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Parthenogenetic and sexual species within the *Haploembia solieri* species complex (Embioptera: Oligotomidae) found in California

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Abstract

Webspinners in the genus *Haploembia* Verhoeff (Embioptera: Oligotomidae) were introduced into California from their native range in the Mediterranean region. In that region the genus has long been thought to comprise a single widespread species *Haploembia solieri* (Rambur) with both sexual and asexual populations and two additional sexual species with restricted ranges in the Mediterranean. For most of its known history in California, only asexual populations of *Haploembia solieri* were collected. However, within the last couple of decades, sexual populations have been discovered. The purpose of this project is to determine whether a single species of *Haploembia solieri* exists in California, which may imply reacquisition of sex, or whether these populations represent two or more species. To resolve this question DNA sequence data were acquired from multiple populations (sexual and asexual) of Californian, New Mexican, and Mediterranean *Haploembia solieri*, including both nuclear (histone 3) and mitochondrial (cytochrome oxidase I) genes. These data were included in a phylogenetic analysis to resolve relationships between these individuals. Our analysis shows asexual California specimens grouping with the asexual specimens from Sardinia; whereas the sexual California specimens group separately from both asexual specimens, and sexual Mediterranean specimens. Therefore, we conclude that there are two species of *Haploembia* introduced into California and at least three species involved in what was historically considered *H. solieri*.

Keywords

parthenogenesis; sexual populations; cytochrome oxidase subunit I; histone 3

Introduction

Embioptera or 'webspinners' are a group of polyneopteran insects found worldwide in tropical and subtropical habitats, but they are among one of the least known insect

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orders. Embioptera are distinguished from other insect groups by the expansion of the basitarsomere on the prothoracic leg which contains numerous silk glands. The silk produced with these glands is used to create galleries in which individuals live, often subsocially. These galleries (Fig. 1) can be found in many microhabitats including rock and tree surfaces, in leaf litter, and under rocks in many areas ranging from tropical forest to dry deserts depending on the species. Many Embioptera species have been artificially distributed throughout the world because of their propensity to place their silk galleries on wood, plants and cargo (Ross 2000).

The classification of this group is primarily based on male characteristics, such as wing venation and terminalia (10th abdominal segment and cerci). Females are nymph-like, without wings, and have very few descriptive characters at all taxonomic levels. This is unfortunate, since it is nymphs and adult females that are most commonly found in nature while adult males are much rarer because they die soon after mating. Fortunately, Embioptera can usually be relatively easily reared, which does allow the possibility of acquiring needed males. Klass & Ulbricht (2009) suggested that using postabdominal characters (which includes internal and external genitalia) can be applied to determining species of Embioptera and aid in the construction of phylogenies for the group.

The majority of Embioptera reproduce sexually, however there are rare cases of parthenogenesis including several species from Africa (Ross 1960). Another case that includes parthenogenetic representatives is the genus *Haploembia* Verhoeff in the family Oligotomidae. Within the family, the genus is distinguished by having two medial-ventral papillae on the hind basitarsi and lacking wings in males. Significant male characteristics were investigated by Ross (1966).

Historically, the genus has comprised ten species including the type species, *Haploembia solieri* (Rambur). Ross (1999) listed each of these taxa and indicated that most of them should be transferred to other genera. To date, *H. algerica* Navas has been transferred into *Embia* (Davis), *H. bourgi* Navas has been transferred to *Chirembia* (Ross), *H. capensis* Esben-Petersen has been transferred into *Apterembia* (Ross), *H. collaris* Navas was moved to *Oligotoma* (Davis), and *H. neosolieri* Marino & Marquez was moved to *Neorhagadochir* and synonymized with *N.* (*Drepanembia*) *salvini* (McLachian) (Ross 2001). Although *H. clypeata* Navas, and *H. verhoeffi* Friederichs from Zaire and British East Africa, and *H. megacephala* Krauss from Syria are still placed in *Haploembia*, Ross (1999) indicated that these species should not be classified in that genus.

Therefore, with the exception of the improperly-placed *H. clypeata*, *H. verhoeffi* and *H. megacephala*, this genus includes only two species, *H. palaui* Stefani and *H. solieri* (Rambur), each of which is historically native to the Mediterranean and Black Sea regions (Stefani 1954; Ross 1966). One of these species, *Haploembia solieri* (Rambur) (Fig. 2) has been consistently regarded as a single widespread species in southern Europe, the Near East and northern Africa (Ross 2000, Fig. 3A).

Stefani (1953, 1956) recognized two forms of *H. solieri*, one sexual and the other parthenogenetic, which he called "biotypes". Historically, the sexual biotype has been recorded from mainland Europe, the Near East and Morocco, and the parthenogenetic biotype has been recorded on isolated islands, including Corsica, Sardinia, Capri, Elba,

