied pairs of bisexual parents. In other groups, many unisexual species are known to have multiple origins, a fact that further explains high genetic diversity and phenotypic variation within these taxa (Vrijenhoek 1998; Simon et al. 2003). Support for multiple origins would have been provided by species-level unisexual polyphyly (Funk and Omland 2003), that is, gene trees in which the different alleles from a unisexual species are most parsimoniously interpreted as having independent origins, rather than exhibiting the monophyly indicative of single origins (Moritz et al. 1992).

In our study, two of the four unisexuals (*C. alnicola* and *C. suturella*) are potentially informative in this regard because they are represented by multiple specimens and localities (four different states/provinces in each case) and thus may be somewhat illustrative of diversity within these species. Mitochondrial data show that each of these species is monophyletic and exhibits exceedingly low genetic variation. In fact, the maximum pairwise divergences among cox1/cox2 sequences in these species (0.3% and 0.1%, respectively) are an order of magnitude lower than those typically found within insect species for these genes (see, e.g., references in Funk 1999). The nuclear trees do not support the monophyly of these species. However, they also do not provide strong statistical support for polyphyly, and maximum nuclear sequence divergences for either of these species for either nuclear gene is also modest (0.7%, compared to a maximum interspecific divergence of 7%). Our findings thus provide no strong evidence of multiple origins for either of these two species. Nor can they completely rule out this scenario, however, as multiple origins may involve extremely closely related bisexual ancestors, and more intensive sampling could provide stronger evidence for polyphyly (Johnson and Bragg 1999). Few animal studies have phylogenetically evaluated these issues within a broad taxonomic framework.

A Biological Mechanism for Unisexual Origins: Hybrid Speciation

Various unisexual animal species are known to be the product of interspecific hybridization between diploid bisexual species (e.g., Suomalainen et al. 1987; Avise et al. 1992; Dufresne and Hebert 1994). This may be the only mechanism by which unisexuality originates in vertebrates (Avise et al.

