

Multiple Parthenoforms of *Empoasca* Leafhoppers from Madeira Island: Where Are These Unisexual Forms Coming From?

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Abstract

There are controversial opinions on whether asexual reproduction is more common on islands than on the mainland. Although some authors consider that the evidences of geographical parthenogenesis support the view that asexual reproduction is more common on islands, comparative data on the modes of reproduction of insular and continental taxa confirming this statement are very limited. In this work, we report the presence of three unisexual forms and three bisexual species of the genus *Empoasca* (Cicadelloidea, Hemiptera, Insecta) from Madeira Island. Experimentally, the unisexual forms reproduced in the absence of males for several generations. The chromosome analysis has shown that the bisexual species differ from one another in chromosome number, and unisexual forms are apomictic and also each have different chromosome numbers. Of parthenoforms, one is triploid and two are of obscure level of ploidy, $2n$ or $3n$. The results obtained show that for this genus unisexual forms are more common on Madeira Island than in the nearby continental areas. It is suggested that unisexual forms may be more plentiful on islands than on the mainland because if an asexual reproduction event occurs, the relaxing competition in these underexploited and enemy-free habitats may favor the establishment of new parthenogenetic lineages.

According to Vandel's rule of geographic parthenogenesis, whenever closely related bisexual and parthenogenetic species occur in a given region, their geographical patterns of distribution tend to be different (Vandel 1928). Generally, unisexuals tend to occur in habitats colder or drier or further north or higher compared to habitats of their related bisexuals. Several authors (Cuellar 1977; Glesener and Tilman 1978) have relatively recently claimed that parthenogenetic forms tend to occupy islands, while related amphimictics occur on the nearby mainland. Unisexuals have stronger dispersal ability and higher prolificity compared to bisexuals. Thus, on islands where both sexes of a bisexual species are not likely to have arrived simultaneously, unisexuals are expected to be more common because a single female is sufficient to start a new population (Cuellar 1977). Despite this, the evidence pinpointing the common occurrence of asexual species on islands is fragmentary, and the affirmation that parthenogens tend to form a higher proportion of the biota on islands than they do on the mainland remains controversial. For example, Cuellar

(1977) after a revision of reptiles from islands suggested that parthenogenetic geckos tended to occupy islands while their amphimictic relatives were present in the nearby continental areas. In contrast, Bell (1982) suggests that the common occurrence of parthenogenesis on islands is dubious for insects and not supported for coccids. This last statement was based on his analysis of Brown's (1965) data for two families of coccids. However, although Bell claims that there is no correlation between parthenogenesis and island life, he did not compare the actual number of unisexual and bisexual coccid species present on islands and continents. Instead, he compared the number of localities for parthenogenetic and sexual coccid species on the mainland and islands and the number of bisexual and unisexual forms within some remote islands.

This controversy can be partly attributed to the limited information available on the modes of reproduction of insular taxa. Apart from a few detailed studies on reptiles, reports of parthenogenesis on arthropods, representing the vast majority of animal species on islands, are only accidental. Most

examples of parthenoforms in invertebrates refer mainly to insects with limited ability for dispersion, such as weevils, stick insects, aphids, or coccids. Depending on their origin, asexual species often have considerable genetic variation (Dowling and Secor 1997; Simon et al. 2003; Suomalainen et al. 1987). To know whether parthenoforms are more capable of colonizing islands than bisexual species, it is necessary to know the modes of reproduction of island biota. However, if parthenogenesis were common on islands, this fact would have deep implications in the explanation of the origin and evolution of island diversity (Rhymer and Simberloff 1996; Seehausen 2004). In this work based on the rearing experiments and chromosome studies, we report multiple cases of parthenogenesis in *Empoasca* leafhoppers from Madeira. We suggest that these parthenoforms have originated within this small oceanic island.

