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Speciation in the asexual realm: Is the parthenogenetic weevil *Naupactus cervinus* a complex of species in *statu nascendi?*

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ABSTRACT

Population genetic theory shows that asexual organisms may evolve into species, which behave as independent evolutionary units. As a result, they form genotypic clusters separated by deep gaps due to geographic isolation and/or divergent selection.

Identification of several genetically divergent groups of weevils embodied in the nominal species *Naupactus cervinus* deserves further study, in order to test if these lineages are evolving independently. In the present paper we tested if the parthenogenetic weevil *N. cervinus*, native to South America and broadly distributed throughout the world, contains more than one evolutionary unit. For this purpose, we applied three different approaches, a multilocus phylogenetic analysis, the GMYC approach and the K/θ method. We accomplished these analyses through a survey of mitochondrial (COI and COII genes) and nuclear (ITS1 sequence) genetic variation and morphometric analysis in a sample which included individuals from different locations within the native geographic range of *N. cervinus*. In addition, we compared the divergence accumulated in this species with that in another weevil of the same tribe (Naupactini) showing identical reproductive mode to see if similar levels of morphological variation matches similar levels of genetic divergence.

We report the presence of two independent evolutionary units living in sympatry in forest areas. The incongruence between mitochondrial and nuclear datasets analyzed herein reflects incomplete lineage sorting of the nuclear marker and different evolutionary rates between genomes.

Ecological divergence driven by natural selection (sympatry) or secondary contact after geographic isolation (allopatry) might explain the deep gaps in mitochondrial phylogenies. Instead, *Wolbachia* infection was ruled out as a causal factor for such differentiation. We conclude that *N. cervinus* is probably a species complex with at least two well differentiated lineages that would represent a cluster of species in *statu nascendi*.

1. Introduction

An "evolutionary unit" is defined as a group of related organisms enjoined by evolutionary processes going on within it, and separated from other groups because of the absence of shared evolutionary processes (Hey, 2001). In trying to match species taxa devised by the mind of the investigators to evolutionary units, confusion can arise because both things are ontologically different. In fact, real evolutionary units might not be morphologically different, but still evolve in an independent way and exist regardless of our recognition of them. Although these entities may be somewhat discrete in time and space, they may also overlap or be nested within one another, while taxa are discrete categories. During its existence, a species is subject to continuous change, but yet remains the same (Rieppel, 2010). This "sameness" could be defined in terms of phenotypic cohesion, i.e. the factors that preserve morphological, genetic and ecological clusters.

Sex has been invoked as the main force behind speciation, with interbreeding maintaining the cohesion within populations and reproductive isolation disrupting it. It creates reproductive boundaries between groups of individuals, leading to discrete entities in nature. However, other factors than sexual reproduction could explain the origin of biological diversity, since many examples of asexual species have been reported (e.g. Fontaneto et al., 2007a,b, 2008, 2012; Johnson et al., 2011; Maraun et al., 2003, 2004; Mark Welch and Meselson, 2001; Barraclough et al., 2003; Mark Welch et al., 2004; Martens et al., 2005; Schön et al., 2009; Schön et al., 2010; Birky et al., 2010; Schwander et al., 2011).

For instance, Fontaneto et al. (2007b) proved the diversification of the genus *Rotaria* (Rotifera, Bdelloidae), which has been

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reproducing without sex perhaps for the last 100 million years. They showed that sex is not a *condition sine qua non* for speciation. Instead, ecological divergence by natural selection was the key element for this clade to diversify, through adaptation of their jaws to different resources à la Darwin's finches. Thus, ecological factors (e.g. Fontaneto et al., 2007b) and/or geographical isolation (e.g. Heethoff et al., 2007) are important forces behind the origin of clusters of asexual individuals.

Templeton understood that sex is not the only factor driving diversification in this planet, and gave a twist to the way of defining species when he postulated the Cohesion Species Concept (CSC) (Templeton, 1989). This concept defines evolutionary groups in terms of the mechanisms that yield cohesion (demographic and genetic exchangeability) rather than the manifestation of cohesion over evolutionary times.