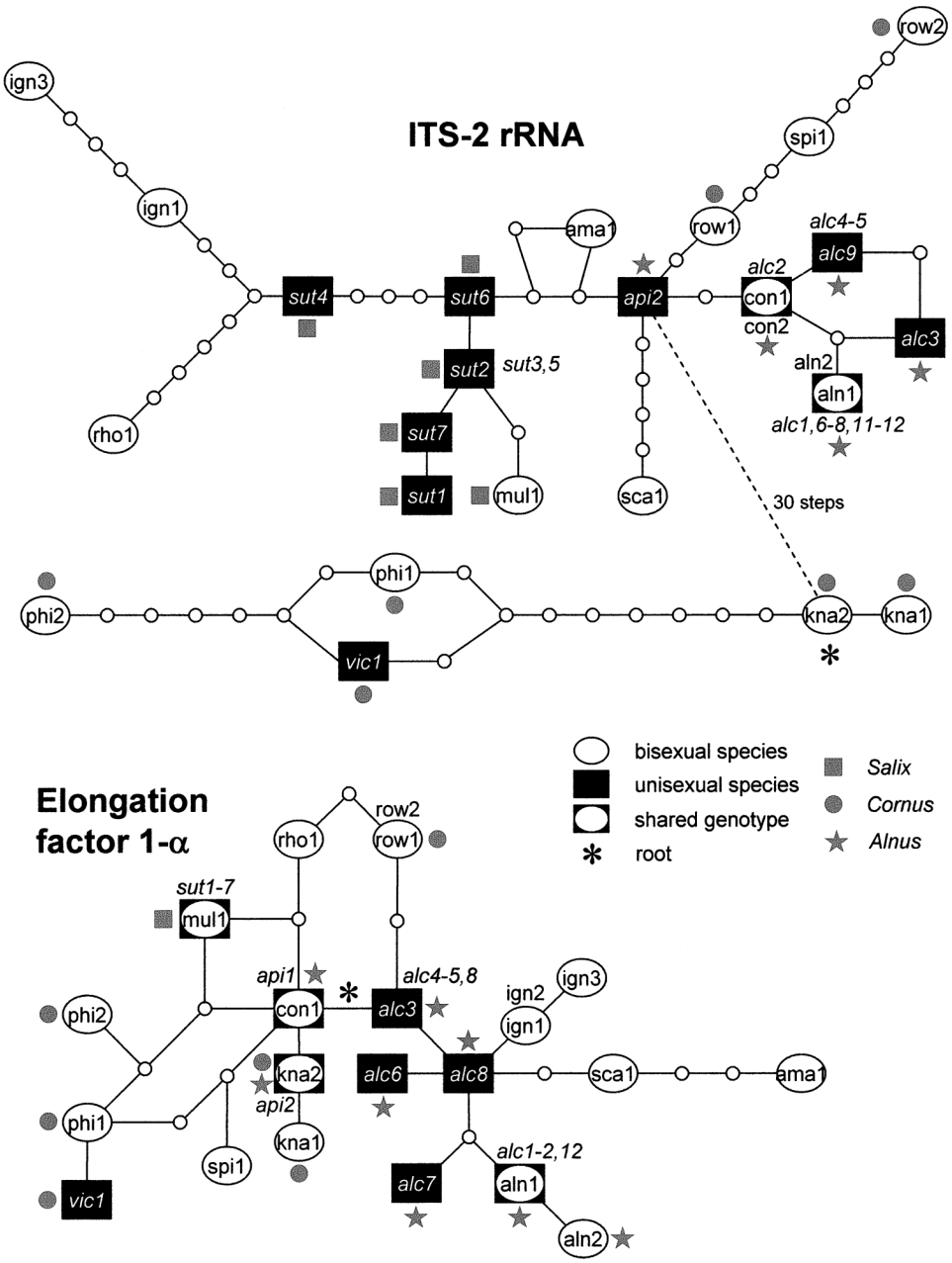

FIG. 3. Nuclear genotype networks of *Calligrapha* specimens belonging to the North American clade. Genotypes obtained from sexually and unisexually reproducing taxa are distinguished with white ovals and black boxes, respectively. When more than one specimen shared the same genotype, these are indicated adjacent to the corresponding symbol. Each species is identified by the three first letters of its specific epithet (except for *C. alnicola* = alc) and italics are used to identify genotypes from unisexual taxa. An asterisk places the inferred root of the network as identified by outgroup rooting with *C. ramulifera*. Species' associations with those host plants shared by multiple *Calligrapha* taxa are indicated with appropriate symbols adjacent to each genotype. Single-species host associations are not indicated.

1992) and it has been demonstrated in several invertebrates as well (Simon et al. 2003). Our findings of conflict between the phylogenetic signals from mitochondrial and nuclear data, and the greater contribution of asexual taxa to this conflict, are highly consistent with this mechanism. This is because hybrid species obtain their mtDNA from their maternal ancestor and at least some of their nuclear alleles from their paternal ancestor. Since maternal and paternal species may be genetically diverged from one another, their hybrid offspring will contain some nuclear alleles with considerably different histories from that of their mitochondrial genome.

The incidence of mtDNA/nuclear historical incongruence is expected to be especially prevalent in the case of asymmetric hybridization. This occurs when successful matings occur more frequently between males of species A and females of species B than between B males and A females.

TABLE 6. Results of the ILD tests. ILD values are corrected by the tree lengths (S) of the corresponding combined analyses: ILD = $[S_{12} - (S_1 + S_2)]/S_{12}$. Below diagonal, analysis treating gaps as a fifth character state; above diagonal, analysis excluding gapped positions.

	cox1	tRNA-Leu	cox2	16S rRNA	ef1-α	ITS-2
cox1		0.006 ns	0.008 ns	0 ns	0.088*	0.040*
tRNA-Leu	0.006 ns		0.010 ns	0.031 ns	0.041*	0.060*
cox2	0.008 ns	0.009 ns		0.007 ns	0.106*	0.049*
16S rRNA	0.002 ns	0.031 ns	0.009 ns		0.122*	0.081*
ef1-α	0.088*	0.070*	0.106*	0.129*		0.077*
ITS-2	0.083*	0.050*	0.086*	0.082*	0.093*	

ns, nonsignificant, * $P < 0.005$.

Hybridization is more commonly asymmetric than symmetric (Dowling and Secor 1997; Wirtz 1999) and has contributed to unisexual origins in taxa such as *Daphnia* (Schwenk 1993 and references therein), *Meloidogyne* root knot nematodes (Hugall et al. 1999), *Darevskia* rock lizards (Murphy et al. 2000), and *Bacillus* stick insects (Mantovani et al. 2001) among others. If asymmetric hybridization is followed by the backcrossing of hybrid offspring with the paternal species, all mtDNA in the resulting progeny will be from the original maternal species whereas the majority of nuclear alleles will be of paternal origin (Harrison et al. 1987; Aubert and Solignac 1990; Saura et al. 1993; Normark and Lanteri 1998).

This scenario might explain the incongruence between mitochondrial and both nuclear loci in *Calligrapha*. It might further provide an alternative explanation for the low intraspecific genetic and phylogenetic variation in the unisexual *C. alnicola* and *C. suturella*. Such asymmetry ensures that mitochondrial and nuclear alleles in the hybrid unisexual each largely or entirely derive from a single parental species and thus are comparatively homogeneous. By contrast, under a scenario of ''symmetrical'' hybridization/backcrossing, mitochondrial and nuclear alleles of both parental species should be distributed among individuals of the new unisexual species, yielding high genetic variation and clear polyphyly for all loci.