Materials and Methods

Species Identification

Males of three bisexual species, *Empoasca fabalis* DeLong 1930, *Empoasca alsiosa* Ribaut 1933, and *Empoasca decedens* Paoli 1932, were collected in the field, the first mentioned having been previously unknown in Madeira. Males of these bisexual species were easily distinguished on the basis of the shape of the genital structures. In addition to these, three all-female morphotypes, which differed from each other and from females of the bisexual species in vertex shape, wing venation, and body size, were regularly found in the field during a year of sampling work without any apparent males. Here, these females are named morphotype A, morphotype B, and morphotype C. The all-female morphotypes were first detected on the basis of morphology, and their distinctiveness was confirmed through rearing experiments (see below). In addition, on the castor plant *Ricinus communis* L., five male specimens different from those of *E. fabalis*, *E. alsiosa*, and *E. decedens* were found. According to genital structures, these males correspond to *Empoasca distinguenda* Paoli 1932, an African species not recorded previously from Madeira. To check if these five males were morphologically similar to the females sampled, they were compared with all unisexual and bisexual females in body size, wing venation, and vertex shape. As a result of these comparisons, these males were found to be clearly associated to females of morphotype C. Records of all unisexual and bisexual species of *Empoasca* from Madeira, together with host plant associations and an identification key, will be published elsewhere (Aguin-Pombo D and Freitas N, in preparation).

Materials

Both unisexual and bisexual species are polyphagous (Freitas and Aguin-Pombo 2004; Freitas N and Aguin-Pombo D, unpublished data). For rearing experiments, females of morphotype A were sampled in Monte, Funchal, at an altitude of 565 m on *Osteospermum barberae* (Harv.) Norl in June; females of morphotype B were sampled in São Vicente at 30 m on

Helianthus annuus L. in June; and females of morphotype C were sampled in Machico at 30 m on *R. communis* L. in June. All samples were collected in 2001. For chromosome analysis, bisexuals and unisexuals were collected in different localities of Madeira Island. The samples were as follows: *E. alsiosa* from Chão dos Louros, São Vicente, at 804 m on *Phyllis nobla* L. and in Garajau on *Coprosma repens* A. Rich.; *E. decedens* from Funchal at 54 m on *Schinus molle* L. and in Caniço de Baixo at 15 m on *R. communis* L.; *E. fabalis* from Faial, at 200, m on *Ipomoea batatas* (L.) Lam. and from Penteada, Funchal, on *Ipomoea* sp.; morphotype C from Machico at 30 m on *R. communis* L. and from Faial at 25 m on *R. communis* L.; morphotype A from Monte, Funchal, at 565 m on *O. barberae* (Harv.) Norl and from Machico at 30 m on *R. communis* L.; and morphotype B from São Vicente at 30 m on *H. annuus* L.

Rearing Experiments

To understand whether morphotypes A, B, and C reproduce parthenogenetically, rearing experiments were started in the summer (June 2001). First rearings were done in a garden, but, when the weather became colder (September 2001), they were transferred to a greenhouse until the end of the experiment. All morphotypes were maintained in plastic rearing boxes under similar environmental conditions. Temperature and humidity were recorded daily. Maximum temperature varied between 20.5°C and 30.2°C and minimum temperature from 17.1°C to 20.7°C, while relative humidity varied from 71% to 86%. Two different plant species were used as host plants, *Phaseolus vulgaris* L. for morphotypes A and B and *R. communis* L. for morphotype C. Plants of *P. vulgaris* were obtained through seed germination, while those of *R. communis* were collected in the field and used in experiments only after new leaves appeared (first leaves were allowed to drop).

Rearings were started with nymphs from the field. Once nymphs reached the adult stage, they were identified, sexed (all were females), and then transferred into a new rearing box. This was considered as the parental generation. Rearing boxes were monitored daily. After oviposition, new nymphs emerged, and after being identified and sexed, some or all of them were transferred again to new boxes to start a new generation. This process was repeated continuously for each new generation during a year.

Chromosome Analysis

For chromosome studies male specimens were collected from the field. Testes were dissected from the abdomen of anesthetized specimens in a drop of 0.9% sodium citrate and squashed on a slide under the coverslip in a small drop of 45% acetic acid. After the removal of coverslips using dry ice, the preparations were dehydrated in fresh fixative Carnoy (1 glacial acetic acid:3 ethanol) for 20 min and air-dried. Staining was performed according to the Feulgen-Giemsas procedure by Grozeva and Nokkala (1996) with minor modifications. The slides were treated in 1 N HCl at room temperature for 20 min, hydrolyzed in 1 N HCl at 60°C for 7 min, stained

Table 1. Bisexual and unisexual *Empoasca* leafhoppers from Madeira with reference to material examined, karyotype, and ploidy level

Species	Number of individuals examined	Number of metaphase plates analyzed	Sex	Chromosome number	Ploidy
<i>Empoasca decedens</i>	23	217	♂♂	14 + X	2n
<i>Empoasca alsiosa</i>	21	185	♂♂	16 + X	2n
<i>Empoasca fabalis</i>	36	230	♂♂	20 + X	2n
Morphotype A	4	4	♀♀	31 ^a	Undetermined ^b
Morphotype B	6	6	♀♀	27	3n
Morphotype C	7	7	♀♀	24	Undetermined

^a Provisional value.