From Templeton's point of view, demographic exchangeability becomes more important for asexual organisms because it may limit the spread of alleles by natural selection and genetic drift in the absence of gene flow, while genetic exchangeability would be of primary importance for sexual organisms. With no interbreeding to maintain cohesion, asexual taxa might not diversify into distinct species, but they could diverge if cohesion and divergence were controlled by factors other than interbreeding, like niche specialization.

Approximately 1% of living organisms are asexual (Suomalainen et al., 1987; Short and Balaban, 1994). Among them, insects are prominent, especially weevils of the subfamilies Scolytinae (bark beetles), Listroderinae, and Entiminae (broad-nose weevils) (Coleoptera, Curculionidae) (Suomalainen, 1962; Suomalainen et al., 1976; Smith and Virkki, 1978; Lokki and Saura, 1980; Lanteri and Normark, 1995; Normark, 2003).

The delimitation of species is particularly difficult in parthenogenetic weevils with broad geographic ranges showing slightly different morphotypes partially congruent with divergent molecular lineages like *Aramigus tessellatus*, which belongs to the tribe Naupactini (Lanteri and Díaz, 1994; Normark and Lanteri, 1998). The "Fuller's rose weevil" *Naupactus cervinus* (Entiminae, Naupactini), native to South America and distributed throughout the world, where it behaves as an invasive species and has a pest status (Rodriguero et al., 2010b), is probably another example of this kind.

Molecular analyses of mitochondrial genes have revealed high levels of genetic variation in most Naupactini native to South America (e.g. Sequeira et al., 2008a,b; Guzmán et al., 2012), especially in both *A. tessellatus* (Normark and Lanteri, 1996, 1998) and *N. cervinus* (Rodriguero et al., 2010b). In these parthenogenetic species mitochondrial diversity is higher than both morphological and nuclear variation, and is also higher than the mitochondrial diversity recorded for some sexual species (e.g. *N. xanthographus*, Guzmán et al., 2012). This information raised questions about the possibility of incipient speciation processes.

The Paranaense forest, a humid subtropical forest covering southern Brazil, eastern Paraguay and north-eastern Argentina and currently reduced to 6% of its original coverage [Laclau, 1994]), is considered the probable geographic area of origin of *N. cervinus* (Lanteri, 1993; Rodriguero, 2009). There, both sexual (Lanteri, 1993) and parthenogenetic forms (Rodriguero et al., 2010b) have been found, although male records were absent since the 1950'. Parthenogens reproduces by apomictic parthenogenesis (Buchanan, 1939; Rodriguero et al., 2010b), and the consequent coevolution between mitochondrial and nuclear genomes was demonstrated to be ancient (Rodriguero et al., 2010b). Moreover, *N. cervinus* is infected with a single strain of the sex ratio distorter bacterium *Wolbachia* (Rodriguero et al., 2010a,b), as many other parthenogenetic Naupactini weevils, while sexual species of this tribe are not (Rodriguero et al., 2010a).

Through a comprehensive sampling in the native area and some places of recent introduction accomplished to study the genetic structure of *N. cervinus*, we identified two divergent lineages within this weevil on the basis of mitochondrial and nuclear loci, called "forest" and "grassland" clades after their geographic location (see Fig. 1 in Rodriguero et al., 2010b). In the present work we report a much more divergent lineage of *N. cervinus* that was identified in Argentina and Brazil while surveying for additional genetic variation across the spatial distribution of this insect. If this highly divergent lineage is an incipient species driven by natural selection, geographic isolation or the result of a strong population subdivision driven by the invasion of different *Wolbachia* strains (see Hurst and Jiggins, 2005 and Ballard and Rand, 2005 for a detailed explanation of this phenomenon) is currently unknown.

The aim of the present report is to test the hypothesis that *N. cervinus* encompasses more than one evolutionary unit in South America, i.e. if this highly divergent lineage, and also the "forest" and the "grassland" clades of the nominal species *N. cervinus* can be considered as independent evolving lineages on the basis of morphology, mitochondrial and nuclear markers.