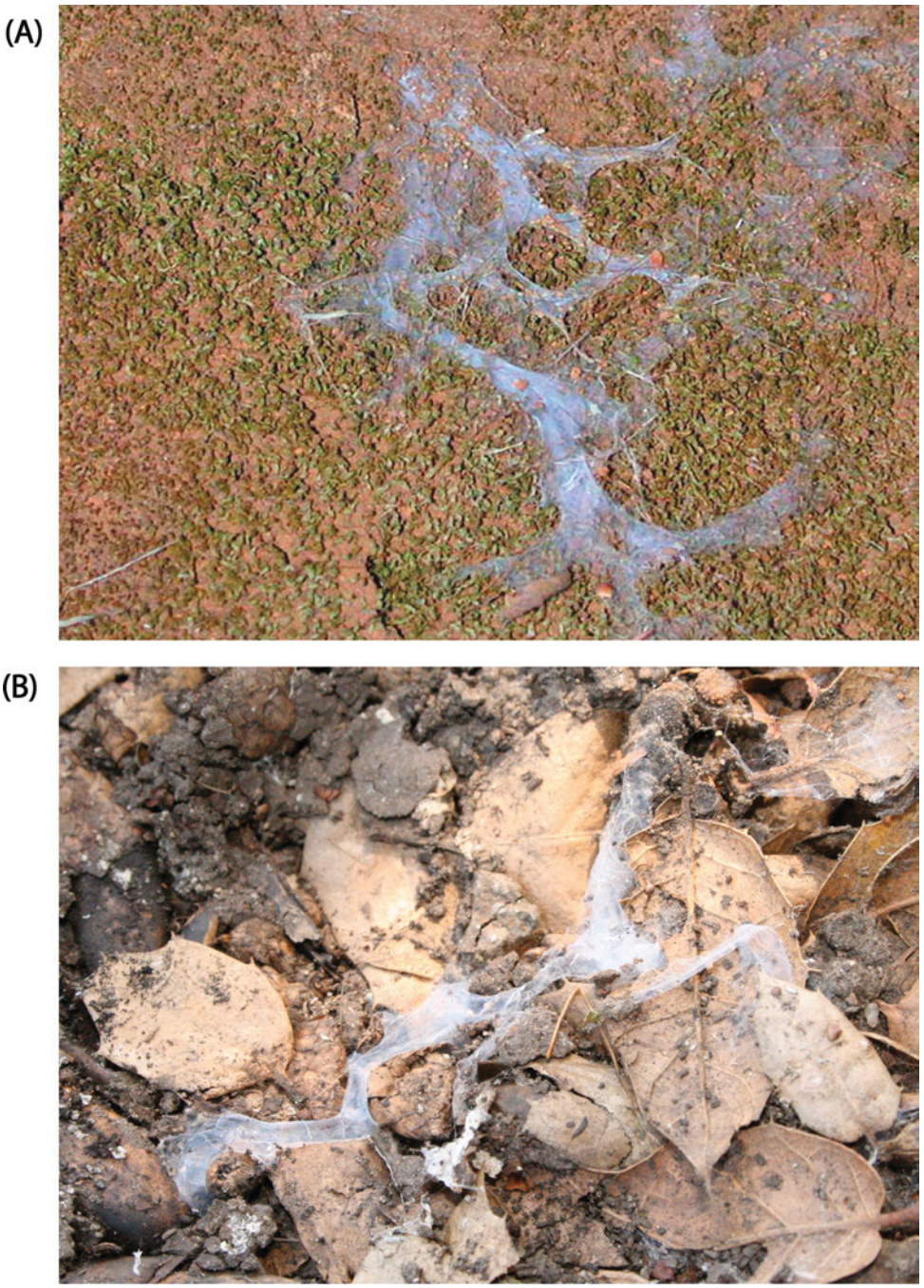

Fig. 1. *Haploembia* silk domiciles. (A) Asexual population from San Jose, CA, USA. (B) Sexual population from Redwood City, CA, USA. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/1876312x.

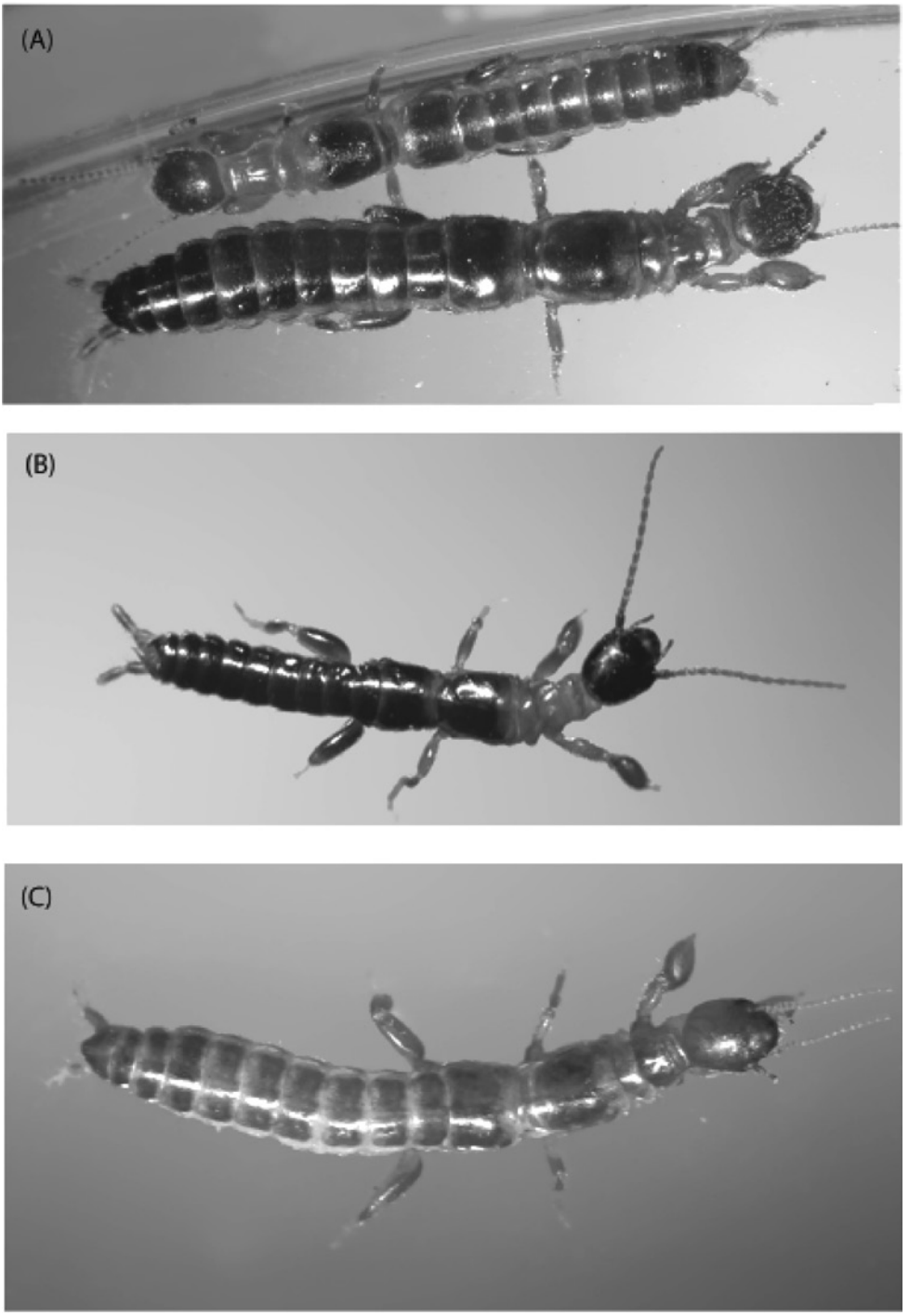

Fig. 2. *Haploembia solieri*. (A) Sexual immature and adult females from Mountain View, CA, USA. (B) Male, same locality. (C) Parthenogenetic female from San Jose, CA, USA.

Giglio, Argentario, the Canary Islands, and in mixed populations (with the sexual biotype) on the mainland of Italy (Nizza, Cervo, Varigotti) (Stefani 1959, Fig. 3A). Neither biotype is known to switch between reproductive syndromes, but Stefani (1956) believed these populations to represent a single species with sexual and asexual populations.

Major differences between the two biotypes include size, body sclerotization, microsculpturing of the hypopharynx and lacinia, shape of the eggs, and their karyotypes (Stefani 1953, 1956). In general, sexual females are shorter (10.0–12.0 mm) and have darker pigmentation (appearing reddish-brown to ferruginous, especially on the head) than the parthenogenetic female. Sexual females also have the hypopharynx with a long groove with short spicules. Near the end the spicules become more complex in form and different from those in parthenogenetic females by being minute and irregular. Sexual females have the microsculpturing of the lacinia consisting of small transverse tubercles in dorsal-lateral aspect. The egg in sexual females is circular in shape, with the operculum raised and thick, and this form has a karyotype of 22 chromosomes (Stefani 1955, 1956; Ross 1966, 2000, Fig. 2A). Females of the parthenogenetic form are larger (12.2–14.0 mm) and paler in pigmentation than the sexual females (especially on the head, pronotum and abdomen), the hypopharynx has a lower region with a long groove along its entire length and microsculpturing of tiny spicules that are irregularly arranged in three rows, and the microsculpturing of the lacinia consists of transverse tubercules that are more pronounced and abundant than in the sexual biotype. The egg of the parthenogenetic form is semicircular in shape with the operculum not raised and thinner than the sexual biotype, and this form has a karyotypes of 22 or 33 chromosomes suggesting possible polyploidy (Stefani & Contini 1961; Fig. 2C). More detailed species descriptions of both biotypes are available in Appendix A, including a description of males.

