

Parthenogenesis in the *Aspidiotus nerii* Complex (Hemiptera: Diaspididae): A Single Origin of a Worldwide, Polyphagous Lineage Associated with *Cardinium* Bacteria

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ABSTRACT *Aspidiotus nerii* Bouché, the oleander scale, is a nearly cosmopolitan pest that attacks >100 families of woody plants. *A. nerii* has long been suspected of comprising multiple cryptic parthenogenetic and sexual species. We amplified and sequenced a 760-bp fragment of cytochrome oxidase 1 and 2 (COI-COII) from 59 individuals representing four laboratory stocks and 16 wild populations from four biogeographic regions (United States, several Mediterranean countries, South Africa, and New Zealand), along with three outgroup species. We also used polymerase chain reaction (PCR) to assay 92 individuals representing five laboratory stocks and seven wild populations for the presence of the intracellular bacteria *Cardinium*. We find three highly (>5%) divergent groups of mitochondrial DNA (mtDNA) haplotypes in *A. nerii*. Two of these are associated with males and not associated with *Cardinium* and are inferred to represent sexual populations. The other haplotype group is not associated with males, is associated with *Cardinium*, and includes laboratory stocks known to be parthenogenetic; this haplotype group is inferred to represent a parthenogenetic lineage. Although *Cardinium* is associated with the parthenogenetic lineage, a “curing” experiment that would demonstrate direct parthenogenesis induction by *Cardinium* has not been done. The parthenogenetic lineage is 5.5–7.3% divergent at COI-II from the closest sexual lineage and comprises five moderately divergent ($\leq 3.5\%$) haplotypes. This amount of mtDNA divergence within the parthenogenetic lineage may imply an ancient (≥ 1 Mya) origin; however, the amount of amino acid sequence diversity within the parthenogenetic lineage is much greater than in the sexual sister-lineage, implying an elevated rate of nonsynonymous substitution in the parthenogenetic lineage. The highest haplotype diversity is found in New Zealand, and the closest outgroup is found in Australia, suggesting a possible Australasian origin of *A. nerii*.

KEY WORDS thelytoky, parthenogenesis, armored scale insects, Aspidiotini, endosymbiosis

Aspidiotus nerii Bouché is one of the most common and widespread scale insects on earth. It is found in 48 countries, representing all zoogeographic regions, and is recorded from 455 hosts in 107 families (Miller and Ben Dov 2004). It is an important pest (Beardsley and Gonzalez 1975, Miller and Davidson 1990) and is the subject of an extensive literature, including records of 54 different species of natural enemies (Miller and Ben Dov 2004). *A. nerii* comprises both sexual and parthenogenetic lineages, and several

authors have suggested that it may constitute a complex of cryptic species (Ferris 1941, Einhorn et al. 1998). In three different parts of the world—California (DeBach and Fisher 1956), Europe (Schmutterer 1952), and Israel (Gerson and Hazan 1979)—sympatric sexual and parthenogenetic populations have been brought into the laboratory and a number of life history characters have been compared under different rearing conditions. These have included generation time, fecundity, larval ability to survive starvation, and success in colonizing different host plants. In each case, significant differences were found between the populations, and the authors have concluded that the sexual and parthenogenetic *A. nerii* of their region represent different subspecies (Schmutterer 1952) or species (DeBach and Fisher 1956, Gerson and Hazan 1979). However, these differences were not consistent between studies or between regions, and the number of different parthenogenetic and sexual lineages encompassed

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within the *A. nerii* complex has remained an open question (Gerson and Hazan 1979).

In some cases, apparently polyphagous parthenogenetic insects have turned out to be communities of diverse monophagous clones (Mitter et al. 1979, DeBarro et al. 1995). Gerson and Hazan (1979) reported that parthenogenetic *A. nerii* in Israel (= *A. paranerii* Gerson) were specialized on a single host, *Pittosporum undulatum*, whereas sexual *A. nerii* used several other hosts. This observation suggested that host-specialized clones may exist within the *A. nerii* complex as well. The occurrence of host-specialized clones would be of great practical interest for the quarantine and control of *A. nerii* as a pest and also would be of general interest for understanding the evolution and ecology of parthenogenetic lineages. *A. nerii* is routinely cultured; indeed, *A. nerii* is a favorite medium for mass-rearing of parasitoids (Hare and Morgan David 1997), and it is thus potentially a model system for the study of parthenogenetic lineages.