We discussed our results on the context of the possible causes for the achieved divergence of *N. cervinus* lineages.

2. Materials and methods

2.1. Sampling and specimens examined

Thirty-six individuals of *N. cervinus* collected from 13 locations in Argentina and Brazil (Fig. 1 and Table 1) were screened for genetic variation in three different gene regions. All samples were composed of females. Ten individuals of *Naupactus dissimulator*, the sister species of *N. cervinus* (Scataglini et al., 2005), and one individual of *Naupactus ambiguus* were used as outgroup. These specimens were collected from several locations in northeastern Argentina and southern Brazil (Fig. 1 and Table 1). Adult weevils were sampled from orange trees, native and introduced plants using a beating sheet (55 cm × 55 cm) and stored in 100% ethanol at 4 °C for molecular analysis.

2.2. PCR Assay and sequencing

Total genomic DNA was extracted following the protocol of Reiss et al. (1995). The negative controls were samples lacking DNA template.

Two mitochondrial genes and one nuclear sequence were used for this analysis. A segment of ca. 700 bp of the Cytochrome Oxidase I (COI) was amplified using the specific primers 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′) (Normark, 1994). A segment of ca. 650 bp of the transfer RNA for leucine (ARNt leu) and Cytochrome Oxidase II (COII) was amplified using the specific primers J3038 (5′-TAA TAT GGC AGA TTA GTG CAT TGG A-3′) and N3668 (5′-GCT CCA CAA ATT TCT GAG CA-3′) (Sequeira et al., 2008b). Additionally, a nuclear region of ca. 1100 bp that include the region 3′ of the 18S rDNA gene, plus the complete ITS1 region (Internal Transcribed Spacer 1) and the 5′ region of the 5.8S rDNA gene was amplified using the primers rDNA2 (5′-TTG ATT ACG TCC CTG CCC TTT-3′) (Vrain et al., 1992) and rDNA1.5.8S (5′-ACG AGC CGA GTG ATC CAC CG-3′) (Cherry et al., 1997).

Wolbachia infection was surveyed through amplification and sequencing of four MLST genes (coxA, fbpA, gatB and hcpA) and the wsp gene using the primers designed by Baldo et al. (2006) and Braig et al. (1998), respectively. Negative controls were performed as previously described (Rodriguero et al., 2010a).