Further support for the asymmetric hybridization scenario

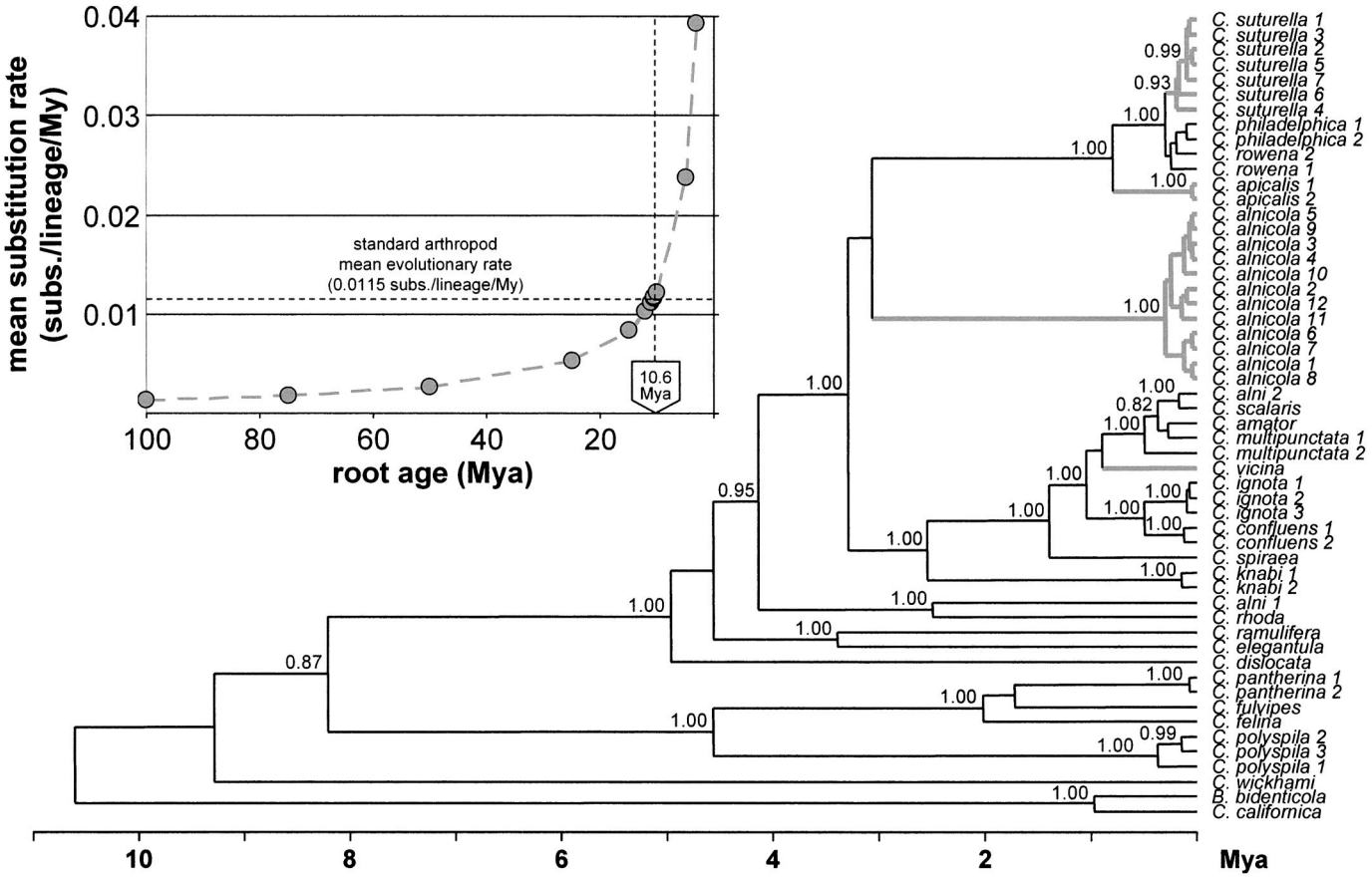

FIG. 4. Linearized maximum-likelihood mtDNA tree indicating inferred divergence dates during *Calligrapha* evolution. Node support is provided by Bayesian posterior probabilities, and gray-shaded branches represent parthenogenetic samples. The inset plot shows the relationship between arbitrarily chosen root ages and average substitution rate across the tree. The tree is scaled using a root age of 10.6 million years ago (Mya), which yields a rate compatible with an arthropod mtDNA clock (see text for details).

TABLE 7. Results of Shimodaira-Hasegawa (SH) and likelihood ratio (LR) tests for the monophyly of asexuality. Note that enforcing the monophyly of subsets of unisexual taxa results in significantly worse fit of the data (at $P < 0.05$) in all cases except those indicated by ''ns.'' *alc*, *C. alnicola*; *api*, *C. apicalis*; *sut*, *C. suturella*; *vic*, *C. vicina*.

	mtDNA			Nuclear DNA			Total data		
	Likelihood	SH test	LR test	Likelihood	SH test	LR test	Likelihood	SH test	LR test
Unconstrained analysis	7318.2216			3780.2090			11,720.3527		
Constrained analyses									
single origin	7509.6795	0.000	0.000	3880.2005	0.000	0.000	11,942.3330	0.000	0.000
(*alc api sut*)	7389.0370	0.000	0.000	3832.6748	0.010	0.000	11,810.5680	0.000	0.000
(*alc api vic*)	7487.2893	0.000	0.000	3865.5747	0.000	0.000	11,912.7598	0.000	0.000
(*alc sut vic*)	7508.0938	0.000	0.000	3878.2093	0.000	0.000	11,940.7298	0.000	0.000
(*api sut vic*)	7478.2103	0.000	0.000	3834.5810	0.003	0.000	11,897.1775	0.000	0.000
(*alc api*)	7377.1531	0.003	0.000	3814.8809	0.087 ns	0.045	11,791.1387	0.002	0.000
(*alc sut*)	7385.8206	0.003	0.000	3829.9995	0.014	0.000	11,807.3918	0.000	0.000
(*alc vic*)	7363.1923	0.030	0.001	3865.3259	0.000	0.000	11,780.3969	0.008	0.000
(*api sut*)	7323.5311	0.735 ns	0.999 ns	3794.4118	0.519 ns	0.996 ns	11,730.4681	0.646 ns	0.999 ns
(*api vic*)	7462.0576	0.000	0.000	3817.4391	0.051 ns	0.018	11,879.2427	0.000	0.000
(*sut vic*)	7475.7530	0.000	0.000	3826.0588	0.020	0.000	11,893.5946	0.000	0.000

