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Colonization ability of two invasive weevils with different reproductive modes

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Abstract The flightless weevils *Naupactus leucoloma* and *Naupactu xanthographus* (Coleoptera: Curculionidae: Naupactini), which are native to and partially co-distributed in South America, apparently have asexual and bisexual reproductive modes, respectively. We used two different molecular markers to elucidate the effects of these reproductive modes on the colonization ability and genetic variability of both species. First, we investigated the occurrence of clonal reproduction in the putative parthenogenetic species (i.e. significant bias in sex ratio) and second, whether parthenogenesis was associated with higher colonization ability and low levels of genetic variability in marginal environments compared with those of the bisexual species. We assessed the central and marginal areas of distribution of these species with ecological niche modeling that includes environmental variables and with landscape interpolation of molecular variability. Our results support the idea that parthenogenetic species are more successful than bisexual ones in colonizing new environments. *N. leucoloma* is most probably apomictic, and would have recently experienced significant population growth concomitant with an important geographic range expansion to distant areas with moderately suitable environmental conditions. On the other hand, the populations of the bisexual species, *N. xanthographus,* seem to have maintained fairly constant sizes, expanding its geographic distribution to locations close to the proposed ancestral area.

Keywords Apomictic parthenogenesis · White-fringed weevil · Fruit-tree weevil · Niche modeling · Landscape genetics · Phylogeography

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Introduction

Thelytokous parthenogenesis has evolved from ancestral sexual lineages in a variety of eukaryotes, providing a unique opportunity for investigating the causes and consequences of diversity in genetic systems. The phylogenetic distribution of thelytoky implies that this reproductive mode represents a general adaptive solution to particular life history patterns (Jensen et al. 2002). The establishment of thelytoky is usually characterized by marked differences in the geographic and/or ecological distributions of sexual ancestral and thelytokous descendents, a pattern known as "geographic parthenogenesis" (Vandel 1928; Glesener and Tilman 1978; Lynch 1984). The high reproductive rates and independence from males for reproduction may confer asexual forms with an enhanced ability to colonize areas of lower biotic diversity (Jensen et al. 2002; Stenberg and Lundmark 2004).

Parthenogenesis is common in many insect groups. It is particularly the case for weevils, which account for about 22% of all known species of asexual insects (Normark 2003). In this group, parthenogenesis has played an important role in evolution and development of biological diversity (Stenberg and Lundmark 2004). Lanteri and Normark (1995) compiled a list of 34 species of Naupactini which are thought to be parthenogenetic due to the absence of males in the samples. Some species of this tribe show "geographic parthenogenesis" (Lanteri 1993; Lanteri and Marvaldi 1995; Lanteri and Normark 1995; Normark and Lanteri 1998). For example, no males have been documented for *Naupactus peregrinus*; males of *Naupactus leucoloma* and *Naupactus cervinus* were recorded long ago in the Paranaense forest (northeastern Argentina and southern Brazil), and gallery forests along the Paraná and Uruguay rivers. In turn, the parthenogenetic lineages of the latter are currently found in grassland and agricultural areas of Argentina and other countries where these species were introduced with crops.

Two main alternative hypotheses have been proposed to explain geographical parthenogenesis: (1) the destabilizing hybridization hypothesis and (2) the general purpose-genotype hypothesis (Lynch 1984; Lanteri and Normark 1995). According to the former, the disjunct distributions of parthenogenetic and sexual lineages may often result from the deleterious genetic effects of hybridization between parthenogenetic females and their sexual relatives. The second hypothesis postulates that parthenogens have more generalized genotypes resulting in less restrictive habitat requirements; this fact would allow them to occupy more extreme environments and broader geographic distributions than their sexual relatives.

The reduction in genetic diversity associated with colonization events may have important ecological consequences for long-term survival. However, the hybridization of clones with closely related species could counteract this process by increasing genetic diversity both within individuals (i.e. increased heterozygosity) and within populations. Replenishment of diversity through such hybridization events could thus provide an escape from inbreeding in colonizing populations, especially when coupled with parthenogenesis (Kearney 2005).

Asexuality in weevils is usually related to polyploidy. In fact, all studied clonal weevils have apomictic parthenogenesis (i.e. lack of meiosis), with most of them being polyploid (Suomalainen et al. 1987; Smith and Virkki 1978). Triploids might have originated from chance fertilization of a diploid gamete by a haploid one, as a result of crosses between parthenogenetic females and males of the same species or closely related species; likewise, these events could have led to increased polyploidy levels (Lanteri and Normark 1995). Stenberg et al. (2003) observed that clones with different ploidy levels show different geographic ranges. These authors, who referred to this pattern as "geographic polyploidy",

suggested that the colonization of extreme environments is facilitated by polyploidy rather than parthenogenesis per se.

On the other hand, the discovery of the vertically transmitted bacterium *Wolbachia* (Werren et al. 1995), which infects 20% of insect species, has opened up new approaches to the study of parthenogenesis in animals (Hurst and Jiggins 2005). It induces several reproductive distortions, including female bias in the host sex ratio, thelytokous parthenogenesis, cytoplasmic incompatibility, feminization of genetic males and killing of male progeny (Werren 1997; Stouthamer et al. 1999). In a recent paper, we reported a strong correlation between the presence/absence of *Wolbachia* and asexual/sexual reproduction in 21 species of the Naupactini tribe, respectively. This suggests that the bacterium is involved in the origin of parthenogenesis in this weevil group (Rodriguero et al. 2010a).

Naupactus leucoloma Boheman (white-fringed weevil) and *N. xanthographus* (Germar) (fruit-tree or grapevine weevil) are flightless broad-nosed weevils (Coleoptera: Curculionidae: Naupactini) native to South America, where they are partially co-distributed. Both species are highly polyphagous and have similar life cycles, with larvae feeding on roots and adults on the green parts of host plants (Lanteri et al. 2002). *N. leucoloma* is native to Argentina—where it is widely distributed—and also to Uruguay and southern Brazil. In addition, it has been introduced into Chile, Peru, USA, South Africa, Australia and New Zealand. It has been reported to feed on 385 species of plants (Young et al. 1950) including crops such as beans and peanuts in Chile and USA (Watson 1937), and pastures in Australia and New Zealand (Hardwick and Prestige 1994; Hardwick et al. 1997). In Argentina, *N. leucoloma* damages alfalfa, beans and soybean (Lanteri and Marvaldi 1995). This is most probably parthenogenetic, as pure female broods have been demonstrated by rearing experiments (Buchanan 1939). Bisexual populations have only been found in a single location on the banks of the Paraná River (Entre Ríos province, Argentina). Most populations are exclusively composed of females (Lanteri and Marvaldi 1995), which were infected with *Wolbachia* (Rodriguero et al. 2010b). *N. xanthographus* is mainly distributed in Uruguay and central Argentina, and has been introduced into Chile including the Juan Fernández and Easter Islands. This species is bisexual and feeds on a great variety of plants. In Chile it is particularly harmful to fruit trees and grapevines (Ripa 1986) and in Argentina to grapevines and alfalfa (Lanteri et al. 2002).