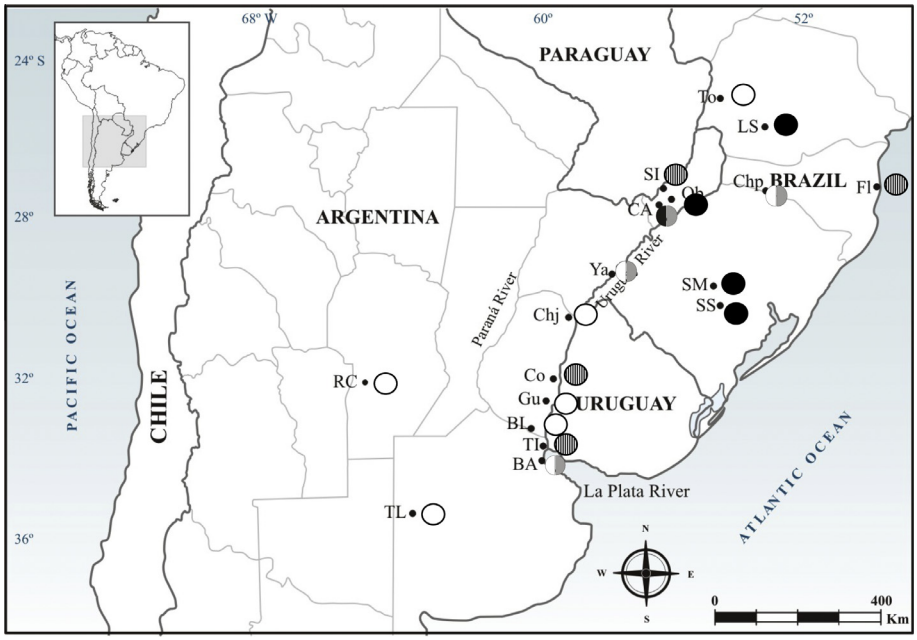

Fig. 1. Sampling locations of *Naupactus cervinus* and *Naupactus dissimulator*. Countries included in this study are indicated in capital letter. Acronyms for locations are as follows: BA = Buenos Aires City; BL = Brazo Largo; CA = Cerro Azul; Chj = Chajarí; Chp = Chapecó; Co = Colón; Fl = Florianópolis; GC = Godoy Cruz; Gu = Gualeguaychú; LS = Laranjeiras do Sul; Ob = Oberá; RC = Río Cuarto; SI = San Ignacio; SM = Santa María; SS = Sao Sepe; TI = Talavera Island; TL = Tres Lomas; To = Toledo; Ya = Yapeyú. After mitochondrial datasets (the only ones that reached reciprocal monophyly), black circles correspond to clade Ia ("forest") weevils, empty circles to clade Ib ("grassland") weevils, grey circles to clade II individuals and striped circles to *N. dissimulator* individuals.

Amplification was carried out in a 50 μl volume reaction with 50–100 ng of DNA used as template, 0.5 μM of each primer, 0.1 mM of each dNTP, 3.0 mM $MgCl_2$, 1.0 unit of Taq polymerase and 1X reaction buffer (Invitrogen). The reactions were performed in a GeneAmp® PCR System 2700 thermal cycler (Applied Biosystems) under the conditions described by Scataglini et al. (2005) for COI and COII fragments, Szalanski and Owen (2003) for ITS1 fragments, Baldo et al. (2006) for MLST genes and Braig et al. (1998) for the wsp gene.

Double-stranded PCR products were separated by electrophoresis on a 1% agarose gel with TAE buffer containing 0.5 mg/ml of ethidium bromide. The bands were excised from the gel and the DNA was purified with a QIAquick Gel Extraction Kit (Qiagen Inc.). DNA was sequenced using a 3130-XL Automatic Sequencer (Applied Biosystems).

2.3. Data analysis

In order to test the independent evolution of *N. cervinus* lineages, first we tested different hypothesis of species delimitation on the basis of "multilocus" (*Beast) and "single locus" (GMYC) coalescent approaches. Second, we studied whether the degree of molecular divergence of *N. cervinus* lineages is comparable to that expected for asexual species (K/θ method, Birky et al., 2010). Third, we compared the degree of molecular divergence achieved by *N. cervinus* lineages to that observed between asexual lineages of a closely related weevil which is in fact a species complex (*Aramigus tessellatus* [Lanteri et al., 1987, 1989; Lanteri and Díaz, 1994; Normark, 1996]). Finally, we typified the *Wolbachia* strains of these groups of weevils to assess if the divergence accumulated could be explained as a consequence of indirect selection acting on different bacterial strains.

2.3.1. Phylogenetic analysis

Alignment was done using CLUSTAL W (Thompson et al., 1994) and adjusted by eye. To check for the presence of pseudogenes, mitochondrial sequences were translated into aminoacid sequences using the invertebrate mitochondrial code with the program MEGA v. 5 (Tamura et al., 2011).

Our complete dataset included 724 (COI), 647 (COII) and 977 (ITS1) aligned nucleotide positions. Phylogenetic analyses were performed on each dataset separately by maximum parsimony using individuals as terminal units.

Maximum parsimony heuristic search consisted of "TBR branch swapping" applied to a series of 1000 random addition sequences, retaining 10 cladograms per replicate using NONA v. 2.0 (Goloboff, 1999), executed through the interface WinClada v. 1.00.08 (Nixon, 2002). All characters were regarded as unordered and unweighted. For the nuclear dataset, gaps were treated as fifth state. Clade stability was assessed by 10,000 parsimony bootstrap replications (Felsenstein, 1985).