In addition to these morphological features, the asexual and sexual females exhibit behavioral differences. They handle their eggs differently during oviposition and vary in their aggressive tendencies (Stefani 1956). Males will not mate with parthenogenetic females, who in turn are not sexually attracted to males according to Stefani (1956) who conducted 122 attempted pairings of sexual males with parthenogenetic females from many locations. He concluded that complete reproductive isolation exists between the two forms, even in locations where they coexist. In addition, Stefani tested the idea that males might be produced sporadically by parthenogens but rejected that proposal when he did not find males after examining more than 10 000 individuals from isolated populations of parthenogens. He also noted that the two biotypes behave like two different species. Despite considerable evidence, however, he was not willing to designate them as such, possibly because of the lack of important character-bearing males in the parthenogenetic biotype.

Although native to the Mediterranean region, populations of *Haploembia solieri* are also found in the southwestern United States, particularly central and southern California (Fig. 3B) presumably introduced through the extensive historical commerce between these two regions. An asexual form of *H. solieri* was first recorded in California by Ross (1940) from 22 localities throughout the Sierra Nevada and Central Valley

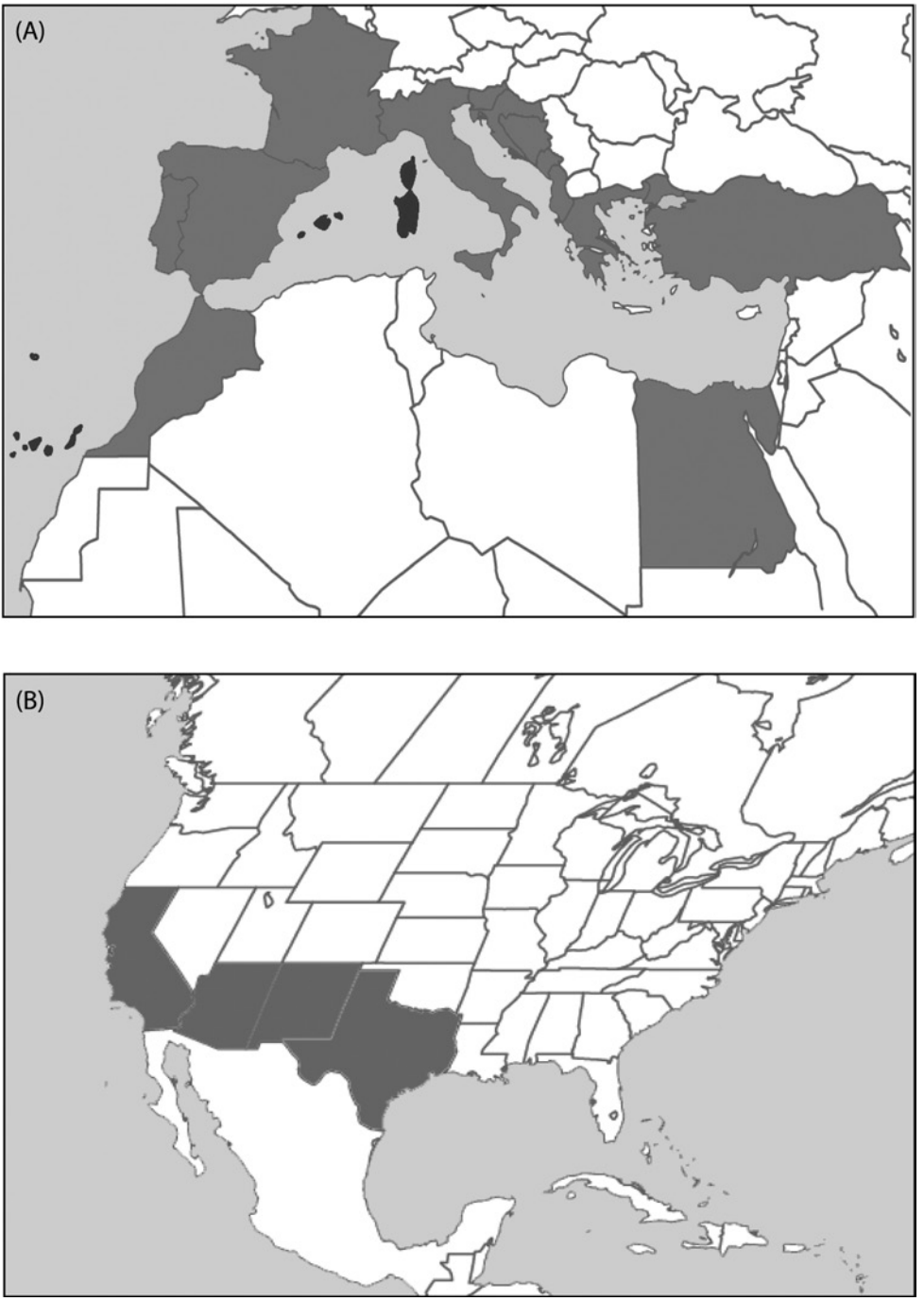

Fig. 3. Geographical distribution of *H. solieri*. (A) Mediterranean, Middle Eastern and northern African distribution. (B) North American distribution.

California and one in Arizona. He did not recognize it as *Haploembia*, however, and described these populations as a new genus and species, *Gynembiatarsalis* Ross. *Gynembiatarsalis* was soon synonymized with *H. solieri* by Stefani (1955). Parthenogenetic *Haploembia solieri* were also recorded from Arizona (Tempe), southwestern Texas (Alpine County), Oregon (Grants Pass), Utah (St. George), and Northern Baja California (Ross 1940, 1957, 1966, 1984). Initially, only a parthenogenetic form was known from North America (Ross 1940, 1957,1966, 1984), however, Ross (1984) later first reported a sexual form in Redwood City, CA, USA.

Discovery of both sexual and asexual populations in California when previously only asexual reproduction appeared to be present (Ross 1940) suggests two possible situations regarding the California populations. The first is that there may be a single species with populations that are either sexual or asexual, which may alternate reproductive modes, or which may have reacquired sexual reproduction in California which would bolster Stefani's assertion that there is only a single species with different reproductive modes. The second possibility is that there are at least two species currently regarded as *H. solieri* which were likely introduced into North America during historical times.