is provided by *Calligrapha* cytogenetics. Work by Robertson (1966) showed a consistent association of unisexuality with tetraploidy in these leaf beetles. This is consistent with findings on invertebrate unisexuals of hybrid origin, most of which are polyploids thought to form via backcrossing of F_1 hybrids with one of the parentals (Suomalainen et al. 1987; Saura et al. 1993; Simon et al. 2003). Unisexual *Calligrapha* furthermore exhibit a form of chromosomal heteromorphy in which one set of chromosomes appears to differ structurally from the other three. This pattern would result, for example, from two generations of backcrossing of an initially diploid hybrid with the paternal species, coupled with unreduced gamete formation in both the F_1 hybrid generation and the F_2 apomictic triploid. A related scenario has been specifically hypothesized for *Otiorhynchus* weevils. In this scenario, diploid unisexuality evolves from diploid bisexuality, and triploid and tetraploid unisexual forms arise from subsequent additions of haploid genomes (Saura et al. 1993; Normark and Lanteri 1998). Because there is no evidence for extant diploid or triploid *Calligrapha* unisexuals, the intermediates would seem to have a brief existence if the above scenario holds. However, intermediate ploidy levels are not a necessary precursor to tetraploidy if unreduced gametes are contributed during any interspecific crosses or backcrosses (Saura et al. 1993). These mechanisms of unisexual polyploid origins are diagrammed in Figure 5.

In sum, data from phylogeny, genetic diversity, and cytogenetics are consistent with the origin of *Calligrapha* unisexuals via asymmetric interspecific hybridization and subsequent backcrossing with the paternal species. For the sake of brevity, this mechanism is hereafter referred to as the asymmetric hybridization scenario.

Identifying Parental Species

Under the asymmetric hybridization scenario, a unisexual species' mtDNA should phylogenetically cluster with that of its maternal parent species, while most of its nuclear DNA should group with that of its paternal species. When both mitochondrial and nuclear gene trees are available, this information can thereby be used to infer putative male and female parental species for a particular unisexual species (Birky 1996; Simon et al. 2003; Fig. 6). When making inferences from such data, two caveats are worth noting. First, their precision may be limited by phylogenetic resolution or the absence of parental species from the analysis. Second, because unisexual species necessarily originated at some point in the past, inferences on parentage are most properly attributed to particular lineages rather than to extant species per se.

This approach provided unambiguous support for paternal and maternal lineages of *C. suturella*, because *C. multipunctata* was embedded within an ITS-2 clade including six of seven *C. suturella* haplotypes, whereas either *C. philadelphica* or *C. rowena* is the sister species of *C. suturella* in the mtDNA tree, respectively. We earlier speculated on such a hybrid origin for *C. suturella* involving just these species based on cox1 sequences and morphological data (Gómez-Zurita et al. 2004). Genealogical evidence similarly supports a paternal ancestor of *C. alnicola* from the lineage of *C. alni* and *C. confluens* (*C. alni* shares an ef1-α genotype with *C. alnicola*, whereas both this taxon and *C. confluens* share ITS-2 variants) and a paternal ancestor of *C. apicalis* from the lineage of *C. confluens* and *C. knabi* (both of which share ef1-α genotypes with the unisexual *C. apicalis*). The *C. philadelphica* and *C. rowena* lineage stands out as the potential maternal lineage based on the mtDNA tree. In the case of *C. alnicola*, which branches off deep in the mtDNA phylogeny, no individual maternal lineage can be inferred given the sampling available; however, until additional evidence is gathered, we can tentatively place its maternal ancestry in the lineage leading to *C. philadelphica* and *C. rowena*. For *C. vicina*, *C. philadelphica* is strongly supported as its closest nuclear relative and possible paternal species, but *C. amator*, *C. confluens*, *C. ignota*, *C. multipunctata*, and *C. scalaris* are all equally phylogenetically related on the mtDNA tree, with *C. scalaris* exhibiting the greatest mtDNA sequence similarity among these. The phylogenetic imprecision of particular parental identifications may partly reflect the absence from our analyses of four bisexual (and thus potentially parental) species whose geographic distributions overlap with those of the unisexuals: *C. amelia*, *C. dolosa*, *C. pnirsa*, and *C. pruni*.