Obtaining same results when applying different methods can be considered as a sign of a strong support for a given analysis. Thus, we also applied Bayesian phylogenetic inference to our datasets through the "metropolis-coupled Markov chain Monte Carlo" (MC3) algorithm implemented in MRBAYES v. 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Two independent analyses were run with a random starting tree over 1,000,000 generations, with a sample frequency of 100. The tree space was explored using four chains: one cold and three incrementally heated chains, with temperature (T) set to 0.20. The first 2500 trees were discarded as burn-in.

We applied several tests to assess stationarity of the cold Markov chain for all MRBAYES analyses implemented in TRACER (Rambaut and Drummond, 2007), and the online convergence program ARE WE THERE YET? (AWTY; Wilgenbusch et al., 2004; Nylander et al., 2008), in addition to the standard deviation of the split frequencies. All posterior samples of a run prior to the burn-in point were discarded. Remaining trees were taken into account to obtain a 50% majority-rule consensus tree and mean branch length estimates. The frequency of all bipartitions was estimated to assess the support of each node (Huelsenbeck and Ronquist, 2001).

Table 1
Multilocus genotype for each individual collected, with its respective clade and subclade (see text).

Species	Location	Acronym	N	Sample ID	COI	COII	ITS1	Clade	GenBank acc. no. COI	GenBank acc. no. COII	GenBank acc. no. ITS1
N. ambiguus	BL, ER, Entre Ríos	BL	1	NaBL1	A	1	I	–	AY790879	JX440469	JX440498
N. cervinus	AR, ER, Brazo Largo	BL	2	NcBL1-NcBL2	M	7	VII	Ib	GQ406839.1[a]	JX440486	GQ406823.1[a]
	AR, MI, Cerro Azul	CA	2	NcCA1	Q	3	IV	Ia	GQ406842.1[a]	JX440488	GQ406821.1[a]
				NcCA2	S	11	IX	II	JX440490	JX440484	JX440499
				NcCA3	T	14	X	II	KC614562	KC614563	KC614561
				NcCA4	T	14	X	II	KC614562	KC614563	KC614561
	AR, ER, Chajarí	Chj	3	NcChj1-NcChj2-NcChj3	F	6	VI	Ib	GQ406832.1[a]	JX440478	GQ406824.1[a]
	AR, ER, Gualeguaychú	Gu	4	NcGu1-NcGu2	F	7	VI	Ib	GQ406832.1[a]	JX440486	GQ406824.1[a]
				NcCGu3	M	6	VI	Ib	GQ406839.1	JX440478	GQ406824.1[a]
				NcGu4	M	6	VII	Ib	GQ406839.1	JX440478	GQ406823.1[a]
	AR, MI, Oberá	Ob	2	NcOb1	Q	2	III	Ia	GQ406842.1	JX440480	GQ406820.1[a]
				NcOb2	R	3	III	Ia	GQ406843.1	JX440488	GQ406820.1[a]
	AR, CB, Río Cuarto	RC	2	NcRC1	A	4	VII	Ib	GQ406827.1	JX440481.1	GQ406823.1[a]
				NcRC2	B	5	VII	Ib	GQ406828.1	JX440482.1	GQ406823.1[a]
	AR, BA, Tres Lomas	TL	3	NcTL1-NcTL2-NcTL3	B	6	VII	Ib	GQ406828.1	JX440478	GQ406823.1[a]
	AR, CO, Yapeyú	Ya	2	NcYa1-NcYa2	C	8	VIII	Ib	GQ406829.1	JX440483	GQ406825.1[a]
	BR, SC, Chapecó	Chp	8	NcChp1	E	10	–	Ib	GU727685.1	JX440487	–
				NcChp2-NcChp3-NcChp4-NcChp5-NcChp6-NcChp7-NcChp8-	U	12	XI	II	GU727685	JX440485	JX440500
	BR, PR, Laranjeiras do Sul	LS	2	NcLS1-NcLS2	R	1	I	Ia	GQ406843.1	JX440479	GQ406818.1[a]
	BR, RS, Santa María	SM	1	NcSM1	Q	3	IV	Ia	GQ406842.1	JX440488	GQ406821.1[a]
	BR, RS, São Sepé	SS	2	NcSS1- NcSS2	P	3	IV	Ia	GQ406841.1	JX440488	GQ406821.1[a]
	BR, PR, Toledo	To	1	NcTo1	C	9	II	Ib	GQ406829.1	JX440489	GQ406819.1[a]
N. dissimulator	AR, BA, Buenos Aires City	BA	3	NdBA1-NdBA2	C	2	III	–	JX440493	JX440471	JX440503
					D	3	II		JX440494	JX440472	JX440504
	AR, CO, Yapeyú	Ya	1	NdYa1	E	4	I	–	JX440495	JX440473	GQ406826
	AR, ER, Colón	Co	2	NdCo1-NdCo2	B	8	I	–	JX440492	JX440477	GQ406826
					F	5	I		JX440496	JX440474	GQ406826
	AR, ER, Talavera Island	TI	2	NdTI1-NdTI2	A	1	I	–	GQ406844	JX440470	GQ406826
	AR, MI, San Ignacio	SI	1	NdSI1	G	6	V	–	JX440497	JX440475	JX440502
	BR, SC, Florianópolis	Fl	1	NdFl1	H	7	IV	–	JX440491	JX440476	JX440501