The purpose of this project is to determine if there are one or more than one species currently regarded as *H. solieri* in California corresponding to sexual and asexual reproductive modes. As described above, the classification of embiopterans is based on male characters making determinations about species limits in parthenogenetic species challenging. Although subtle differences in coloration and other features exist between sexual and asexual female biotypes of *H. solieri*, it is not clear whether these represent intra- or intersexual variation. The differences between eggs of the two biotypes could be useful in interpreting phylogeny but not enough is known about how this character system functions in separating closely related species (Edgerly et al. 2007). To better address the problem of species limits in these populations, we used molecular techniques. DNA sequence data has become popular in phylogenetic analyses, and, more controversially, for species-level decisions (e.g., Blaxter 2004). We agree with modern critics of DNA taxonomy (e.g., Will & Rubinoff 2004; Wheeler 2005; Will et al. 2005) that DNA sequence data cannot by itself be the source of information for all species decisions and that a comprehensive approach to species delimitation using various data sources is the best approach (e.g., Will et al. 2005). In this particular case, however, with species depauperate in characters, such as parthenogenetic Embioptera, sequence data may be among the few characters available.

Materials and Methods

Collecting and rearing Haploembia *specimens*

Embioptera specimens were collected in the field in leaf litter, under rocks, in soil crevices, and in detritus where they can often be found in large numbers and collected into containers using soft forceps. Embioptera, including *Haploembia*, are usually easily reared in captivity in containers with dried leaves and fed lichen and lettuce

(Ross 2000). Whenever possible, populations were observed through more than one generation to determine whether males were produced indicating sexual reproduction.

Taxon sampling

Ingroup. Specimens of *Haploembia solieri* were collected from several general areas within its known distribution: (1) California (Santa Clara, Riverside and Mountain View), (2) near Pisa, Italy, (3) Sardinia, Italy and (4) Albuquerque, NM, USA (a new state record for the taxon). These included specimens from both sexually reproducing (California and Pisa, Italy) and asexually reproducing (California, Sardinia and New Mexico) populations. A total of 33 specimens were used in the analysis (Table 1).

Outgroup. Thirteen outgroup species were included from two other genera within Oligotomidae (*Aposthonia* Krauss and *Oligotoma* Westwood) (Table 1). Multiple individuals were included from the same species in several cases to examine intraspecific variation among taxa closely related to *Haploembia*. In some cases these included other species artificially dispersed by humans such as *Oligotoma saundersii* (Westwood) and *O. nigra* Hagen.

DNA

Whole genomic DNA was extracted from whole specimens using the Qiagen DNeasy Blood & Tissue Kit (Valencia, CA, USA). An incision was made along the lateral margin of the thorax of the specimen allowing buffer to enter the cavity. After incubation the remaining portions of the specimen were removed and retained as vouchers. These are deposited in The Museum of Southwestern Biology Division of Arthropods, University of New Mexico.

Two genes were sequenced: *Cytochrome c oxidase I* (COI, approx. 1294 bp, except for one specimen from Pisa) and *Histone 3* (H3, 328 bp, all specimens) (Table 2). DNAs were amplified using polymerase chain reaction (PCR) using Nova Taq (VWR, West Chester, PA, USA), Takara Taq (Takara Bio, Otsu, Japan), or KOD Hot Start Master Mix (EMD Chemicals, Darmstadt, Germany) on an Eppendorf Mastercyclerep thermal cycler (Eppendorf, Hamburg, Germany). Conditions for DNA amplification are presented in Table 3. To ensure amplification efficiency and reduce contamination both positive and negative controls were used and examined along with PCR fragments using an electrophoresis gel. PCR product was purified by using ExoSap-IT PCR clean up kit (Affymetrix, Santa Clara, CA, USA) or filtered through G-50 Sephadex medium (GE Healthcare, Amersham, UK) and Montage multiscreen filter plates (Millipore, Billerica, MA, USA). Sequencing was then conducted using Applied Biosystems Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) kit using the same primers as in amplification. Sequencing reaction products were then purified using G-50 Sephadex medium then sequenced by an ABI 3130 xl Genetic analyzer (Molecular Biology Facility, Department of Biology, University of New Mexico, Albuquerque, NM, USA). Gene regions were sequenced in both forward and reverse directions and then resulting data were edited in Sequencher 4.2.2 (GeneCodes,

Table 1. Taxa and specimens used in analysis including locality data, specimen voucher numbers and GenBank accession numbers for DNA sequences.

Species	Locality	Voucher number	GenBank accession number	
			COI	H3
Aposthonia ceylonica[1]	India, Karnataka, Siddapur, 14°20.307'N 74°52.881'E 07 Oct 2004, K.B. Miller, colr.	UNM KBMEB58	JQ907051	JQ907105
A. ceylonica	Same	KBMCEB98	KC014542	–
A. glauerti[1]	Australia, New South Wales, Tantawango Mountain Road, 36°47.268'S 149°36. 206'E, 23 Nov 2005, K.B. Miller, colr.	UNM KBMEB71	JQ907056	JQ907109
A. gurneyi[1]	Australia, Northern Territory, 6km W Alice Springs, 23°44.3'S 133°44.484'E, 08 Oct 2002, K.B. Miller, colr.	UNM KBMEB60	JQ907053	JQ907107
Oligotoma humbertiana	Mexico, El Ocotillo, 10km SE Tepic, 17 Dec 2005, J. Edgerly, colr.	KBMCEB97	KC014535	KC014571
O. nigra	United States, Nevada: Clark Co., Muddy River at Palm Valley RV Park, 6-7 Aug 2000, Waite & Winkler, colrs.	KBMCEB91	KC014537	KC014574
O. nigra	United States, Arizona: Maricopa Co., 1 mi W Canyon Lk, Tonto NF, 10 Aug 2000, Waite & Winkler, colrs.	KBMCEB100	–	KC014572
O. nigra	United States, Arizona, Maricopa Co., Pioneer Park, Mesa, 15 Jun 2000.	KBMCEB41	KC014536	KC014573
O. saundersii[1]	Trinidad and Tobago, Trinidad, J. Edgerly, colr.	KBMCEB50	EU157062	EU157031
O. saundersii	India, Karnataka, Siddapur, 14°20.307'N 74°52.881'E, 07 Oct 2004, K.B. Miller, colr.	KBMCEB82	KC014539	KC014576
O. saundersii	Ghana, Volta Region, Nkwanta, nr Wildlife Division office; 8°15' 32.5"N 000°31'08.2"E, 13-17 Jun 2005, K.B. Miller, colr.	KBMCEB92	KC014538	KC014575

(*Continued*)

Table 1. (*Cont.*)