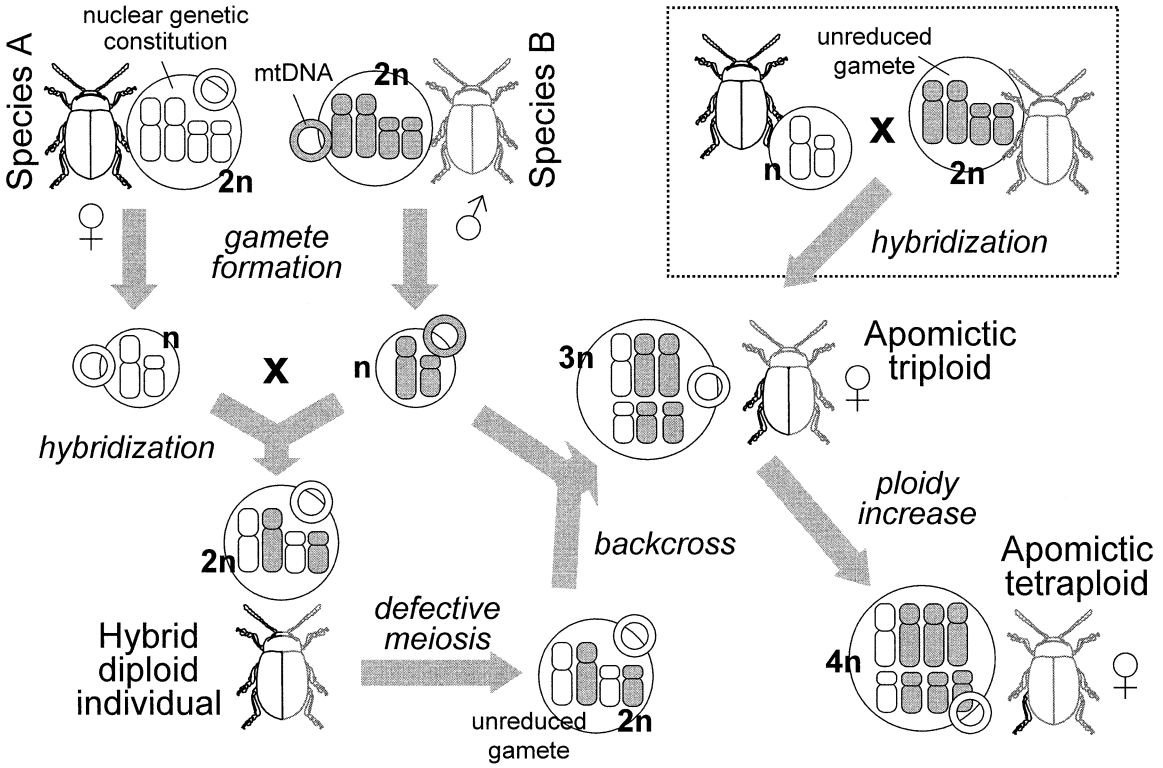

FIG. 5. Schematic representation of a scenario describing the origins of polyploid apomixis in *Calligrapha* (adapted from Saura et al. 1993). This scenario is consistent with cytogenetic, molecular, and phenotypic findings described in this and previous studies. Via this mechanism, unisexuality results from interspecific matings and subsequent asymmetric backcrossing of the hybrid with the paternal parental species. Through an unknown process that may involve unreduced gametes in intermediate stages, this newly formed unisexual lineage ultimately attains a stable tetraploid condition. The resulting apomictic tetraploid is phenotypically and ecologically similar to the paternal parent from the initial interspecific hybridization event, reflecting overrepresentation of paternal nuclear genes (color of beetle outlines schematically indicates phenotype). Thus, nuclear alleles tend to be indicative of unisexual ancestry through the paternal species while the mtDNA of the unisexual hybrid lineage should be informative of the maternal species from which it descended.

Most nuclear alleles drawn from unisexual individuals appear closely related in the networks (with the exception of those of *C. vicina*), a pattern (Fig. 3) that could be interpreted as a single gene pool contributing nuclear alleles to the interspecific matings with divergent mtDNA maternal lines. However, as mentioned above, lower variation in the nuclear markers compared to their mtDNA counterpart and correspondingly reduced phylogenetic resolution is possibly a better explanation for this clustering. Moreover, at least two of the unisexual taxa share nuclear alleles simultaneously with each of two different bisexual species (*C. alnicola* with *C. alni* and *C. confluens* for ITS-2, and *C. apicalis* with *C. confluens* and *C. knabi* for ef1-α; see Fig. 3). Assuming the model described above and mtDNA monophyly for each unisexual species, this could be explained as a single maternal line mating with at least two different paternal lines to produce the same kind of unisexual allotetraploid. We think this is an implausible scenario, and allele sharing with multiple extant species most likely reflects an ancestral origin of these particular unisexual taxa from a single lineage currently showing incomplete lineage sorting among its derived species.

Our genetically based identifications of parental species lineages are consistent with the overlapping geographic and habitat distributions of each unisexual species and with those of its putative parents in northeastern North America. Phenotypic information further informs and corroborates our conclusions. Because the asymmetric hybridization scenario would suggest that unisexuals receive most of their nuclear alleles from their paternal species and because nuclear genotypes produce phenotypes, we predict that our genetically identified paternal species should also be quite phenotypically similar to their putative unisexual offspring, and much more similar to them than are the maternal species. This prediction is borne out with respect to the elytral and pronotal markings that are most diagnostic of *Calligrapha* species. We have previously described in detail the resemblance of *C. suturella* to *C. multipunctata* (Gómez-Zurita et al. 2004). Such similarities are likewise obvious between *C. alnicola* and *C. alni* or *C. confluens* and between *C. vicina* and *C. philadelphica*. For example, in the latter case the morphological distinction between these taxa is based largely on the slightly heavier elytral markings in *C. vicina*. The exhibition of markings similar to but heavier than those of the putative paternal species also occurs in the other unisexuals and presumably reflects the production of additional paternal gene products in tetraploid organisms in which three of four gene copies are of paternal origin. Intriguingly, this observation allows us to predict that if the paternal species for *C. apicalis* is actually among the unsampled *Calligrapha* bisexuals, then