To investigate the evolution of parthenogenesis in *A. nerii*, we have undertaken a phylogenetic study by using mitochondrial DNA (mtDNA) sequences. Because mtDNA is maternally inherited, it provides an estimate of maternal-lineage genealogy within a species, an important component of any study of the origins of parthenogenetic lineages. Because parthenogenetic lineages of *A. nerii* had recently tested positive for the presence of *Cardinium*, an intracellular bacterium known to manipulate reproductive biology in various insects (Weeks et al. 2003, Zchori-Fein et al. 2004), we also surveyed several populations for the presence of these bacteria.

Materials and Methods

A. nerii specimens were obtained from widely scattered populations and laboratory stocks, primarily by soliciting specimens from generous colleagues around the world. Collection data and sample sizes are given in Table 1. Some of the laboratory stocks were known to be parthenogenetic, and some of the populations could be seen to contain sexual lineages because males were present. Through the generosity of Uri Gerson, our sample included specimens of *Aspidiotus paranerii* Gerson, the putative parthenogenetic species he described from *Pittosporum undulatum* in Israel, which most subsequent authors have regarded as a synonym of *A. nerii* (Miller and Ben Dov 2004). We also included three outgroup species in our phylogenetic analysis of the mtDNA sequences. One is a possibly undescribed Australian species, identified by D. J. Williams of The Natural History Museum as "*Aspidiotus* near *nerii*," and the others are two other species in the subtribe Aspidiotina [*Hemiberlesia lataniae* (Signoret) and *Aonidia aurantii* (Maskell)]. All specimens were preserved in 95–100% ethanol and stored at -20°C . Voucher specimens of each sample have been preserved and are held by the University of Massachusetts Insect Collection.

Nucleic acids were extracted from whole insects by using the salting out method of Sunnucks and Hales

(1996) as modified by Normark (1999). A region of mtDNA encompassing partial sequences of cytochrome oxidase I (COI) and cytochrome oxidase II (COII) was amplified under the following reaction conditions: 2.5 μM MgCl_2 , 200 μM each dNTP (QIAGEN, Valencia, CA), $1\times$ QIAGEN buffer, 1.25 U of *Taq* polymerase (QIAGEN), 10 pmol of each primer, and 2 μl of scale insect nucleic acid solution in a total volume of 50 μl . The primers used were c1-J-2753ywr (gtaaactaacatttttyccwcarca) and c2-N-3662 (Simon et al. 1994). The basic temperature profile (in an MJ Research thermocycler) was as follows: denaturation at 95°C for 30 s, annealing at $40\text{--}50^{\circ}\text{C}$ for 1 min, and extension at 72°C for 2 min, for a total of 33 cycles. We used a 2°C stepdown procedure for annealing, with the first cycle at 50°C for 1 min, and annealing in later cycles reduced by 2°C after every second cycle until reaching 40°C . We also used an initial 1-min denaturation at 95°C before the first cycle, and a final 10-min extension at 72°C after the last cycle. Each set of reactions included negative (no-DNA) controls. Double-stranded polymerase chain reaction (PCR) amplification products were visualized on 1.5% agarose gels and purified using microcentrifuge columns (Qiaquick, QIAGEN) and then were shipped to the Brigham Young University DNA Sequencing Center for direct cycle sequencing.

A PCR assay for the presence of *Cardinium* was carried out for samples of all four laboratory colonies and five of the 16 wild populations from which mtDNA haplotypes were obtained, as well as additional populations from which mtDNA haplotypes were not obtained (Table 1). DNA was extracted from single *A. nerii* specimens by using a CTAB-phenol-chloroform extraction method with RNase and proteinase K treatment (Weeks et al. 2003). These extractions were then screened for the presence of *Cardinium* by using the hemi-nested protocol described in Weeks et al. (2003). Briefly, DNA extractions were screened for the presence of eubacteria by using the 16S rDNA primers 27 F and 1513R (Weisburg et al. 1991) in a PCR. PCR products were then diluted in water (1 μl of PCR product:500 μl of sterile water) and re-screened again with *Cardinium*-specific 16S rDNA primers (CLOf and CLOr; Weeks et al. 2003) that amplify a product of ≈ 450 bp. Appropriate negative and positive controls were included in all PCR reactions, with 5 μl of each PCR product run on a 1.0% agarose gel stained with ethidium bromide to determine the presence of an amplified product. To further reduce the chance of false negatives due to genomic DNA extraction failures, all DNA extractions were screened with general 28S rDNA primers that will amplify a product in all insects of between 500 and 600 bp, as described in Werren et al. (1995).