One crucial task of phylogeographic studies is to untangle the contribution of historical *vs* contemporary ecological processes in shaping the current distribution of species. In particular, studies of insects with parthenogenetic reproduction can add substantially to our knowledge of how asexuality can shape the distribution of genetic variability within a species. Furthermore, taking into account that phylogeography is useful in elucidating the central and marginal areas of a species' distribution and colonization routes (Lanteri and Confalonieri 2003; Scataglini et al. 2006), the comparison of species with different reproductive modes may provide evidence to test the hypothesis that asexual species have greater colonization capacities in marginal habitats.

Here we carried out a comparative phylogeographic study of *N. leucoloma* and *N. xanthographus* to test the following hypotheses: (1) *N. leucoloma* exhibits geographic thelytokous parthenogenesis; then, a thorough sampling of this species along its geographic distribution is expected to reveal the presence of males and females in central populations and only females in marginal ones. *N. xanthographus* has sexual reproduction; then, a thorough sampling of this species along its geographic distribution is expected to show a fairly similar proportion of males and females in all populations. (2) *N. leucoloma* reproduces by apomictic parthenogenesis; then, nuclear markers are expected to be clonally transmitted together with mitochondrial ones, rather than being affected by

recombination. Conversely, nuclear markers of *N. xanthographus* are expected to show evidence of genetic recombination. (3) Levels of genetic variability are expected to be lower for *N. leucoloma* than for *N. xanthographus* as a consequence of apomictic parthenogenesis. (4) The asexual *N. leucoloma* is expected to be more capable of colonizing marginal areas than sexual *N. xanthographus* because of its putatively higher reproductive rate and independence from males for reproduction. Then, *N. leucoloma* populations are expected to show signs of exponential growth and to occupy wider areas of less suitable (marginal) environments than *N. xanthographus* populations.

Materials and methods

Sampling scheme

We collected 184 specimens of *N. leucoloma* and *N. xanthographus* for molecular studies throughout their range of distribution in Argentina and Brazil, including areas of native vegetation and cropfields. We made samplings between 2005 and 2009 and stored specimens at −70°C until DNA extraction. The analysis also included samples from Chile, Uruguay and Australia stored in 100% ethanol. Tables 1 and 2 show the number of

Table 1 Geographic localities (abbreviations between brackets), number of individuals (N_{AFLP}: number of individuals analyzed in AFLP studies; N_{COI}: number of COI-sequenced individuals), and COI haplotype (L1–L4) frequencies (N) of *N. leucoloma*

Source location	N_{AFLP}	N_{COI}	Haplotypes (N)
AR, Bs As, Arrecifes (AR)	–	4	L1 (4)
AR, Bs As, Cañuelas (CA)	6	6	L1 (6)
AR, Bs As, Chillar	1	–	–
AR, Bs As, Colón (COL)	–	1	L1 (1)
AR, Bs As, Delta (DE)	4	4	L1 (4)
AR, Bs As, La Plata (LP)	7	7	L1 (7)
AR, Bs As, O'Brien (OB)	7	8	L1 (3), L4 (5)
AR, Bs As, Otamendi (OT)	3	7	L1 (4), L2 (3)
AR, Bs As, Pergamino	3	–	–
AR, Córdoba, La Carlota (LC)	10	7	L1 (7)
AR, Córdoba, Río Cuarto (RC)	3	1	L1 (1)
AR, Chubut, Trelew (TR)	12	6	L1 (6)
AR, E Ríos, Concordia (CN)	2	3	L1 (3)
AR, E Ríos, Gualeguay (GA)	1	1	L2 (1)
AR, E Ríos, Gualeguaychú (GU)	13	8	L1 (8)
AR, Mendoza, Godoy Cruz (GC)	9	5	L1 (1), L3 (2)
AR, Santa Fe,Venado Tuerto (VT)	–	1	L1 (1)
AR, Tucumán, Los Leales (TU)	–	1	L1 (1)
BR, R Grande do Sul, Santa María (SM)	–	1	L1 (1)
CH, Santiago (ST)		1	L1 (1)
AU, Capital Territory, Black Mountain	–	1	L1 (1)
AU, New South Wales	–	1	L3 (1)

Table 2 Geographic localities (abbreviations between brackets), number of individuals (N_{AFLP}: number of individuals analyzed in AFLP studies; N_{COI}: number of COI-sequenced individuals) and COI haplotype (X1–X7) frequencies (N) of *N. xanthographus*

Source location	N_{AFLP}	N_{COI}	Haplotypes (N)
AR, Bs As, Arrecifes (AR)	5	7	X1 (6), X2 (1)
AR, Bs As, Chillar (CH)	2	1	X1 (1)
AR, Bs As, Buenos Aires (CB)	9	9	X (1), X3 (3), X6 (5)
AR, Bs As, Hortensia (HT)	3	3	X3 (3)
AR, Bs As, La Plata (LP)	16	16	X3 (15), X6 (1)
AR, Bs As, Otamendi (OT)	15	15	X1 (3), X3 (2), X7 (10)
AR, Bs Ad, Pergamino (PG)	15	15	X1 (3), X2 (3), X3 (7), X6 (2)
AR, Bs As, Zárate (ZA)	4	4	X1 (1), X3 (3)
AR, Corrientes, Yapeyú (YP)	6	5	X1 (5)
AR, E Ríos, Colón (CO)	16	13	X4 (13)
AR, E Ríos, Gualeguaychú (GU)	2	3	X5 (3)
AR, Mendoza, Godoy Cruz (GC)	9	6	X3 (6)
AR, Santa Fe, Venado Tuerto (VT)	3	3	X1 (1), X3 (1), X6 (1)
CH, Vicuña (VI)	3	3	X1 (1), X3 (2)
CH, Eastern Island	1	1	X3 (1)
UR, Paysandú, Paysandú (PS)	3	3	X3 (3)

individuals examined from each sample location. Tables S1 and S2 of Supplementary Material give sample location details.