^b Most likely 3n (see the text).

with Schiff's reagent for 30 min, and rinsed in distilled water and Sorensen's phosphate buffer pH 6.8 for 5 min in each. Finally, the slides were stained with 5% Giemsa in Sorensen's buffer for 20–30 min. After staining, the preparations were rinsed briefly with distilled water, air-dried, and mounted in Entellan.

Females were fixed in Carnoy. Mature eggs were dissected from ovarioles in a drop of 50% propionic acid and were kept there from 3 to 6 min. After removing the chorion, eggs were placed in a drop of 45% acetoorcein for about 4 h or overnight. When adequate staining was achieved, one or two eggs together were squashed under a coverslip and examined. The number of individuals of each species and of each morphotype that were cytogenetically studied and number of metaphase plates examined are presented in Table 1.

Results

The rearing experiments showed that all offspring of the morphotypes A, B, and C were females. As in other species of *Empoasca*, eggs of each morphotype eclose into first-instar nymphs, which moult into adults after a five-instar period each. The offspring of each morphotype were always phenotypically similar in vertex shape and body size to the virgin females of each parental generation, and this similarity was maintained along all generations. For morphotypes A and C, continuous reproduction allowed several asexual generations per year. Morphotypes A and C reproduced asexually during at least 10 generations, while morphotype B reproduced only during two generations. This fact was probably due to two different reasons: the environmental conditions used, which seemed to be unsuitable for morphotype B, and high sensitivity of the latter to handling during nymphal stage. Details of rearings for the first three generations are shown in Table 2.

The study of three unisexual forms and three bisexual species of *Empoasca* from Madeira showed that they have different chromosome numbers (Table 2). In metaphase I (MI) of male *E. alsiosa* (Figure 1A), eight autosomal bivalents, three distinctly larger than the others, and a univalent X were found (2n = 16 + X). In male *E. decedens* (Figure 1B), MI showed seven autosomal bivalents, two larger than the others, and a univalent X (2n = 14 + X). Males of *E. fabalis*

(Figure 1C) had 10 autosomal bivalents, three to four larger than the others, and a univalent X (2n = 20 + X). In each species the largest bivalent was readily distinguishable; X was one of the smaller chromosomes and tended to be positioned at a distance from bivalents that is characteristic of Auchenorrhyncha as a whole (Halkka 1959).

The all-female forms reproduced by apomictic parthenogenesis, that is, meiosis was suppressed completely in females, and eggs went through a mitosis-like cell division. Females of morphotype B showed 27 univalent chromosomes in eggs, and the largest chromosomes were three in number and similar in size (Figure 1D). In the morphotype A, all chromosomes of eggs were also univalent, and their number was most likely 31 (Figure 1E). However, it requires further confirmation. The eggs of morphotype C showed 24 univalent chromosomes; of these six, as a minimum, are larger than the others (Figure 1F). In addition to females, two males of the last-mentioned morphotype were studied. In one male, testicular follicles showed early meiotic pro-phases but neither spermatids nor sperms. In the second male, early pro-phases as well as spermatids and sperms were found. Both spermatids and sperms seemed to be normal; however, they were far from being as numerous as in males of bisexual species studied.