Sequences were compiled and edited using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI). There were no insertions or deletions except in a small intergenic spacer region (see *Results*) that was excluded from the analysis; therefore, alignment was trivial. Parsimony analysis was conducted using PAUP 4.0b10 (Swofford 2002). Due to the small number of

Table 1. Collection data, haplotype distributions, results of bacterial PCR assays, and evidence bearing on sexuality vs parthenogenesis, for *A. nerii* specimens used in this study

Normark catalog no.	Locality	Date collected	Host plant	Collector	Sex?	n haplo-typed	<i>Cardinium</i>	Haplotypes found
D404	Italy: Palermo (laboratory colony)	25-III-2002	<i>Cucurbita maxima</i> Duchesne	S. Colazza	T	7	10/10	H1,H2
D410	Hungary: Budapest (laboratory colony)	20-II-2001	<i>Cucurbita</i> sp.	F. Kozár	M	0	0/12	
D504	USA: Florida, Homestead (laboratory colony)	2-IV-2001	<i>Cucurbita</i> sp.	H. Glenn	T	8	6/7	H2
D527	USA: California, Riverside (laboratory colony)	I-2002	<i>Citrus limon</i> (L.)	J. Hiare	T	2	10/10	H2
D530	Turkey: Adana-Gar (laboratory colony)	X-2000	<i>Cucurbita</i> sp.	L. Erkişçi	M	4	0/8	H1
D405	Italy: Sicily, Trapani Provinces	25-III-2002	<i>C. limon</i>	S. Colazza	M	6	0/10	H1
D406	USA: California, San Diego	31-XII-2001	<i>Pittosporum</i> sp.	L. M. Provencher		1		H406B
D408	USA: California, Vacaville	27-VIII-2002	<i>Nerium oleander</i> L.	L. M. Provencher		1		H2
D409	South Africa: Nelspruit	2001		J. Daneel		1		H1
D411	Greece: Athens	12-IV-2001	<i>Olea</i> sp.	C. Stathas	M	4	0/9	H411
D418	Israel: Rehovot	10-IX-2001	<i>Pittosporum undulatum</i> Vent.	U. Gerson	Ap	2		H2
D420	Israel: Rehovot	10-X-2001	<i>Pelargonium</i> sp.	U. Gerson	Ap	1		H1,H2
D423	Spain: Grenada: Castle of Alhambra	20-XI-2000	<i>Citrus</i> sp.	L. M. Provencher	An	1		H1
D424	Spain: Madrid	21-XI-2000	<i>N. oleander</i>	L. M. Provencher		1		H1
D426	New Zealand: Waitakere Range	12-XII-2000	<i>Dysoxylum spectabile</i> (C. Forst.)	R. C. Henderson		2		H1, H426N
D430	USA: California: Sonoma Co.: Penngrove	3-X-2000	<i>Schefflera</i>	M. Vernon		1		H430A
D492	France: Paris	7-XII-2000		D. J. Williams		1		H1
D493	New Zealand: Auckland	12-XII-2000	<i>Trachycarpus fortunei</i> (Hook.)	A. Martin		2	6/6	H493D, H493J
D498	Italy: Poggiore	18-III-2001	<i>Citrus</i> sp.	A. Garrona	M	0	2/6	
D499	Italy: Portici	3-III-2001	<i>Citrus</i> sp.	A. Garrona		1	0/5	H1
D505	Italy: Amalfi	5-X-2001	<i>Citrus</i> sp.	A. Garrona		6	0/4	H1
D506	Cyprus: Nicosia	X-2000	<i>N. oleander</i>	C. Orpanides	M	3		H1
D529	USA: Nevada Co.: North San Juan	14-X-2000	<i>Cytisus scoparius</i> (L.)	R. Wall	M	1	0/5	H1