Species	Locality	Voucher number	GenBank accession number	
			COI	H3
O. saundersii	India, Karnataka, 13°40.807'N 74°41.172'E, 06 Oct 2004, K.B. Miller, colr.	KBMCEB95	KC014540	KC014577
O. saundersii	Madagascar: Toliara, 08 Jul 2003, A.Y. Kawahara, colr.	KBMCEB99	KC014541	KC014578
Haploembia solieri	United States, California, Riverside.	KBMCEB29	KC014566	KC014604
H. solieri	Same	KBMCEB131	–	KC014603
H. solieri	Same	KBMCEB132	KC014544	KC014580
Haploembia sp.	United States, California, Mt. View, 14 Jul 2006, N. Calvert, colr.	KBMCEB52	KC014562	KC014599
Haploembia sp.[1]	Same	UNM KBMEB93	JQ907068	JQ907117
Haploembia sp.	Same	KBMCEB94	KC014563	KC014600
Haploembia sp.	Same	KBMCEB96	KC014564	KC014601
Haploembia sp.[1]	Italy, Pisa Region, Santa Luce, 43°28'N 10°34'E, 30 Apr 2006, K.B. Miller, colr.	UNM KBMEB86	JQ907065	JQ907115
Haploembia sp.	Same	KBMCEB88	KC014565	KC014602
H. solieri	Italy, Sardinia, Sassari Prov., 5km W Valledoria, 40°54.157' N 8°46.358'E, 15 Apr 2006, K.B. Miller, colr.	KBMCEB87	KC014568	–
H. solieri	Same	KBMCEB83	KC014567	KC014605
H. solieri	United States, California: St Clara Co., 19 Mar 2002, J. Edgerly, colr.	KBMCEB104	–	KC014606
H. solieri	Same	KBMCEB11	KC014569	KC014607
H. solieri	Same	KBMCEB48	KC014570	–
H. solieri	United States, California, Sutter Butte Co. Sutter Buttes, Entrance Slope, 39°15'11.97"N 121°48'05.40"W, 06 Apr 2008, Coll: A. Sutherland & I. Pearse, colrs.	KBMCEB193	KC014561	KC014598
H. solieri	United States, New Mexico, Bernalillo Co. University of New Mexico, Albuquerque, 35.082844°N 106.622360°W, 26 May-20 Jun 2008, A.M. Hodson, colr.	KBMCEB189	KC014557	KC014594

Table 1. (*Cont.*)

Species	Locality	Voucher number	GenBank accession number	
			COI	H3
H. solieri	Same	KBMCEB190	KC014558	KC014595
H. solieri	Same	KBMCEB191	KC014559	KC014596
H. solieri	Same	KBMCEB192	KC014560	KC014597
Haploembia sp.	United States, California, Santa Clara Co. Winstar Open Space, Jan 2008, J. Edgerly, colr.	KBMCEB175	KC014543	KC014579
Haploembia sp.	Same	KBMCEB176	KC014545	KC014581
Haploembia sp.	Same	KBMCEB177	KC014546	KC014582
Haploembia sp.	Same	KBMCEB178	KC014547	KC014583
Haploembia sp.	Same	KBMCEB179	KC014548	KC014584
Haploembia sp.	Same	KBMCEB180	KC014549	KC014585
Haploembia sp.	Same	KBMCEB181	KC014550	KC014586
Haploembia sp.	Same	KBMCEB182	KC014551	KC014587
Haploembia sp.	Same	KBMCEB183	KC014552	KC014588
H. solieri	Same	KBMCEB184	KC014553	KC014589
Haploembia sp.	Same	KBMCEB185	–	KC014590
Haploembia sp.	Same	KBMCEB186	KC014554	KC014591
H. solieri	Same	KBMCEB187	KC014555	KC014592
Haploembia sp.	Same	KBMCEB188	KC014556	KC014593

[1]Sequences from Miller et al. (2012).

Table 2. Primers sequenced for COI and H3.

Gene	Primer	Direction	Sequence (5′→3′)
COI + COII	C1-J-1718 (Mtd6)[1]	Forward	GGAGGATTTGGAAATTGATTAGTT CC
COI + COII	C1-J-2183 (Jerry)[1]	Reverse	CAACATTTATTTTGATTTTTTGG
COI + COII	TL2-N-3014 (Pat)[1]	Forward	TCCAATGCACTAATCTGCCATATTA
COI + COII	C1-J-1751 (Ron)[1]	Forward	GGATCACCTGATATAGCATTCCC
COI + COII	C1-N-2191 (Nancy)[1]	Reverse	CCCGGTAAAATTAAAATATAAACTTC
COI + COII	EBCO1F1[2]	Forward	GTWATACCMATYATAATTGGWGG
COI + COII	EBCO1F2[2]	Forward	CCMATYATAATTGGWGGWTTYGG
COI + COII	EBCO1F3[2]	Forward	GAAGTYTAYATTCTWATYYTACCKGG
COI + COII	EBCO1R1[2]	Reverse	CCMGGTARRATWAGAATRTARACTTC
COI + COII	EBCO1R2[2]	Reverse	GRGTWATRATRTGRGARATTATWCC
COI + COII	EBCO1R3[2]	Reverse	RGTWGCTGAWGTRAARTARGCTC
COI + COII	EBCO1R4[2]	Reverse	RTGRCGTACTACATARTAWGTRTC
H3	Haf[3]	Forward	ATGGCTCGTACCAAGCAGACGGC
H3	Har[3]	Reverse	ATATCCTTGGGCATGATGGTGAC

[1]From Simon et al. (1994).
[2]From Miller &Edgerly (2008).
[3]From Colgan et al. (1998).

Table 3. Amplification conditions used in step-down PCR reactions for H3 and COI.

	Taq (Hot start temp/ time)	Number of cycles	Denaturation temperature (time)	Annealing temperature (time)	Extension temperature (time)	Final extension temp (time)
H3	Nova Taq (95°C/ 8–10 min)	5	94°C (30 s)	52–56°C (30 s)	58–72°C (1–1.5 min)	60–72°C (7–10 min)
		5	94°C (30 s)	49–53°C (30 s)		
		25–30		45–50°C (30 s)		
	Takara (94°C/1 min)	5	94°C (30 s)	56°C (30 s)	68°C (1–1.5 min)	68°C (7 min)
		5		53°C (30 s)		
		30		50°C (30 s)		
	KOD (95°C/2 min)	5	95°C (20 s)	53°C (30 s)	70°C (1–1.5 min)	60°C (7 min)
		5		50°C (30 s)		
		30		50°C (30 s)		
COI	Nova Taq (95°C/ 8–10 min)	5	94° C (30 s)	54–56°C (30 s)	72°C (1–1.5 min)	60–72°C (7–10 min)
		5		50–53°C (30 s)		
		25–30		45–50°C (30 s)		
	Takara (94°C/1 min)	5	94°C (30 s)	56°C (30 s)	68°C (1–1.5min)	68°C (7 min)
		5		53°C (30 s)		
		30		50°C (30 s)		
		5	94°C (20 s)	56°C (30 s)	68°C (1–1.5min)	68°C (7 min)
		5		53°C (30 s)		
	KOD (95°C/2 min)	25		52°C (30 s)		

Ann Arbor, MI, USA). All sequences were BLASTed against GenBank to mediate for possible contamination.

Embioptera DNA is difficult to amplify compared with many other insect taxa for reasons that are not clear but probably having to do with primer mismatch in these unique taxa. Best results were obtained in PCR with step-down amplifications (Table 3), though no technique found gave universally high PCR yield. For this reason, some of the taxa were not sequenced for one of the two genes. This is indicated in Table 1.

Analysis

Alignment was unambiguous for these sequences. The regions amplified are not length variable in these specimens and alignment was done in Sequencher 4.2.2. (GeneCodes).