Ecological niche modeling

We inferred the central and marginal areas of both species by estimating the probability distribution for their occurrence based on environmental constraints. This analysis is useful to test: (a) if the species compared have similar environmental requirements; and (b) if parthenogens occupy more extensive areas of less suitable (marginal) environments than do sexual individuals. We used MAXENT version 3.1 (http://www.cs.princeton.edu/~schapire/maxent/) to estimate the probability distribution for the species' occurrence (Phillips et al. 2006). This program uses presence-only data and environmental variable layers (continuous or categorical) for the studied area. It generates an estimate of probability of species presence ranging from 0 to 1, where 0 is the lowest and 1 the highest probability. Testing or validation is required to assess the predictive performance of the model (Kumar and Stohlgren 2009). To this aim we implemented a jackknife (also called "leave-one-out") procedure (Pearson et al. 2007).

We performed two separate analyses for each species to develop present-day niche models, one including samples from South America (142 for *N. leucoloma* and 72 for *N. xanthographus*), and the other including also samples from Australia, New Zealand, USA and South Africa. Some of the localities coincided with those used in the molecular analysis; in other cases, we obtained data from specimens deposited in different entomological collections, mainly at the Museo de La Plata (Argentina) and the Universidad de la República (Uruguay), and from the literature (Tables S1 and S2, Supplementary Material). All bioclimatic variables available (20 layers) were tested for multicollinearity

by examining cross-correlations among variables (Pearson correlation coefficient, r) in geographical space, based on occurrence records of both species. Only one variable from a set of highly correlated variables was included in the analysis ($r \geq 0.80$), based on its potential ecological influence on species' distribution. Then, we selected the following 11 environmental variables for both *N. leucoloma* and *N. xanthographus*: Annual Mean Temperature; Mean Diurnal Range (mean of monthly (max temp–min temp)); Isothermality; Max Temperature of Warmest Month; Temperature Annual Range; Mean Temperature of Wettest Quarter; Mean Temperature of Driest Quarter; Annual Precipitation; Precipitation of Wettest Month; Precipitation of Driest Month; Altitude. These were downloaded from WORLDCLIM version 1.4 (http://www.worldclim.org/) (Hijmans et al. 2005) and were combined and analyzed in a Geographic Information System (GIS). All layers were at 30 arc-sec (~ 1 km^2) resolution for the South American analysis, and 2.5 arc-min for the worldwide analysis. In MAXENT, we used the default convergence threshold (10^{-5}) and increased maximum iterations to 1,000; the program selected automatically regularization values and functions of environmental variables. For each run, we used 75% of the localities to train the model and randomly selected 25% of the localities to test the model. Models were generated using 10,000 background points (as pseudo-absences). The geographical region from which these random background points are selected to represent the environmental background may affect model quality, but guidelines for the appropriate selection are still a matter of research (VanDerWal et al. 2009; Wilson et al. 2009). To accomplish this task we decided to select the whole South American region because the predicted distributions obtained were uninterpretable when background points were restricted to narrower areas or even to the sampling extent. We evaluated the model performance using a threshold-independent method based on the Area Under the Curve (AUC) of Receiver Operating Characteristics Curve (ROC) (Swets 1988). The AUC value ranges from 0.5 (random accuracy) to a maximum value of 1 (perfect discrimination). We transformed the output into a map representing probabilities of occurrence because MAXENT produces a continuous probability. Finally, we show response curves for the three top predictors for the occurrence of both weevil species. These curves are automatically generated by MAXENT and illustrate how predictions depend on the environmental variables.

Analysis of molecular markers

We extracted whole genomic DNA using lysis buffer with Proteinase K, according to the protocol described in Reiss et al. (1995). Individuals preserved in 100% ethanol were dried before extraction. Extracted DNA was amplified via PCR for sequencing analysis (Cytochrome oxidase I (COI) mitochondrial gene) or digested and further amplified for fragment analysis (Amplified Fragment Length Polymorphisms (AFLP)).

Mitochondrial DNA

We amplified a fragment of COI gene via PCR using primers already assayed for other Naupactini species (Scataglini et al. 2006): S1718 5′-GGA GGA TTT GGA AAT TGA TTA GTT CC-3′ and A2442 5′-GCT AAT CAT CTA AAA ATT TTA ATT CCT GTT GG-3′. Amplifications were performed in a volume of 50 µl with 50–100 ng of DNA used as template, 100 ng of each primer, 0.1 mM of each DNTP, 3 mM $MgCl_2$, 1 unit of *Taq* polymerase and buffer 1X provided by Invitrogen. Amplification was carried out in a thermocycler (MyGenie 96 Thermal Block, Bioneer) under the following conditions: initial

denaturation at 94°C for 1 min, 35 cycles at 94°C for 60 s, at 49°C for 60 s, at 72°C for 90 s, and final extension at 72°C for 10 min. PCR products were purified with Accuprep® PCR Purification Kit (Bioneer), following the manufacturer's instruction. Samples were run on an ABI 3130XL automated sequencer (Applied Biosystems) with Big Dye terminator sequencing kit (Applied Biosystems). Haplotypes were identified manually, and standard indices of genetic variation such as nucleotide diversity (π) and haplotype diversity (H_d) were computed using the program DNAsp version 4.10.9 (Rozas et al. 2003). The haplotype network was constructed by a parsimony method using the program Winclada version 1.00.08 (Nixon 2002).

Nuclear DNA

The AFLP method was performed as described by Vos et al. (1995), with a few modifications. For digestion with EcoRI and MseI enzymes, 250 ng of genomic DNA were used, and PCR reaction was carried out in a total volume of 25 µl (pre-selective amplification) and 20 µl (selective amplification). For selective amplification, EcoRI primers were labeled with fluorescent 6-FAM (6-carboxyl-fluorescein) to enable automated analysis with an ABI 3130XL automated sequencer (Applied Biosystems). One of eleven primer combinations was chosen for selective amplification because it gave clear and reproducible electrophoretic patterns and showed variation within and between populations. The AFLP adapter sequences were as follows: 5′-CTCGTAGACTGCGTACC-3′ and 3′-CATCTGACGCATGGTTAA-5′ for Eco RI; 5′-GACGATGAGTCCTGAG-3′ and 3′-TACTCAGGACTCAT-5′ for MseI. Pre-amplification primer sequences and selective amplification primer sequences were EcoRI- AAG/MseI-CAC (EcoRI 5′-GACTGCGTACCAATTC -3′; MseI 5′-GATGAGTCCTGAGTAA-3′). Each pre-amplification and amplification contained 2.5 ng of template. Selective amplification products were separated with an ABI 3130XL (Applied Biosystems) automated sequencer. A 1,000 bp internal size standard (LIZ 500 (−250 bp)) was run with samples, and data were collected and analyzed using the Local Southern Size Calling method (with minor modifications) in GeneMapper version 3.7 (Applied Biosystems), a polynomial degree of 5 and a peak window size of 13. A peak (i.e. a fragment) was scored as present (1), otherwise it was scored as absent (0). Results of scoring were exported to a presence/absence matrix and used for further analyses.