Collection dates are given as day, month (in roman numerals), year. T, laboratory colony known to be parthenogenetic (thelytokous); M, males observed; Ap, putative parthenogenetic species *A. parameritii* Gerson, from its type locality; An, sexual *A. nerii* sensu Gerson. The column "*Cardinium*" gives the number of individuals that tested positive for *Cardinium* over the total number of individuals tested. Collection data for outgroups are as follows: *Aonidiella aurantii*: Argentina, Tucumán, Yerba Buena, 30-XII-2001, ex *Citrus limon*, coll. Lucia Claps, D290; *Hemiberlesia latantiae*: New Zealand, North Island, Auckland, Glen Eden, 12-V-2001, ex *Rosa*, coll. Rosa C. Henderson, D066; *Aspidiotus near nerii*: Australia, A.C.T., Canberra, Black Mt., Aust. Nat. Bot. Carduus, 35° 16'36" S, 149° 06'26" E, 16-VII-2001, ex *Macrozamia lucida*, coll. P. J. Gullan & S. R. Donaldson, D043.

haplotypes found, an exhaustive search was used. To assess confidence, bootstrap percentages were calculated using branch-and-bound search for 10,000 replicates.

To explore the evolution of parthenogenesis and of bacterial infection status, evidence bearing on genetic system (presence of males, known parthenogenesis of laboratory colonies) and *Cardinium* infection was mapped onto the most parsimonious cladogram. To explore consequences for molecular evolution, coding regions of DNA were translated to predicted amino acid sequence in MacClade 4.0 (Maddison and Maddison 2000) by using the *Drosophila* mtDNA code, and amino acid changes were mapped onto the most parsimonious tree obtained from the analysis of the nucleotides.

Results

Among the 59 individuals of *A. nerii* sequenced, eight different haplotypes were found at COI-CO₂ (GenBank accession nos. DQ119748-DQ119758). The total length of the aligned sequences was 777 bp, including 234 bp of COI, 531 bp of CO₂, and 2–12 bp of what is apparently an intergenic spacer between them. The gene encoding leucine tRNA, normally found between COI and COII in the mitochondrial genomes of related insects (Normark 2000, Thao et al. 2004), was absent. It also is missing in >90 other species of diaspidid scale insects (G.E.M., M. E. Gruewell, and B.B.N., unpublished data) and has presumably been translocated to a different position in the mitochondrial genome, similarly to the mitochondrial genome rearrangements seen in other paraneopteran orders such as thrips, lice, and barklice (Shao et al. 2001).

Of the 59 individuals sequenced, more than half (31) shared the most common haplotype, H1. Individuals with H1 were found in two of the four laboratory colonies and in 12 of the 17 natural populations examined. These include populations from the Mediterranean area (Spain, France, Italy, Cyprus, and Israel), the United States (California), South Africa, and New Zealand (Table 1). Males were found in three of these populations (in Italy and Cyprus). The second most common haplotype, H2, was found in a total of 19 individuals, representing three parthenogenetic laboratory colonies and two natural populations (California and Israel). One of these populations is from the type locality and typical host plant of *Aspidiotus paranerii* Gerson, as identified and supplied by U. Gerson. H2 was not found in association with males.

All other haplotypes are known only from a single locality. One of these (H411) was found in four individuals from Greece. H411 is only a single base-pair (0.14%) different from H1 and is found in association with males. Five other haplotypes were found, each in association with a single individual from California (H430A and H406B) or New Zealand (H426N, H493J, and H493D). The parsimony analysis produced a single most parsimonious tree of haplotypes (Fig. 1).

The intergenic spacer between COI and COII is 3 bp long in haplotypes H1, H2, H411, and H430A, and only 2 bp long in H406B and the three unique haplotypes found in New Zealand. These are the shortest intergenic spacers yet found in this position among >90 spp. of armored scale insects (G.E.M., M. E. Gruewell, and B.B.N., unpublished data). The outgroups used here have intergenic spacers of 12 bp (*A. aurantii*), 8 bp (*H. lataniae*), and 11 bp (*Aspidiotus nearnerii*).

The PCR assays for the presence of the *Cardinium* intracellular bacterium were consistent with the hypothesis that the bacterium is associated with parthenogenesis. All three parthenogenetic laboratory colonies were infected with the bacterium, and both sexual laboratory colonies lacked it (Table 1). Four natural populations containing males were tested for the bacterium: three of these were completely negative, and one was mixed (two positives of six individuals tested). Three natural populations lacking evidence of males (and thus having unknown genetic system, given the short lifespan of *A. nerii* males) also were tested: one of these tested positive for all six individuals tested, and the other two were negative.