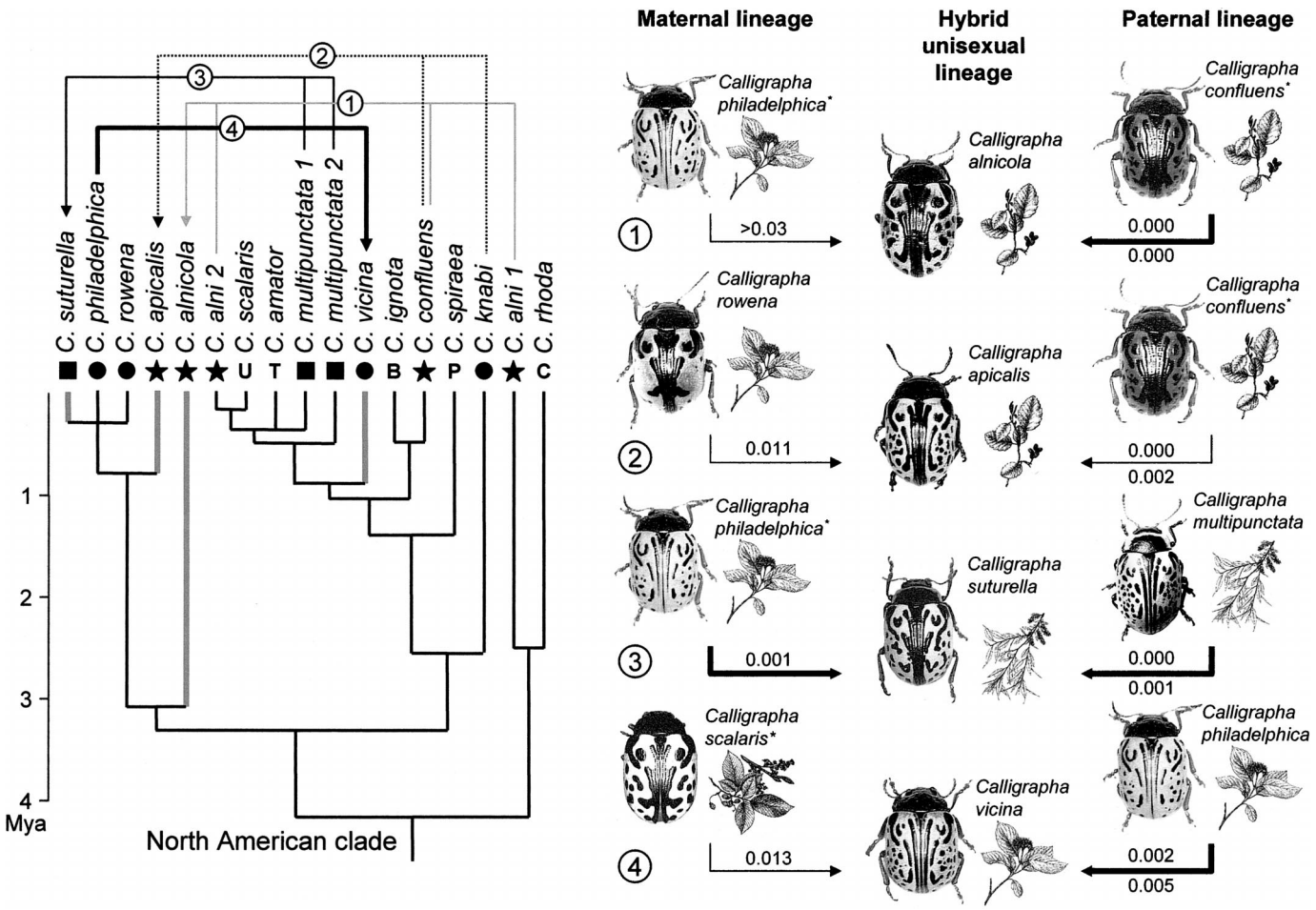

FIG. 6. (Left) Clock-enforced mtDNA North American clade showing four origins of unisexuality as thick gray branches and host-plant associations (for symbols see Fig. 2 caption). The arrows signal the inferred origin of nuclear alleles for each unisexual *Calligrapha* from phylogenetic trees and networks. (Right) Inferred parental species for unisexual *Calligrapha* taxa of putative hybrid origin. Thicker arrows indicate greater confidence in the inference of a specific parental species lineage. Thinner arrows indicate that additional sampled or unsampled species from a particular lineage offer comparably plausible parents. Numbers indicate levels of sequence divergence between unisexuals and putative parents with respect to mtDNA (maternal parents) and the two nuclear DNA genes (paternal parents). Parental inferences were based on phylogenetic relatedness and genetic and phenotypic similarity (inferences with an asterisk denote that other alternatives are possible; see text for details). Notably, inferred paternal species always share their host plant with their unisexual descendents, whereas inferred maternal species never do. These latter patterns also hold with respect to plausible alternative choices of parental species. (Plant drawings used with permission of the U.S. Dept. of Agriculture Forest Service Collection, Hunt Institute for Botanical Documentation, Carnegie Mellon University, Pittsburgh, PA.)

C. amelia is the most likely culprit. This is because *C. apicalis* is more similar in its markings to *C. amelia* than to *C. confluens*. *Calligrapha apicalis* and *C. amelia* also share the *Alnus* host association.