We estimated variability by applying the Shannon Index (SI) because it does not assume HW equilibrium and hence can be applied for both species having different reproductive modes. We calculated SI using the Popgene program version 1.32 (Yeh and Boyle 1997).

Indirect evidence of asexual reproduction in *N. leucoloma*

Before the application of genetic tools to whole-organism biology, the detection of clonality was only possible through morphological observations (absence of males) and/or rearing experiments with virgin females (offspring production without previous fertilization). The presence of rare males does not necessarily imply sexual recombination (Halkett et al. 2005). To investigate meiotic recombination—or lack thereof—is thus the best way to assess the presence of clonality (i.e. the occurrence of parthenogenetic reproduction).

To test for non-random associations among AFLP markers, we measured multilocus linkage disequilibrium by calculating the Index of Association (I_A, Maynard Smith et al. 1993) and $\bar{r}_d$ (a modification of I_A index), using the program Multilocus version 1.3 (Agapow and Burt 2001). We compared the observed I_A and $\bar{r}_d$ for each species with an expected I_A and $\bar{r}_d$ under random mating simulated through the reshuffling of data over 100

permutations. I_A and $\bar{r}_d$ have an expected value of zero if there is no association of alleles at unlinked loci, as expected in a randomly mating population.

Asexual reproduction was also assessed by the R index (Dorken and Eckert 2001) with Genclone software (Arnaud-Haond and Belkhir 2007).

$$R = \frac{(G - 1)}{(N - 1)}$$

where G is the number of distinct multilocus genotypes and N is the number of individuals. These statistics were estimated from a randomly chosen set of 42 AFLP loci because Genclone software can analyze a maximum of number of 42 loci.

Demographic analyses

We conducted demographic analyses by means of a mismatch distribution (Rogers and Harpending 1992), Fu's F_S (Fu 1997) and Tajima's D_T (Tajima 1989) tests using the program DNAsp version 4.10.9 (Rozas et al. 2003). We carried out the significance of the tests with 10,000 coalescent simulation replicates based on Monte Carlo process (Hudson 1990) and assuming no recombination in the data sets.

Geographic distribution of nuclear and mitochondrial variation: landscape genetics

To relate genetic diversity and reproductive mode to geographic occupancy, we mapped genetic distances for both nuclear and mitochondrial markers on the landscape using Alleles in Space version 1.0 (Ais) (Miller 2005). We used a Delaunay triangulation-based connectivity network to identify midpoints between the sample sites and then calculated the raw genetic distance (Dij) at each midpoint (Miller 2005). This genetic distance measure is similar to Nei's standard genetic distance (Ds; Nei et al. 1983). By this method, a landscape of genetic distances between sampling sites is expressed as "surface heights" and is displayed as a 3-dimensional graph. To better visualize the Ais height output, we imported the output file into Diva-Gis version 7.1 (http://www.diva-gis.org/) and created a 2-dimensional color hot-spot map overlaid on the geographic study area. Colors correspond to "heights" of genetic distance between points.

Results

Potential geographic distributions

The potential geographic distributions of *N. leucoloma* and *N. xanthographus* predicted by Maxent using samples from South America are displayed in Fig. 1. Models performed better than random predictions with high ROC (AUC) values: 0.955 for *N. leucoloma* and 0.957 for *N. xanthographus*. The highest level of probability ($P > 0.69$) for the geographic distribution of *N. leucoloma* (Fig. 1a) and *N. xanthographus* (Fig. 1b) covered in Argentina an area close to the banks of the Paraná and Uruguay rivers near the confluence with La Plata river (Paraná- La Plata Delta), the northeastern area of Buenos Aires province and also a central area of Argentina and Chile at about the same latitude. The three environmental variables with the highest training gain (i.e. variables containing the most useful information by themselves) for both species were Annual Mean Temperature,

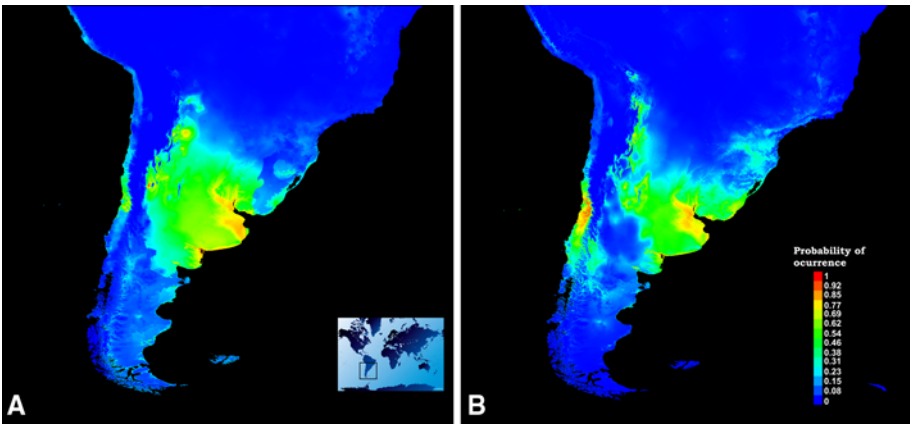

Fig. 1 Predicted distribution from Maxent Ecological Niche Modeling for **a** *N. leucoloma* and **b** *N. xanthographus* obtained from localities in South America. Distributions are defined by probabilities of species occurrence above the minimum predicted probability (lower limit of the area *shaded in blue*). The *color code* in the *vertical bar* on the *lower right corner* corresponds to the probability of species occurrence (from 0 to 1)

Isothermality and Temperature Anual Range. Altitude and Annual Mean Temperature were the variables that decreased the gain most when omitted for *N. leucoloma* and *N. xanthographus*, respectively. On this basis, both species are likely to share environmental requirements, and hence their most probable central area of distribution, modeled in relation to environmental and geographic variables, would be almost the same.

When we included samples from outside of South America in the analyses, models also performed better than random predictions with high ROC (AUC) values: 0.982 for *N. leucoloma* and 0.99 for *N. xanthographus*. Figure 2a, b show that the areas of the world suitable for both species would be very similar, encompassing a narrow strip along the Pacific coast of North America, all the southeastern States of USA (*N. leucoloma*), northern Mexico (*N. xanthographus*), some European countries (Portugal, France, England and Ireland), southern Africa (mainly South Africa) and Oceania (New Zealand and vast areas of Australia).