Colonies and populations in which only H1 (or the closely related haplotype H411) was found always tested negative for *Cardinium*. These included one sexual laboratory colony and four natural populations (all in Italy or Greece). Conversely, laboratory colonies and populations containing H2 individuals (or those with the closely related haplotypes H493D and H493J), always tested positive for *Cardinium*. These included three laboratory colonies (with H2) and one natural population (with H493D and H493J).

The reconstruction of the minimum number of amino acid replacement substitutions on the MP haplotype tree estimated that the most common haplotype, H1, had the ancestral amino acid sequence from which the others were derived. There have been 16 unambiguous amino acid replacements in the evolution of *A. nerii*; five of these are in the branch leading to the most-divergent (male-associated) haplotype H430A; one separates H1 from the closely related (male-associated) haplotype H411, and the majority (10) occur within the parthenogenetic lineage.

Discussion

The *A. nerii* complex comprises at least two different lineages, highly (5.5–7.3%) divergent in mtDNA sequence. One of them (characterized by haplotypes H1 and H411) is predominantly or entirely sexual, with cytoplasm uninfected by *Cardinium*; the other (characterized by H2 and related haplotypes) is predominantly or entirely parthenogenetic and infected by *Cardinium*. Both lineages are worldwide in distribution. These results tend to support the Gerson and Hazan (1979) recognition of a separate parthenogenetic species, *Aspidiotus paranerii* Gerson. Of the samples supplied to us by Gerson, the *A. paranerii* had the widespread parthenogenetic haplotype H2, and the

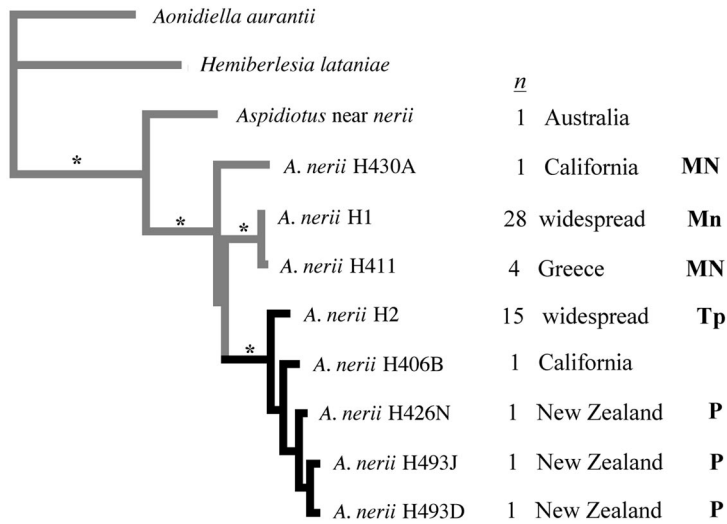


Fig. 1. The single most-parsimonious cladogram for the haplotypes of *A. nerii* and outgroups. Treelength, 344. Consistency index, 0.846. Branchlengths are proportional to the number of changes along the branch. Branches marked with an asterisk (*) had bootstrap scores of 99.6% or higher (10,000 replicates, branch and bound). There are three columns of information about the haplotypes. The first column (*n*) indicates the number of individuals in our sample that had that haplotype. The second column indicates roughly where that haplotype was found (for more geographic detail, see Table 1). The third column summarizes evidence bearing on the genetic system: M, males found; T, parthenogenesis (thelytoky) confirmed by rearing; N, population tested negative for *Cardinium*; n, for the H1 haplotype, eight of nine populations tested negative and one tested mixed for *Cardinium*; P, population tested positive for *Cardinium*; and p, for the H2 haplotype, two of three populations tested positive and one tested mixed for *Cardinium*. The color of the branches indicates the most parsimonious reconstruction of the evolution of the genetic system in *A. nerii*: gray, sexual; black, parthenogenetic.

typical *A. nerii* had the widespread sexual haplotype H1.

In addition to these widespread and common haplotypes, there are a number of other, “singleton” haplotypes, found only once in our sampling, and each bearing one or more nonsynonymous nucleotide substitutions. This finding suggests that more extensive and intensive sampling would turn up considerably more haplotype diversity. The singleton haplotypes have been found in California and in New Zealand. The native range of *A. nerii* is unknown, but North America has very few native *Aspidiotus* species and seems an unlikely candidate. New Zealand is more likely, there being several *Aspidiotus* species endemic to various islands in the Australasian region (Williams and Watston 1988). It is also possible that haplotype diversity is even higher in some unsampled part of the range of *A. nerii*. A good candidate for future sampling is Australia, which has endemic species of *Aspidiotus* that are similar to *A. nerii*, e.g., *A. kennedyae* (Boisduval) and the “*Aspidiotus near nerii*” of this study (Ben Dov and German 2003), and it is a plausible source for the rarer haplotypes found in New Zealand and California.