Ecological Aspects of Hybrid Speciation

One unforeseen finding of our study was that each unisexual *Calligrapha* species shared its host plant with its inferred paternal lineage. This is especially noteworthy because *Calligrapha* species tend to be quite host specific and different species tend to use different hosts (Brown 1945). We calculated the probability of this pattern occurring by chance based on the observed proportions of bisexual (and thus potentially parental) species associated with each *Calligrapha* host. To provide a conservative estimate, we restricted our analysis to the 10 bisexual species within the North American clade that includes all unisexual species. Since two of these 10 species are associated with *Alnus*, one with *Salix*, and three with *Cornus* (the hosts of *C. alnicola* and *C. apicalis*, *C. suturella*, and *C. vicina*, respectively), this probability is calculated as $0.2 \times 0.2 \times 0.1 \times 0.3 = P = 0.0012$. Moreover, in both of the cases where the paternal species lineage could only be narrowed to two possibilities, both potential paternals use the same host as the unisexual species. In stark contrast, none of inferred alternative species of the maternal line for a given unisexual shares its host with its putative descendant. Following the above reasoning, the chance probability of this pattern is $P = 0.056$ and the combined probability of both patterns is $P < 0.0001$.

By providing additional evidence for the hypothesis that unisexual phenotypes take after the paternal species, these patterns further corroborate our inferences on paternal species

and of the asymmetric hybridization scenario of unisexual origins. The underlying host-use biology of these beetles is quite relevant in this regard. Most fundamentally, because these host-specific beetles ordinarily mate on their host plant and because male and female parental species always use different hosts, a host choice ''mistake'' (or possibly a new host preference allele) is necessary to provide an opportunity for hybridization. These hybrid-mated females will then be expected to deposit eggs on the maternal host plant. Since *Calligrapha* larvae typically mature entirely on their natal host plant, these hybrids must be capable of developing on the maternal host if the hybrid lineage is to persist. Under the favored scenario that is consistent with all our results, only hybrids that subsequently backcross with the paternal species ultimately generate stabilized unisexual lineages. This requires either that hybrids backcross with both maternal and paternal species, but only the paternal backcrosses prove evolutionarily successful, or that hybrids backcross only with the paternal species. Since there is no a priori reason to believe that maternal backcrosses would be less successful in this regard, the latter alternative seems more likely. This backcrossing of hybrids with the paternal species in turn requires either that the paternal species mate with hybrids on the maternal species' host plant, or that hybrids visit the host of the paternal species. Given the host fidelity mentioned above, the latter alternative seems more likely, especially if hybrids inherited dominant alleles for host preference from the paternal parent. This would be even more likely if host preference were a male sex-linked trait. However, because male *Calligrapha* males have an XO sex determination (Robertson 1966), they do not pass on sex chromosomes (and potentially associated host preferences) to offspring.

The above scenario illustrates how the host-use ecology of *Calligrapha*, and its genetic architecture, must play critical roles in determining the likelihood that particular interspecific matings will occur and yield viable hybrids and stabilized unisexual lineages. Factors determining the likelihood of a particular hybrid mating include the degree to which maternal and paternal host plants tend to grow in the same microhabitats and the degree to which each species is willing to temporarily accept the host of the other. The likelihood of producing viable offspring will depend on the degree to which divergent host adaptation has resulted in intrinsic and extrinsic hybrid inviability, reflecting fundamental incompatibilities of the species' genomes and a reduced capacity to survive on parental hosts, respectively. The likelihood of producing stabilized lineages will depend, for example, on the capacity of hybrid populations to persist on their hosts in the face of potential competition from paternal species and other insects.

A further intriguing pattern is the fact that in no case do inferred parental lineages both use the same host plant. In a purely statistical context, this is not as unlikely as only five of 45 possible interspecific pairings involve species using the same host. However, in view of the ecological hurdles to unisexual formation and establishment described above, it would seem that the opportunities for and success of same-host parentage should be much greater. Specifically, two species using the same host will regularly encounter each other as potential mates and would be expected to produce offspring that do not suffer from host-associated extrinsic inviability. Perhaps this curious pattern indicates that divergent host-associated adaptation has pleiotropic effects on meiosis that make the production of unreduced gametes more likely. Or perhaps species sharing the same host are only able to coexist and avoid genetic homogenization if they are so strongly reproductively isolated that they are highly unlikely to mate and produce hybrid offspring. Clearly, the ecology of unisexual speciation is critical to understanding this process. As specialized insect herbivores, *Calligrapha* provide highly appropriate systems for evaluating these issues (Funk et al. 2002).

Additional Correlates and Causes of Unisexuality

In addition to host association, three other factors that may bear on the likelihood of unisexual origins in *Calligrapha* deserve mention: phylogenetic affiliation, genetic divergence, and space/time.