The response curves of the most important environmental variables in the predictions are shown in Fig. S1 a-f. The ecological niche of the two weevil species appears to be defined by Mean Annual Temperature values of 15–18°C, Isothermality values of $\leq$40 and a Temperature Annual Range of 26–34 (*N. leucoloma*) and 26–28 (*N. xanthographus*). Such environmental conditions are characteristic of humid temperate climate where the species are known to occur (Table S1, S2 and references therein).

Genetic diversity of mtDNA and its geographic distribution

Naupactus leucoloma

We used 72 individuals sampled from 20 populations for COI sequence analysis (Table 1). All populations were exclusively composed of females. Alignment of mtDNA sequences (595 bp long) yielded only three polymorphic sites and four mitochondrial haplotypes (arbitrarily named L1, L2, L3 and L4 [GeneBank Accession No. JF811692-JF811695]) (Table 1). The geographic distribution of these haplotypes is shown in Fig. 3a and their

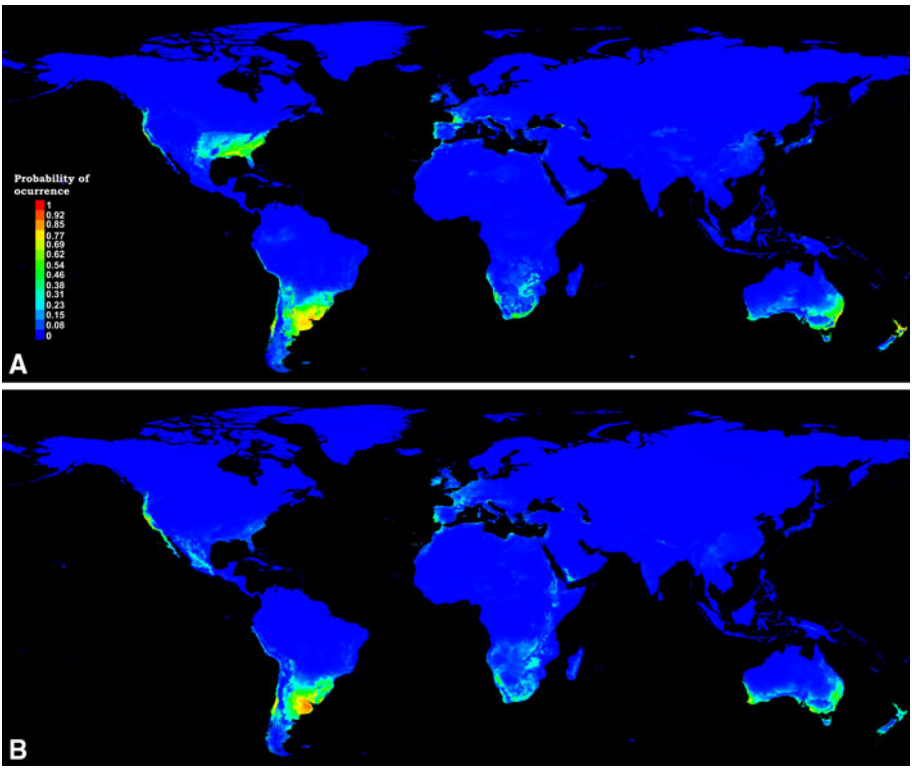

Fig. 2 Predicted distribution from Maxent Ecological Niche Modeling for **a** *N. leucoloma* and **b** *N. xanthographus* obtained from localities in and out of South America. Distributions are defined by probabilities of species occurrence above the minimum predicted probability (lower limit of the area *shaded in blue*). The *color code* in the *vertical bar* on the *lower left corner* corresponds to the probability of species occurrence (from 0 to 1)

population frequencies in Table 1. One particular haplotype (L1) was present in 83% of the sampled individuals. Mean haplotype diversity (H_d) was 0.319 ± 0.00475 and mean nucleotide diversity (π) was 0.00072 ± 0.00017.

Phylogenetic analysis of haplotypes generated one tree with three mutational steps. Haplotype L1 occupied a central position in the network; the three remaining haplotypes derived from L1 through a single mutational step (Fig. 4a). Individuals collected in geographic areas far away from their putative central distribution (i.e. Australia) had the most frequent haplotype (L1) and another one (L3) which was also present in western Argentina (Table 1).

Naupactus xanthographus

All populations of *N. xanthographus* showed fairly similar proportions of both sexes. Alignment of COI sequences (653 bp long) analyzed in 107 individuals from 16 populations yielded seven different haplotypes (arbitrarily named X1-X7 [GeneBank Accession No. JF811696-JF811702]) (Table 2), differentiated by 14 polymorphic sites. The geographic distribution of these haplotypes is shown in Fig. 3b and their population

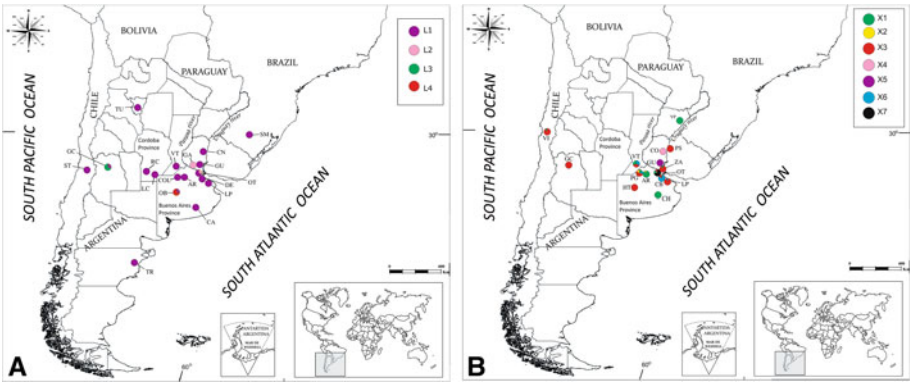

Fig. 3 Geographic distribution of mtDNA haplotypes in **a** *N. leucoloma* (L1-L4) and **b** *N. xanthographus* (X1-X7) populations (see Table 1, 2 for abbreviations). Haplotype identification is shown on the *upper right corner*. The pie chart in each locality shows relative frequencies of haplotypes