There seems to be a strong association between parthenogenesis and the presence of *Cardinium*. It is possible that this bacterium induces parthenogenesis in *A. nerii* as it does in several other host insects (Zchori-Fein et al. 2004), and our results are consistent with a single origin of bacterial infection and parthenogenesis within the complex (Fig. 1), although

this conclusion is tentative pending further testing. The gold standard for demonstrating that a bacterial infection induces parthenogenesis in its host is a curing experiment in which a parthenogenetic insect lineage reverts to sex after being fed antibiotics (Stouthamer et al. 1990). Such curing experiments are most simple and dramatic in arrhenotokous lineages such as Hymenoptera, in which the cured virgin females begin to produce males rather than females. In an armored scale insect such as *A. nerii*, reproduction depends upon mating, and a clear reversion to sex after an antibiotic “cure” would depend on the female’s interfertility with some sexual lineage, in this case with the sexual *A. nerii* lineage. However, the sexual *A. nerii* lineage is divergent enough in mtDNA sequence that its conspecific status and potential for interfertility is dubious. Indeed, the parthenogenetic lineage has ceased to produce sex pheromone (E. Peri, S. Ramirez, F. Saiano, and S. Colazza, unpublished data). This is a serious obstacle to designing a curing experiment appropriate for *A. nerii*. Another challenge for the design of a curing experiment is the dependence of armored scales on primary endosymbiotic bacteria (Tremblay 1990), although the successful selective curing of secondary endosymbionts in aphids by Fukatsu (2004) has shown that this is not an insurmountable problem. Further indirect evidence of parthenogenesis induction may be obtained by showing that there is a consistent association between the presence of this bacterial lineage and parthenogenetic reproduction across a wider sample of insects.

Weeks et al. (2003) showed that the *Cardinium* endosymbiont of *A. nerii* is nested within a larger clade of *Cardinium* strains isolated from the wasp genus *Aphytis*, many species of which parasitize *A. nerii*. This clearly implies transmission from parasitoid to host, and further implies a mechanism for the wide horizontal transfer of *Cardinium* across insect lineages.

It seems from our results that there has been a very uneven pattern of amino acid replacement at COI-COII across the *A. nerii* complex. In the most common sexual lineage, there have been essentially no amino acid replacements at all since their divergence from the most recent common ancestor of the *A. nerii* complex. Most of the unambiguous reconstructed amino acid replacements (10 of the 16 seen in the *A. nerii* complex) occurred within the *Cardinium*-associated parthenogenetic lineage. A similar pattern—increased mtDNA substitution rate in maternal lineages whose cytoplasm is infected with *Wolbachia*—has been reported previously in several dipteran, hymenopteran, and isopteran species (Shoemaker et al. 2004). It is usually interpreted as reflecting an increased deleterious substitution rate resulting from a series of mitochondrial population bottlenecks associated with the infection. The invading bacterial lineage is effectively linked to a particular mtDNA haplotype and carries this haplotype with it to fixation. Any subsequent infections by competing bacterial strains, and any new adaptive bacterial mutants, are likewise linked to whatever mtDNA haplotype they are initially associated with, and carry those mtDNA haplotypes as they spread to fixation. The resulting series of population bottlenecks severely reduces the effective population size of mtDNA and increases the rate of fixation of slightly deleterious mutations (Shoemaker et al. 2004). In a parthenogenesis-inducing bacterium, this phenomenon may be even more severe because the mtDNA and bacterial genomes also are effectively linked to a particular nuclear genome (Normark et al. 2003).

The possibility of an accelerated rate of nonsynonymous nucleotide substitution within the parthenogenetic lineage makes it problematic to apply any sort of molecular clock calibration. But the divergences seen within the parthenogenetic lineage of *A. nerii* (up to 3.5%) are among the highest seen in any parthenogenetic insect and qualifies parthenogenetic *A. nerii* as one of the best candidates to be considered an ancient parthenogenetic insect lineage (Normark et al. 2003).

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