A combined, equal-weights parsimony analysis was conducted using NONA (Goloboff 1995) and the commands "hold 10000," "h/50," "mu*50" and "max*". Trees were examined and the consensus was calculated in WinClada (Nixon 2002). Bootstrap values were also calculated in NONA as implemented by WinClada using 1000 replicates and saving the consensus of each replicate.

Genetic distances

In addition to phylogenetic analyses, genetic distances were measured to compare species divergences within the *H.solieri* species complex with published measurements of distances between other species of insects and between other conspecifics included in our analysis. Uncorrected *p*-distances between COI and H3 sequences were calculated using MEGA 2.1 (Kumar et al. 2005). The *p*-distance is the proportion of nucleotide differences between sequences. One of two specimens of *Haploembia* from Pisa was not sequenced for COI because of difficulties in amplifying a clean sequence from this specimen. Therefore, only a single specimen from Pisa was included in calculations of *p*-distances.

Results

Parsimony

The analysis resulted in 854 equally parsimonious trees (CI = 66, RI = 88 and length = 1168; Figs 4, 5). The strict consensus of these is shown in Fig. 5 and one of the most parsimonious trees (showing branch lengths) is presented in Fig. 4. *Haploembia* was recovered as monophyletic with high support (bootstrap = 100; Fig. 5). Each species of outgroups, for which multiple specimens were included, were also recovered as monophyletic with high support (bootstraps = 92–100; Fig. 5). Within *Haploembia*, three separate clades were recovered subtended by relatively long branches, each with high bootstrap support (59–99). Within each of these groups, the branches are considerably shorter (Fig. 4) and each group is unresolved in the consensus (Fig. 5).

Genetic distances

Select genetic distances are shown in Table 4 for COI and Table 5 for H3. For COI, genetic distance across Oligotomidae ranged from 0–21.40% (average 14.30%). Between non-*Haploembia* species the distance ranged from 9.10–21.40% (average 15.34%), and within non-*Haploembia* species the distance ranged from 0–0.20% (average 0.06%). Within *Haploembia*, the distance was 0–14.50% (average 7.52%). However, three groups are resolved that exhibit genetic distance less than 0.20% within the groups and more than 14.30% between the groups (Table 4). For H3, genetic distances across *Oligotoma* ranged from 0–20.40% (average 9.50%). Between non-*Haploembia* species the distance ranged from 1.20–16.70% (average 9.40%), and within non-*Haploembia* species ranged from 0–0.07% (average 0.02%). Within *Haploembia*, the genetic distance was 0–6.10% (average 3.00%). Similar to COI three

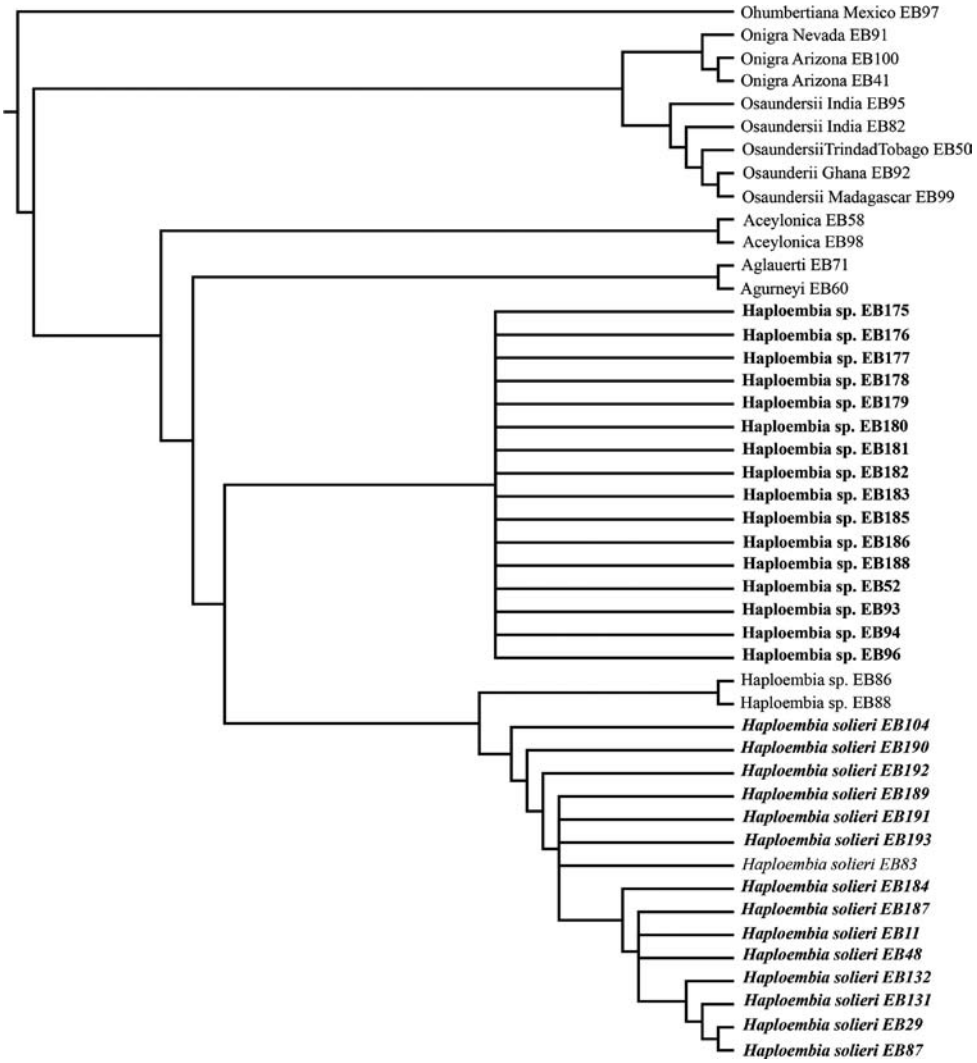

Fig. 4. One of 854 most parsimonious trees chosen at random. Branch lengths shown are proportional to the number of changes mapped using "fast" optimization in WinClada. Asexual species are in italics. Sexual species are not in italics. Specimens collected in the United States are in boldface.

groups are resolved that exhibit genetic distance less than 6.10% within groups and more than 5.50% between groups (Table 5).