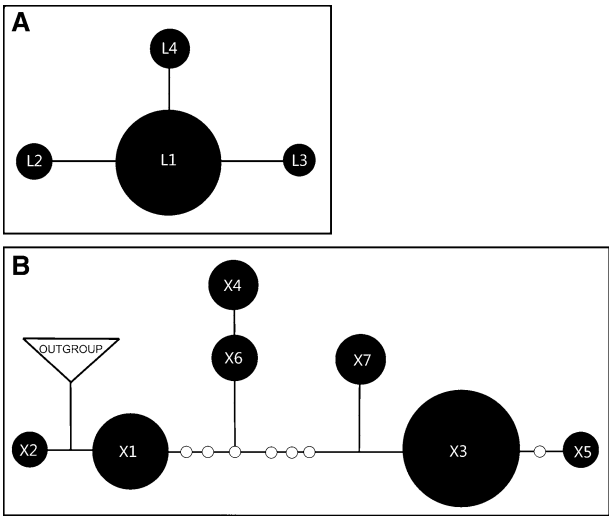

Fig. 4 mtDNA haplotype network of *N. leucoloma* (**a**) and *N. xanthographus* (**b**). *Circle* sizes are proportional to the haplotype frequency. *White circles* correspond to absent haplotypes (see Table 1, 2 for identification of haplotypes)

frequencies in Table 2. Mean haplotype diversity (H_d) was 0.724 ± 0.001 and mean nucleotide diversity (π) was 0.00686 ± 0.00038. This result demonstrates that mitochondrial diversity in this species is much higher than that in *N. leucoloma*.

Phylogenetic analysis of the seven haplotypes produced one tree with 14 mutational steps (CI = 100, RI = 100) (Fig. 4b). According to the position of the outgroup (*N. verecundus*), X1 and X2 were basal haplotypes. Moreover, X1 and X3 were the most frequent haplotypes (Table 2; Fig. 3b). Polymorphic populations were distributed throughout northeastern Buenos Aires province, near the Paraná-La Plata Delta (Fig. 3b). Outside this range, populations were generally monomorphic, which is characteristic of

marginal populations. Based on the geographic distribution of variability and location of the basal haplotypes (i.e. X1 and X2) (Fig. 3b), the north of Buenos Aires province is proposed herein as the most plausible primary center of diversification for *N. xanthographus*.

The haplotypes X1 and X3 were also found in locations far from the putative central area [e.g. X1 and X3 in Vicuña, continental Chile; X3 in Easter Island, Chile; and X3 in Godoy Cruz, in central-western Argentina (Table 2, Fig. 3b)].

Demographic events

Both D_T and F_S tests revealed no significant patterns of demographic or selective sweep events. For *N. leucoloma*, Fu's F_S and Tajima's D_T values were -1.646 ($P > 0.10$) and -0.927 ($P > 0.10$), respectively, indicating no significant deviations from the expected allele frequency spectrum. For *N. xanthographus*, Fu's F_S and Tajima's D_T values were 6.267 ($P > 0.10$) and 1.7984 ($P > 0.10$), respectively, showing no evidence of population expansion events.

For *N. leucoloma* the pairwise mismatch distribution was unimodal. However, no conclusion can be drawn because 86% of the individuals sampled shared the same haplotype. According to coalescence theory, such a pattern of extremely low variability is expected for populations that have gone through a very recent severe bottleneck or selective sweep, in which case none of the statistics will detect these processes (Hein et al. 2005).

For *N. xanthographus*, the pairwise mismatch distribution did not differ significantly from the distribution expected under a constant population size model ($r = 0.1157$). Furthermore, it had a bimodal distribution, as predicted by the coalescent model for a population with random mating and constant size. Therefore, this species shows no signs of population growth.

Nuclear DNA: AFLP

To analyze genetic variability we assayed 81 and 112 individuals of *N. leucoloma* and *N. xanthographus*, respectively (Tables 1, 2). The primer combinations generated 291 fragments for *N. leucoloma* and 257 for *N. xanthographus*, with sizes ranging between 50 and 300 bp. *N. leucoloma* showed slightly lower levels of genetic diversity ($SI = 0.3847 \pm 0.23$) in comparison with *N. xanthographus* ($SI = 0.3991 \pm 0.2175$).

Indirect evidence of asexual reproduction in *N. leucoloma*

The I_A and $\bar{r}_d$ statistics measuring linkage disequilibrium revealed significant levels of non-random association among AFLP markers for both species (*N. leucoloma:* $I_A = 11.05$ ($P < 0.01$), $\bar{r}_d = 0.044$ ($P < 0.01$); *N. xanthographus:* $I_A = 2.05$ ($P < 0.01$), $\bar{r}_d = 0.0089$ ($P < 0.01$)). However, both statistics were approximately five times higher in *N. leucoloma* than in *N. xanthographus*.

The analysis of clonal richness and genotype diversity revealed 22 groups of clones for *N. leucoloma* ($R = 0.2625$). Conversely, all individuals of *N. xanthographus* were genetically different ($R = 1$).

Population expansion

The linkage disequilibrium and clonal richness analysis of nuclear AFLP markers suggested at least some level of asexual reproduction for *N. leucoloma.* These results are also supported by the rearing experiments of Buchanan (1939) and by the fact that only females were found in 141 of 142 populations analyzed in this and previous studies (see Table S1 of Supplementary Material for references). Therefore, it is assumed lack of recombination in *N. leucoloma* permitting to estimate the statistics based on the coalescence theory (Fu's F_S and Tajima's D_T) for nuclear markers. Fu's F_S was significantly negative ($F_S = -24.88$, $P < 0.001$), suggesting a process of population expansion or rapid increase in effective population size. This event was not detected by Tajima's D_T ($D_T = 1.6379$, $P > 0.10$), which is less sensitive to demographic events than Fu's F_S (Ramos-Onsins and Rozas 2002).

Landscape genetics

Figure 5 shows the topography of genetic variation for both species and markers inferred by the Ais program; the highest genetic distance values (i.e. regions with highest levels of genetic variation) are represented by red-colored areas.