First, it may be pertinent that our phylogenetic analyses consistently placed all unisexuals within what we have called the North American clade (Fig. 2). To test whether this phylogenetic association was nonrandom, we estimated its chance probability using formula (1), where m is the number of species in the clade of interest (M), n is the total number of taxa in the ingroup, and j is the number of observed unisexual taxa within M:

$$P(j \text{ occurrences of unisexuality in clade } M) = \prod_{i=0}^{j-1} \frac{m - i}{n - i} \quad (1)$$

At $P = 0.17$, this pattern proves not to significantly deviate from the random expectation. However, this result likely reflects the paucity of Central and South American species in our study. The Latin American species we did include were all placed outside of the North American clade. These are phenotypically similar to excluded Latin American species that, for example, tend to have herbaceous hosts rather than using trees as do the species of the North American clade. No Latin American *Calligrapha* are suspected of parthenogenesis. Given these facts, it seems likely that unisexuality is indeed restricted to a particular modest-sized clade within *Calligrapha*, raising the question of whether certain clade-specific traits might promote the production or persistence of unisexual species.

Second, we considered the balance hypothesis of hybrid unisexual origins. This is the idea that unisexual daughter species are more likely to be produced when hybridization involves potential parental species that are sufficiently genetically diverged that the control of meiosis is disrupted, but not so divergent as to produce entirely inviable hybrids (Moritz et al. 1989, 1992). To test this idea, we evaluated mtDNA sequence divergences between the four pairs of inferred unisexual parent species with respect to the distribution of all possible pairwise divergences between ingroup bisexual species. For this analysis, we randomly chose one individual to represent each species (one exception was our inclusion of both of the mitochondrially divergent *C. alni* individuals). This analysis showed the sequence divergences of unisexual

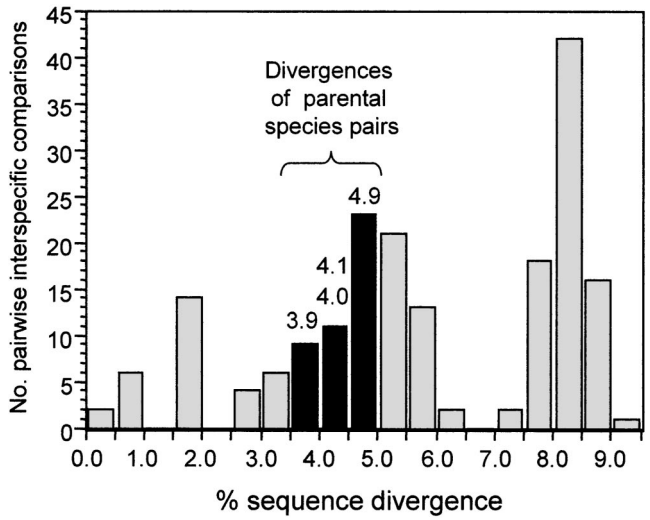

FIG. 7. Distribution of pairwise mtDNA sequence divergences among bisexual *Calligrapha* species. Black bars indicate those bins including divergences for the four pairs of inferred parental species of *Calligrapha* unisexuals, with absolute values of these sequence divergences indicated above. Note the narrow and intermediate distribution of parental divergences, as predicted by the balance hypothesis. See text for details.

parental species (3.9%, 4.0%, 4.1%, 4.9%) to be quite homogeneous and consistently intermediate with respect to the overall distribution (Fig. 7). None of these fell within the lowest 21% or the highest 62% of species divergences. These findings are thus highly consistent with both elements of the balance hypothesis.

Third, our molecular clock analyses date all unisexual origins to the Pleistocene, and the geographic distribution of these unisexuals and their parent species corresponds to previously glaciated areas (Dyke et al. 2002) in the northern United States and Canada. These young species clearly do not represent ancient asexuals (Judson and Normark 1996). However, the high latitudes and Pleistocene origins of these species are indeed very typical of hybrid unisexuals (e.g., White 1978; Dufresne and Hebert 1997; Johnson and Bragg 1999; Mantovani et al. 2001; Law and Crespi 2002; Kearney 2005). Both historical and adaptive processes have been invoked to explain these patterns (Kearney 2005). One hypothesis explains the prevalence of polyploid unisexuals of hybrid origin in formerly glaciated areas in terms of increased opportunities for hybridization via secondary contact between species (Stebbins 1984; Vepsäläinen and Järvinen 1979). Alternatively, greater dispersal and increased survivorship of unisexual organisms in ecologically marginal habitats—such as previously glaciated areas (Stearns 1987)—might explain their distribution. This same pattern, however, has recently been attributed to hybrid origins rather than reproductive mode per se (Kearney 2005). This latter view argues that hybridization increases phenotypic diversity, thus increasing opportunities for adaptation to suboptimal habitats. Because many asexuals are of hybrid origin, this might then lead to mistaken assumptions about which factor is actually responsible for the distributional patterns.

Conclusions and Future Directions

In this first study of unisexual evolution in *Calligrapha* leaf beetles, we have used sequences from mitochondrial and nuclear loci to evaluate questions about the origins of four unisexual species within an otherwise bisexual group of insects. Our findings indicate that each unisexual species represents an evolutionarily independent Pleistocene origin. They provide diverse evidence that these unisexuals originated through asymmetric interspecific hybridization and subsequent backcrossing with the paternal species. They allowed the inference of parental species lineages and associated insights on the genetic and ecological factors that may favor the production and persistence of unisexual hybrid species. These inferences call for further investigations into the mechanisms and evolution of *Calligrapha* unisexuality. Because *Calligrapha* includes seven unisexual species and has ecology and genetics that are amenable to study, this work further illustrates the more general value of these leaf beetles as a potential model system for comparative and empirical investigations of outstanding questions on the evolution of sex.

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