Table 2. Results of rearing unisexual morphotypes of *Empoasca*. Each generation was started with some or all individuals obtained from the previous generation. Morphotypes A and C reproduced asexually for 10 generations, while morphotype B reproduced only two generations

Morphotypes	Generations	Number of parental individuals	Number of descendants
Morphotype A	P	11	55
	F1	22	53
	F2	34	37
	F3	13	2
Morphotype B	P	30	150
	F1	30	5
	F2	2	0
Morphotype C	P	9	32
	F1	9	84
	F2	10	87
	F3	9	26

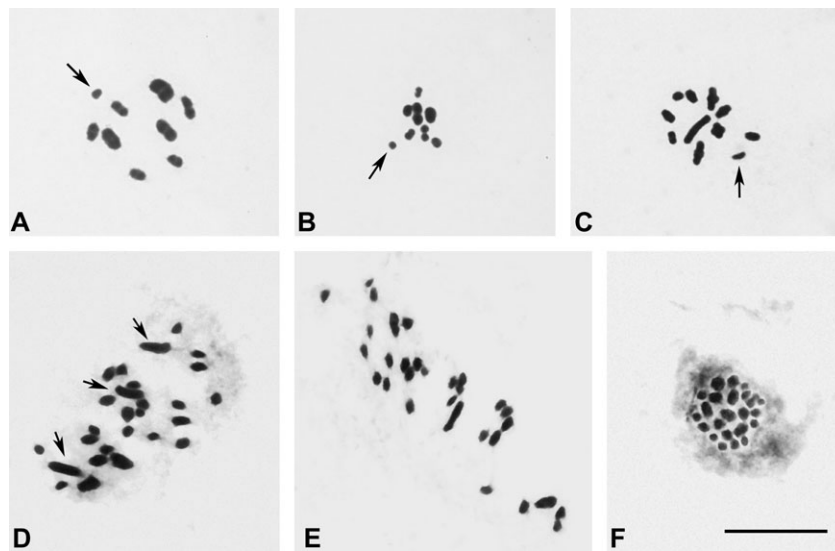


Figure 1. Metaphases I of bisexual species (A–C) and oocyte metaphases of unisexual morphotypes (D–F) of *Empoasca*. (A) *Empoasca alsiosa* (♂), (B) *Empoasca decedens* (♂), (C) *Empoasca fabalis* (♂), (D) morphotype B (♀), (E) morphotype A (♀), and (F) morphotype C (♀). Arrows show sex chromosomes in (A), (B), and (C) and three largest chromosomes in (D). Scale 2 μ m.

Discussion

Parthenoforms are usually considered to have originated mainly by three different mechanisms: (1) an internal chromosomal event, such as endomitosis (2) the fertilization of an unreduced egg by a conspecific male sperm, or (3) by interspecific hybridization (Bell 1982; Simon et al. 2003; Suomalainen et al. 1987). Induction of parthenogenesis is also possible through infection by vertically inherited microorganisms, mainly by *Wolbachia* (Proteobacteria) (Simon et al. 2003). The present data on *Empoasca* leafhoppers from Madeira are still insufficient to learn the origin of parthenoforms A and C, but the triple number (triplet) of the largest chromosomes in morphotype B females suggests that it may be the result of the triplication of a haploid chromosomal set. Because these females were $3n = 27$, this could be due to the triplication of the haploid chromosomal set of *E. alsiosa* ($n = 9$ in females); that is, it may have been the result of the fertilization of an unreduced egg. In morphotype A, we failed to discover convincing proof of the triplication of a particular haploid chromosomal set as in morphotype B. Our provisional conclusion is that it has $3n = 31$. Because in none of the 316 cytogenetically studied species of the superfamily Cicadelloidea does chromosome number exceed 28 (Emeljanov and Kirillova 1989), the morphotype A is most likely also triploid. If its chromosome number is actually 31, we could assume that morphotype A has originated by hybridization between two species with different chromosome numbers. The fact that the largest chromosomes in morphotype A are of different sizes also counts in favor of this hypothesis. Hybridization in animals is more common than generally believed (Arnold 1997; Delmotte et al. 2003). In the case of morphotype C with 24 chromosomes in the eggs, it is not possible to know at present if it is triploid or diploid.