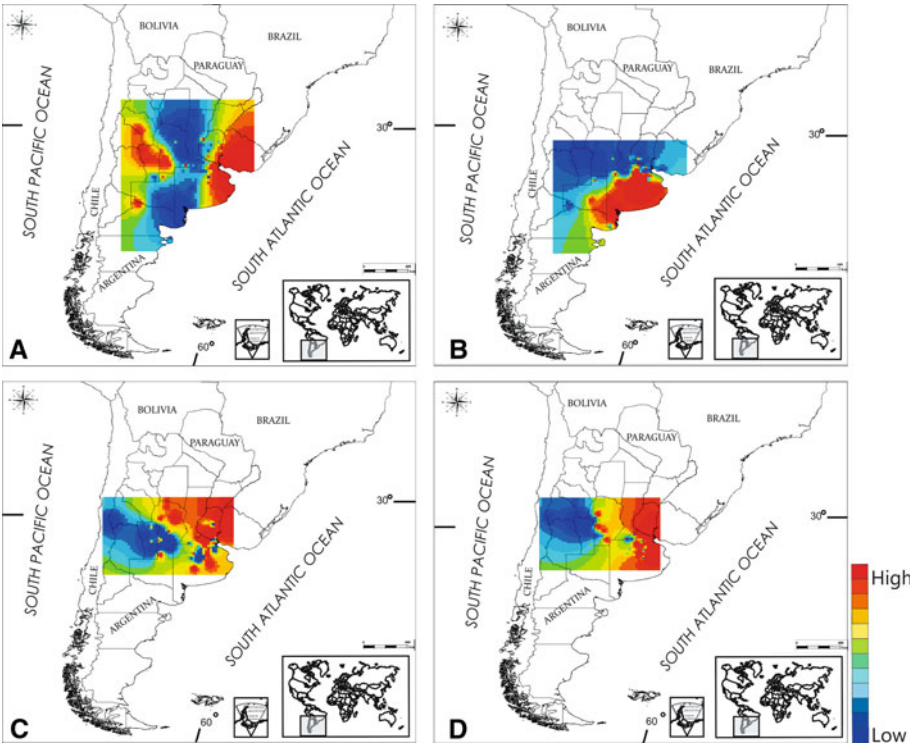

Fig. 5 Genetic heat maps generated from mtDNA (**a**, **c**) and AFLP markers (**b**, **d**) data, using AIS software for *N. leucoloma* (**a**, **b**) and *N. xanthographus* (**c**, **d**). Genetic topography varies from low (*blue*) to high (*red*). Interpolated genetic distances were arbitrarily assigned to twelve bins. Scales are not comparable because genetic distances generated from mitochondrial sequences versus allelic data are not equivalent

The asexual species *N. leucoloma* exhibited different topographic patterns for both genetic markers. The COI marker showed higher levels of diversity in Uruguay and eastern Buenos Aires province, near both margins of La Plata River, and also in central-western Argentina (including Córdoba province, Fig. 5a). The AFLP revealed high genetic distances throughout the Buenos Aires province (Fig. 5b). Despite the extremely low level of mitochondrial variability, the Aɪs results for COI showed areas of highest genetic distances overlapping those with the most suitable environmental conditions for the occurrence of *N. leucoloma* (Fig. 1a). Furthermore, both markers showed that the highest levels of genetic diversity were in northern Buenos Aires province, where environmental conditions would be optimal for the occurrence of this species.

In the bisexual *N. xanthographus*, the geographic distribution of genetic variation was quite similar for both AFLP and mitochondrial markers (Figs. 5c, d). The highest levels of genetic diversity were in Entre Rios province (bounded by the Paraná and Uruguay rivers), northeastern Buenos Aires province, and part of Córdoba province. These areas also overlapped with the most suitable areas for this species (Fig. 1b).

Discussion

Apomictic parthenogenesis, bisexuality and levels of genetic variability

Three different lines of evidence indicate that *N. leucoloma* reproduces by apomictic parthenogenesis; (1) complete absence of males in the comprehensive sampling performed between 2005 and 2009 in Argentina and neighboring countries; (2) presence of many individuals with identical multilocus genotypes (i.e. individual clones); and (3) significant levels of linkage disequilibrium between nuclear markers. On the other hand, all the populations of *N. xanthographus* have an even proportion of males and females, with all individuals being genetically different ($R = 1$), as expected for species with sexual reproduction. However, it shows a significant level of linkage disequilibrium among nuclear markers.

Animals that reproduce by apomictic parthenogenesis lack mechanisms of genetic recombination (Suomalainen and Saura 1973). In this case, it is expected the occurrence of linkage disequilibrium among nuclear loci and co-segregation of nuclear and mitochondrial markers. Although the statistical analyses reported herein show significant levels of linkage disequilibrium for both species, the Indexes of Association (I_A and $\bar{r}_d$) of *N. leucoloma* were more than five times higher than those of *N. xanthographus*. These high $I_{A,}$ and $\bar{r}_d$ values may indicate non-random association among most—if not all—nuclear loci (i.e. they cosegregate as if they were in the same linkage group), as expected for species with apomictic parthenogenesis. The significant values obtained for *N. xanthographus* is not surprising considering that several AFLP markers are expected to map on the same linkage group (i.e. on the same chromosome).

As expected, the level of mitochondrial diversity was much lower for the parthenogenetic *N. leucoloma* than for *N. xanthographus*. This result is nonetheless surprising, since values of haplotype diversity similar to those of the bisexual *N. xanthographus* have been obtained for other parthenogenetic species of the same genus such as *N. cervinus* (Rodriguero et al. 2010b). The extremely low level of mitochondrial diversity found in *N. leucoloma* is compatible with either the hypothesis of a recent severe population bottleneck or the hypothesis of a selective sweep.

The first hypothesis of reduction in population size can be associated with the origin of parthenogenesis and infection with *Wolbachia*, a symbiont recently found in several Naupactini with this type of reproduction (Rodriguero et al. 2010a). However, this demographic event is likely to affect all neutral loci of the genome in a similar way, which was not observed for *N. leucoloma*. In this species, the level of AFLP variation (estimated through the Shannon Index) was much higher than that of mitochondrial genome variation, but very similar to that of AFLP variation in the bisexual species. A reduction of genetic variability for asexual lineages compared to sexual ones is not always the rule (Atchley 1977). Parthenogenesis is known to enhance diversity because each clone would accumulate mutations and heterozygosity at a speed reflecting the age of the clone and its overall mutation rate. Such individuals become gradually heterozygous in regard to more and more gene pairs, since the elimination of recessive mutations is impossible (Suomalainen 1962). However, in several insects the mitochondrial mutation rate is higher than the nuclear mutation rate (Brown 1985; Mander et al. 2003). If this would be the case for *N. leucoloma* parthenogens, the rate of diversity recovery since the bottleneck event is expected to be higher -instead of lower- for these loci compared to nuclear ones.

Another possible scenario includes hybridization of clones with closely related species (Kearney 2005), or with individuals of the same species [as recently demonstrated for *N. cervinus* (Rodriguero, Lanteri and Confalonieri, manuscript in preparation)]. This could have differentially affected the variability of nuclear *vs* mitochondrial markers, because such phenomenon is expected to result from mating of clones with males who do not pass on their organelle genomes. If hybridization is presumed to have occurred many times since the putative bottleneck in *N. leucoloma*, then there would have been an increase in the variability of nuclear markers compared to that of mitochondrial markers. However, hybridization has not yet been demonstrated for this species.

Therefore, unless hybridization of clones with individuals from sexual populations is demonstrated, the second hypothesis including natural selection seems more appropriate because of the selective sweep of variability in the mitochondrial markers.