Discussion

These results suggest that there are at least three well defined groups within what has historically been recognized as *Haploembia solieri* even with our very limited sampling of *H. solieri* from the Mediterranean; (1) asexual individuals from California,

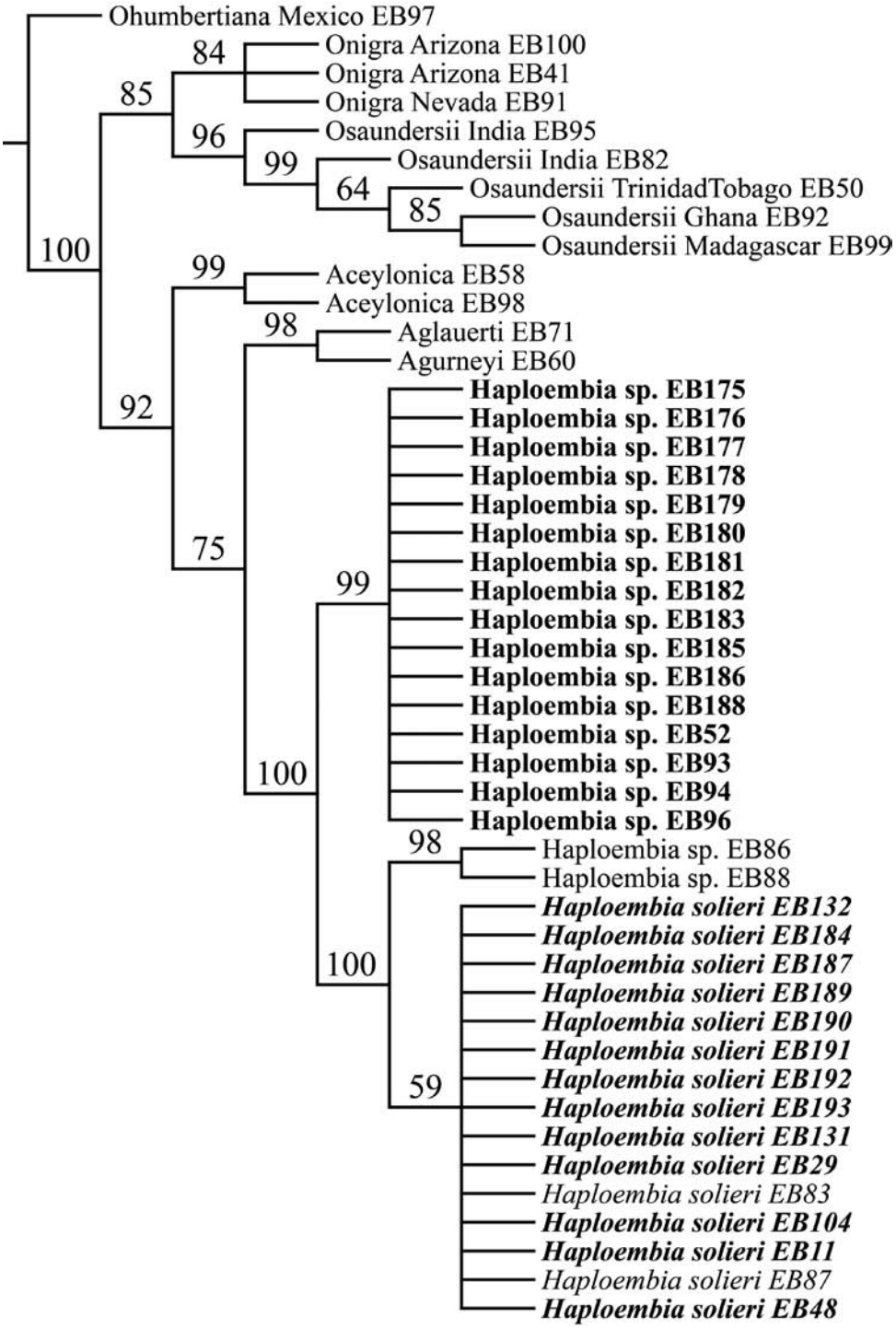

Fig. 5. Strict consensus of 854 most parsimonious trees. Bootstrap support values are below branches. Asexual species are in italics. Sexual species are not in italics. Specimens collected in the United States are in boldface.

Table 4. Uncorrected *p*-distances (percentages) between COI sequences.

	All	*Haploembia*	*Haploembia*					Non-*Haploembia*		*Oligotoma*	*Aposthonia*
			CA Sex	Asex	CA Sex/ Pisa	CA Sex/ Asex	Pisa/ Asex	Between species	Within species		
Average	14.30	7.52	0.00	0.10	14.30	14.44	9.34	15.34	0.06	7.41	17.30
Max	21.40	14.50	0.00	0.20	14.30	14.50	9.40	21.40	0.20	14.60	21.40
Min	0.00	0.00	0.00	0.00	14.30	14.30	9.20	9.10	0.00	0.00	0.00

CA Sex, Californian sexual populations; Asex, Asexual populations; Pisa, Italian sexual populations. Only one of the two specimens from Pisa was sequenced for COI because of difficulties in amplification so it is not included in these calculations.

Table 5. Uncorrected *p*-distances (percentages) between H3 sequences.

	All	*Haploembia*	*Haploembia*					Non-*Haploembia*		*Oligotoma*	*Aposthonia*
			CA Sex	Asex	CA Sex/ Pisa	CA Sex/ Asex	Pisa/ Asex	Between species	Within species		
Average	9.50	3.00	0.00	0.10	5.90	5.40	1.40	9.40	0.02	1.10	17.30
Max	20.40	6.10	0.00	0.30	6.10	5.50	6.10	16.7	0.07	2.80	20.60
Min	0.00	0.00	0.00	0.00	5.80	5.20	1.20	1.20	0.00	0.00	12.00

CA Sex, Californian sexual populations; Asex, Asexual populations; Pisa, Italian sexual populations.

New Mexico, and Sardinia, Italy, (2) sexual individuals from California and (3) sexual individuals from Pisa, Italy. The parsimony analysis supports this with long branches subtending each of these groups with high support and short branches (and poor resolution) within each of these groups (Figs 4, 5). Genetic distances within each of these groups ranges from 0–0.20% (COI) and 0–0.30% (H3), which is comparable to distances within other conspecifics of *Oligotoma* and *Aposthonia* (0–0.20% COI; 0-0.07% H3).Distances between these groups of *Haploembia* (9.20–14.50% COI; 1.20-6.10% H3) are comparable to distances between other species in the analysis (9.10–21.40% COI; 1.20-16.70% H3) and comparable to other species of living things in general (Avise 2000) and other Embioptera in particular (Miller & Edgerly 2008). Based on these results, it seems clear that these three groups represent three separate species of *Haploembia* rather than a single species with two different reproductive modes.

The discovery of three separate species contradicts Stefani's (1956) assumption that both sexual and asexual forms are the same species. Recognition of the parthenogenetic populations as a distinct species is not unreasonable or entirely unexpected and is consistent with the observation that these individuals do not appear to interbreed with sexual individuals (Stefani 1956) and the relatively subtle coloration, body form character states, karyotype information and behaviors (Stefani 1955, 1956; Ross 1957; Stefani & Contini 1961). An example of this is in the walking stick genus *Timema* in California, where some sexual species form pairs with very similar appearing asexual

species (Vickery & Sandoval 1999; Law & Crespi 2002).

Evidence of two sexual species, however, is less expected. What seems clear is that the sexually-reproducing species present in California is not the same sexually-reproducing species found in the area around Pisa, Italy (Figs 4, 5; Tables 4, 5). This suggests that the species-level situation in *Haploembia* may be considerably more complex than is currently recognized. *Haploembia* is widespread throughout southern Europe and the Black Sea Region. Although at least one (or perhaps two) other *Haploembia* species are recognized, with relatively limited distributions (see Stefani 1955; Ross 1966), many additional cryptic species may exist as well. Given the propensity for specimens to be introduced artificially, the distributions of these species may be extremely complex, particularly given the extensive history of human commerce and travel throughout the Mediterranean.