Regardless of its origin, the asexual mode of reproduction is a fairly rare event in nature. In the suborder Auchenorrhyncha, for which more than 30,000 species have been described, only two cases of true parthenogenesis from two different families are known: *Delphacodes* planthoppers (Den Bieman and De Vriejer 1987) and *Agallia quadripunctata* leafhoppers (Black and Oman 1947). In the genus *Empoasca* with approximately 450 nominal species, many of which are largely distributed, no case of parthenogenesis has been so far known. To learn whether unisexual forms are more common on islands than in nearby continental areas, as is predicted by geographical parthenogenesis (Vandel 1928), it is necessary to compare the number of unisexual and bisexual European species represented on the continent and nearby islands (Glesener and Tilman 1978). However, for this estimation, it should be taken into account (1) that parthenogenesis is unequally distributed among taxa, so comparisons should be restricted to the same taxon ex. same genus, and (2) that insular faunas tend to have different balance of species compared to equivalent patches of mainland—known also as disharmonic faunas—and that insular diversity is much lower as compared with the nearby continental areas. Therefore, the proportion of unisexual and bisexual species represented on islands and in continental areas should be calculated separately. Madeira is a true oceanic island which never has been joined to the continent, and its fauna has mainly its origin in Europe (Baez 1993). Taking this into account and based on the knowledge of the actual distribution of the species of the genus *Empoasca* in Europe, we accept that in mainland Europe there are 11 bisexual species of this genus and 0 unisexuals. On the other hand, in nearby islands, including the Arquipelagos of Madeira and Canary Islands, there are nine *Empoasca* species (D'Urso 1995; Nast 1972, 1987; our unpublished data),

of which three are unisexuals. Hence, unisexual *Empoasca* leafhoppers are associated only with European islands, which represent 33% of all insular species, but 0% are associated with the mainland. These results clearly suggest that in this case unisexuals are comparatively more common on islands than in nearby mainland regions as is predicted by geographical parthenogenesis.

Parthenogenesis being an extremely rare phenomenon within this genus and to a greater extent in the Auchenorrhyncha suborder as a whole, these results raise the question why so many parthenoforms are represented in Madeira. It has been suggested that parthenoforms are more able to colonize new habitats because a single female is sufficient to start a new population. Therefore, parthenoforms would have greater chances to colonize and establish themselves in insular habitats. However, unisexual populations of the same species should be present also in closer continental areas. Instead, indirect evidences suggest that parthenoforms of *Empoasca* leafhoppers in Madeira did not come from nearby continental areas but probably have originated within this island. Firstly, if unisexual forms known from Madeira had originated outside this volcanic island, they should have been found also in mainland areas. Nevertheless, no such case has come to our notice. Secondly, although it could be assumed that parthenoforms may have gone unnoticed within this large genus, this seems not to be the case because the unisexual leafhoppers are very common on agricultural fields (Aguin-Pombo D and Freitas N, in preparation). Host plant associations are very important for leafhoppers because the plants represent not only a food resource for adults and nymphs but also a place for mating and laying eggs (Claridge 1993). Thus, if these parthenoforms have derived from a crop-associated sexual species, as seems to be the case, they would have retained some similar plant associations also in mainland. The possible mainland areas where these parthenoforms could be found are Europe, America, and Africa; this is supported by the fact that two of the three bisexual species are pests in America and Mediterranean countries, respectively, while *E. distinguenda* (indistinguishable morphologically from morphotype C) is a pest in Africa and Middle East. However, despite being the bisexual species of *Empoasca* relatively well known in countries of these regions, no case of asexual reproduction is known there. Finally, the fact that bisexual forms reproduce only sexually in their mainland distribution areas likewise argues for the insular origin of unisexuals. For example, *E. alsiosa*, to which morphotypes A and B are similar in morphology (Freitas N and Aguin-Pombo D, unpublished data), reproduces sexually outside Madeira, and the same is true for *E. distinguenda*, whose males are common in Africa and Middle East where this species is common (Webb 1987). The above indirect evidence suggests that these parthenoforms have evolved within Madeira.

Several features of islands may help to successfully establish new parthenoforms. Colonization of true oceanic islands like Madeira is possible through long dispersion events. Under these circumstances insular habitats are underexploited with a low number of competitor species, parasites, and predators. If an accidental mistake in meiosis produces

an unreduced egg that is fertilized, this new triploid would be easier to establish on islands because the high prolificity of unisexuals together with empty niches on islands and a reduced number of predators will allow their establishment more easily than on the mainland.

The asexual species can increase variability through mutations, through occasional sex, or due to a multiple origin (Bengtsson 2003). Thus, the coexistence of different modes of reproduction in insular species may be important not only in maintaining but also in increasing genetic variability that has been lost due to drift during colonization. In this context, the study of the modes of reproduction may give us some clues for understanding the origin of species on islands.

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