Geographic distribution of sexual and asexual lineages, colonization ability and dispersal routes

Typically, the (ancestral) sexual population and parthenogens occupy the center and marginal areas of the species' distribution, respectively. There is no ecological explanation for this pattern of geographic parthenogensis. Lynch (1984) proposed that parthenogens might fail to establish themselves in the presence of sexuals, as backcrosses with the ancestral sexual forms may hamper the independence of purely parthenogenetic forms. But parthenogens may have better colonizing capacities: they show higher intrinsic growth rate and do not pay the deleterious effects of population bottlenecks (e.g. inbreeding) that affect sexual populations. As a result, they may colonize areas where sexuals have difficulties in establishing a population (Pongratz et al. 2003).

The central and marginal areas of distribution of the studied species were inferred using two different approaches: an analysis of the ecological niche requirements and genetic variability distribution. The former indicated a quite similar probability distribution for the occurrence of both species in humid temperate climates. On this basis, the patterns of geographic distribution of these two flightless weevils with different reproductive modes are most likely to depend on the same climatic variables (mainly Mean Annual Temperature, Isothermality and Temperature Annual Range). Coincidentally, the ecological and genetic approaches pointed to northern Buenos Aires and the area near the confluence of

the Paraná and Uruguay rivers as the center of the geographic distribution for both species (Figs. 1, 3, 5). A fundamental assumption of phylogeographic analysis is that geographic areas with high genetic diversity correspond to the center of the species' geographic distribution (Avise 2000). Results indicate higher levels of genetic variability in areas with optimal environmental conditions for species occurrence, which is in line with the principle of central area location of phylogeography.

The pattern of geographic parthenogenesis could not be confirmed for *N. leucoloma*, as no males were found. However, the fact that they had been found in the locality of Paraná (Entre Rios province, Argentina, Table S1, Supplementary Material) 30 years ago, suggests the extinction of bisexual populations. Paraná is close to the area with the highest probability of species occurrence according to the ecological-niche modeling, thereby supporting the hypothesis of a central/marginal distribution pattern for sexual/asexual lineages, respectively. The areas adjacent to the Paraná River have suffered environmental degradation and parthenogenetic lineages, probably more tolerant to changes in ecological conditions, would have outcompeted sexual lineages leading to their extinction. Likewise, other phylogenetically close parthenogenetic species (e.g. *N. peregrinus* (Buchanan) and *N. minor* (Buchanan)) (Lanteri and Marvaldi 1995) could have been involved in ecological competition (Rodriguero et al. 2010b).

The higher colonizing ability of parthenogenetic species is partly due to the potential of a single colonist (parthenogenetic female) to start a new population at any developmental stage (Lanteri and Normark 1995). Moreover, parthenogens avoid the cost of producing males, and their populations can potentially increase twice as rapidly as species that have balanced sex ratios because of the production of all-female progeny (Mayr 1963). In this regard, the significant Fu's F_S value for nuclear markers indicates population growth. Both types of markers help explain the recent history of *N. leucoloma*: the lack of variation in mitochondrial markers would indicate the occurrence of a selective sweep, and the accumulation of rare mutations detected by AFLP markers suggests an increase in population size, most probably caused by higher reproductive success and geographic expansion. On the contrary, bisexual species showed no signs of exponential growth.

The fact that the putative central area (in terms of distribution, location and requirements of climatic conditions) of the asexual species was similar to that of the bisexual one, allowed us to test if the former was able to colonize more extreme environments and/or to extend its distribution to other countries with environments similar to those in the central distribution area. Both phenomena were indeed observed. First, *N. leucoloma* occupies larger areas of less suitable (marginal) environments than the bisexual species because it was able to extend its distribution to higher latitudes in Argentina (i.e. Trelew, Patagonia) (Figs. 1, 3 and 5). In these areas, which correspond to the southernmost limit of the species distribution, the mitochondrial and AFLP markers of parthenogens show lower levels of genetic distance (blue-green color surrounding Trelew in Figs. 5a, b, respectively), as estimated by the Ais program. Second, *N. leucoloma* was able to colonize countries neighboring its putative central area (e.g. Chile) and distant continents (e.g. USA, Australia, New Zealand and South Africa). The ecological niche model for the species predicts that these distant areas have moderately suitable environmental conditions (probability of species occurrence between 0.66 and 0.46). *N. xanthographus* is expected to survive in these areas but its presence has not been reported (Table S2, SupplementaryMaterial). The occurrence of L1 and L3 haplotypes of *N. leucoloma* in Australia and Argentina (Table 1) supports colonization through commercial trade in historical times. Conversely, *N. xanthographus*, which is a serious pest of *Vitis vinifera* and other fruit trees, has only colonized Chile (Table 2; Fig. 3b), probably due to a steady commercial trade of grapes for wine

production. Females of this species can store viable sperm in the spermatheca for at least 3 months (González 1983), a period possibly permitting the colonization of neighboring rather than distant areas.

Finally, it is worth to mention that *N. leucoloma* is much more widespread than its closest related species, *N. peregrinus* and *N. minor*, also capable of parthenogenetic reproduction (Lanteri and Normark 1995; Lanteri and Marvaldi 1995; Rodriguero et al. 2010a). The two latter "white-fringed weevils" are only found in their original distribution area (i.e. central Argentina, southern Brazil, Paraguay and Uruguay) and USA, where they show a restricted distribution range. In Argentina *N. leucoloma* was able to colonize Pampean prairies and Patagonian steppes (e.g. Chubut province), whereas the other two species are restricted to the margins of the Uruguay and Paraná rivers in the Mesopotamian region. This suggests that factors other than parthenogenetic reproduction would have contributed to the remarkable dispersal of *N. leucoloma,* such as the presence of a genotype adapted to survive under the cooler, more seasonal and arid conditions of the marginal habitats. In turn, this phenomenon would have led to a selective sweep reducing variability. However, *N. leucoloma* is distributed worldwide but in areas considered as moderately suitable for its occurrence and therefore the putative selected genotype does not agree with the definition of a high generalized genotype given by Lynch (1984).

Further studies on hybridization and ploidy level determination in members of the "*N. leucoloma* species group" (Lanteri and Marvaldi 1995) will contribute to elucidate the origin of parthenogenesis and to interpret the evolutionary history of the species. Moreover, the significant correlation between asexual/bisexual conditions and presence/absence of *Wolbachia* reported for several species of Naupactini such as the "white-fringed weevils" (Rodriguero et al. 2010a), justifies the inclusion of *Wolbachia* infection in their evolutionary scenario. Therefore, hybridization and polyploidy should be analyzed jointly with *Wolbachia* infection to better understand the origin and evolutionary significance of parthenogenesis in this weevil group.

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