Given our results regarding the number of species in *Haploembia*, it is clear that the species-level nomenclature needs revision. The type specimen of *H. solieri* (Rambur) is problematic since it is a nymph (from near Marseille, France), which is missing its head, prothorax and appendages (Ross 1966). There are several additional synonyms, as well. The classification of available names is as follows:

Embia solieri Rambur, 1842. Type locality: near Marsaille, France.
= *Embiataurica* Kusnezov, 1903. Type locality: Southern coast of the Crimea peninsula (type specimen and locality not fixed)
= *Embia* (*Haploembia*) *grassii*Friederichs, 1906. Type locality: Sicily.
= *Embiacephalotes*Navás, 1908. Type locality: Spain, Orihuela (Alicante).
= *Haploembia codinae*Navás, 1922. Type locality: Morocco, Ceuta.
= *Gynembiatarsalis* Ross, 1940. Type locality: USA, California, Clayton.

Of these, the only name that definitely applies to the parthenogenetic species (whether from Sardinia, other Mediterranean Islands or California) is *Haploembia tarsalis* (Ross 1940). It is possible that *H. grassii* (Friederichs) from Sicily applies to this species, but the type of this species also appears to be a nymph (Friederichs 1906; Enderlein 1912) which may make it impossible to identify, and the parthenogenetic form of *Haploembia solieri* has not been recorded from Sicily. Therefore, in the interest of improving the taxonomy in this group we hereby resurrect the name *Haploembia tarsalis* (Ross 1940) for those *Haploembia* in (at least) Southwestern United States and Sardinia, Italy that reproduce using parthenogenesis (*new status*). Other than this, the assignment of other available names to sexually-reproducing species in *Haploembia* (including the two in this study from California, USA and Pisa, Italy, Table 1) cannot be adequately assessed. Nothing less than a full revision of the genus along with collection of fresh material from throughout the range of the species and examination of type specimens will be required, however this is well beyond the scope of this study.

The presence of two species of *Haploembia* in California can be explained by introduction of two species, *H. tarsalis* derived from one or more asexual Mediterranean populations and a sexual species derived from an unknown source.

Other unusual reproductive syndromes have evolved on Mediterranean islands (Normark 2003), and it is well known that these alternatives to typical sexual reproduction often have a distinctive biogeographic pattern (Law & Crespi 2002). Stefani wrote volumes on his hypotheses to explain the evolution and maintenance of asexuality in *Haploembia*. His themes ranged from sympatric speciation to selective pressures imposed by protozoan parasites to aberrations during cell division that lead to the unusual chromosome numbers of the asexuals, which can be 22 or sometimes 33. His key hypothesis was that parasitism effectively sterilized males lending a selective advantage to female *H. solieri* who could reproduce without mating. Other authors have suggested that parthenogenesis is induced by the infection of *Wolbachia* (Stouthamer & Werren 1992, Baldo et al. 2006). Baldo et al. (2006) tested for *Wolbachia* using 16S, MLST and *Wsp* primers. Their results showed that *Wolbachia* could be present in *H. solieri* from the 16S primers, but no evidence was found for the MLST or *Wsp* primers suggesting that there needs to be more evidence for the presence of *Wolbachia* within *H. solieri*. With current molecular tools and our ability to locate populations in the field, it may now be possible to address questions that remain about the evolution of parthenogenesis in this genus, especially given the insight that *H. tarsalis* has a different ancestry than proposed by Stefani.

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Appendix A

Full species descriptions of sexual and Parthenogenetic biotypes of *Haploembia solieri* (Rambur) translated from below cited text

Female of the sexual biotype description (translated from Stefani &Contini 1961; Fig. 2A)

Length 10.0–12.0 mm. Overall coloration reddish-brown to ferruginous; head darker than rest of body.

Head (dorsal view) on opposite sides with symmetrical pigmented to slightly depigmented areas from the vertex, occiput, and parietal areas.

Hypopharynx. Lower region with long groove along entire length with microsculpting of tiny spicules; spicules short except last segment where they become more complex in form but different from parthenogenetic biotype by being minute and irregular.

Microsculpturing of the lacinia. Small tubercles present transversely (dorsal-lateral view).

Pronotum. Dark red; Acrotergite (between criponotum and metanotum) a sclerotized band broad, extending laterally on thorax, deeply pigmented.

Abdomen. Streaks (irregular depigmented areas) present longitudinally along pleural sclerites (lateral view).

Egg. Operculum raised, thick and circular in shape.

Sexual male biotype description (Stefani 1955; Ross 1966; Fig. 2B)

Length 10.6–11.5 mm. Apterous. Overall coloration dark brown with thorax and legs yellow-brown; head dark brown with symmetrical pattern similar to female; Gula and margins of occipital formen golden-brown; Eyes black with pale ring around them; First and second antennomere dark brown; Antennomeres III–XI tan with reddish-brown apices; Anteclypeal membrane purple-white; Labrum dark brown; Mandibles amber; submentum light chestnut brown, other mouthparts tan; Prothorax and legs amber; Meso- and metathorax and abdomen dark reddish-brown, membranes purple; Hind femora medium brown. Abdominal terminalia and cerci medium brown with ferruginous maculae.

Mandibles. Large, elongate; longitudinal prominence along dorsal side, extends from base of mandible to proximal joint of mandible. Condylar region of mandible two times greater than the distal half. Mandibular teeth present.

Terminalia. Hemitergites fused at base (dorsal view); right hemitergite tapers regularly, facing left, ending in a minute hook; Epiproct fused between two membranous hemitergites; Left hemitergite highly sclerotized, not articulated to base, acuminate, styliform, apex rotated right; Hypandrium (ventral view) convex, robust, extending caudally in a conical process. Ventral view convex and sclerotized, dorsal view membranous and concave. Left cercus somewhat clavate (Davis 1939).

Asexual female biotype description (Stefani &Contini 1961; Fig. 2C)

Length 12.2–14.0 mm. Overall coloration depigmented and paler in color than sexual biotype; head same color as rest of body.

Head (dorsal view) on opposite sides with symmetrical depigmented areas from vertex, occiput, and parietal areas.

Hypopharynx. Lower region with long groove along its entire length with microsculpturing of tiny spicules; spicules irregularly arranged in three rows; basal row with spicules minute; middle row spicules longer and pointed; distal third row of spicules more complex, lash-like with 2–4 points.

Microsculpturing of the lacinia. Tubrecules present transversely and more pronounced and abundant than in sexual biotype (dorsal-lateral view).

Pronotum. Depigmented medially; meso- and metanotum more sclerotized; acrotergite (between criponotum and metanotum) sclerotized, extending medially on thorax, deeply pigmented, reduced.

Abdomen. Streaks (irregular depigmented areas) indistinguishable longitudinally along pleural sclerites (lateral view).

Egg. Operculum not raised, thinner than sexual biotype, and semicircular in shape.