AR: Argentina; BR: Brazil; BA: Buenos Aires; CB: Córdoba; CO: Corrientes; ER: Entre Ríos; MI: Misiones; PR: Paraná; RS: Rio Grande do Sul; SC: Santa Catarina.
[a] Extracted from Rodriguero et al. (2010b).

2.3.2. Species delimitation

*2.3.2.1. Species delimitation I: multilocus coalescent-based approach using *Beast.* We applied the Bayesian method *BEAST (Heled and Drummond, 2008) implemented in the computer program BEAST v. 1.6.1 (Drummond and Rambaut, 2007) to estimate the species tree from multilocus data. This method emphasizes incomplete lineage sorting as the main source of inconsistency between gene trees and species trees and assumes free recombination between genes and no recombination within genes. Species tree and all gene trees are coestimated in one Bayesian Markov Chain Monte Carlo analysis, as well as divergence times and population sizes.

The optimal substitution models were selected using MrModeltest software v. 2.2 (Nylander, 2004), on the basis of the Akaike information criterion, as suggested by Posada and Buckley (2004). The GTR + I + G model was selected as the best fit model of nucleotide substitution for the COI and COII sequences, and the HKY + G for the ITS1 fragment.

Terminal taxa were defined a priori representing every possibly independently evolving lineage. Accordingly, we proposed several hypotheses of species delimitation (H1–H4), as follows: H1: *N. cervinus* is in fact a complex of two species, one including forest and grassland clades defined for mitochondrial sequences by Rodriguero et al. (2010a), another one including the highly divergent individuals sampled at Cerro Azul and Chapecó; H2: *N. cervinus* comprises three species, one including the forest clade, another one including the grassland clade and the last one including the the highly divergent individuals sampled at Cerro Azul and Chapecó; H3: this hypothesis recognizes two species within *N. cervinus*, one comprising forest and the highly divergent individuals sampled at Cerro Azul and Chapecó, and another one including only grassland individuals; H4: under this hypothesis *N. cervinus* embodies two species, one including grassland and the highly divergent individuals sampled at Cerro Azul and Chapecó, and another one including forest weevils.

Chains were run for 500,000,000 generations, sampling every 50,000 with a Yule Process tree prior. A strict clock model was assumed, based on the results obtained with the programs Dnaml and Dnamlk (Felsenstein and Churchill, 1996) from the PHYLIP package v.3.69 (Felsenstein, 2005).

Convergence of the chains was assessed in TRACER (Rambaut and Drummond, 2007), and the first 5000 trees sampled before reaching stationarity were discarded as burn-in. Plausible trees were summarized in a single species tree that best represents the posterior distribution using TreeAnnotator (Drummond and

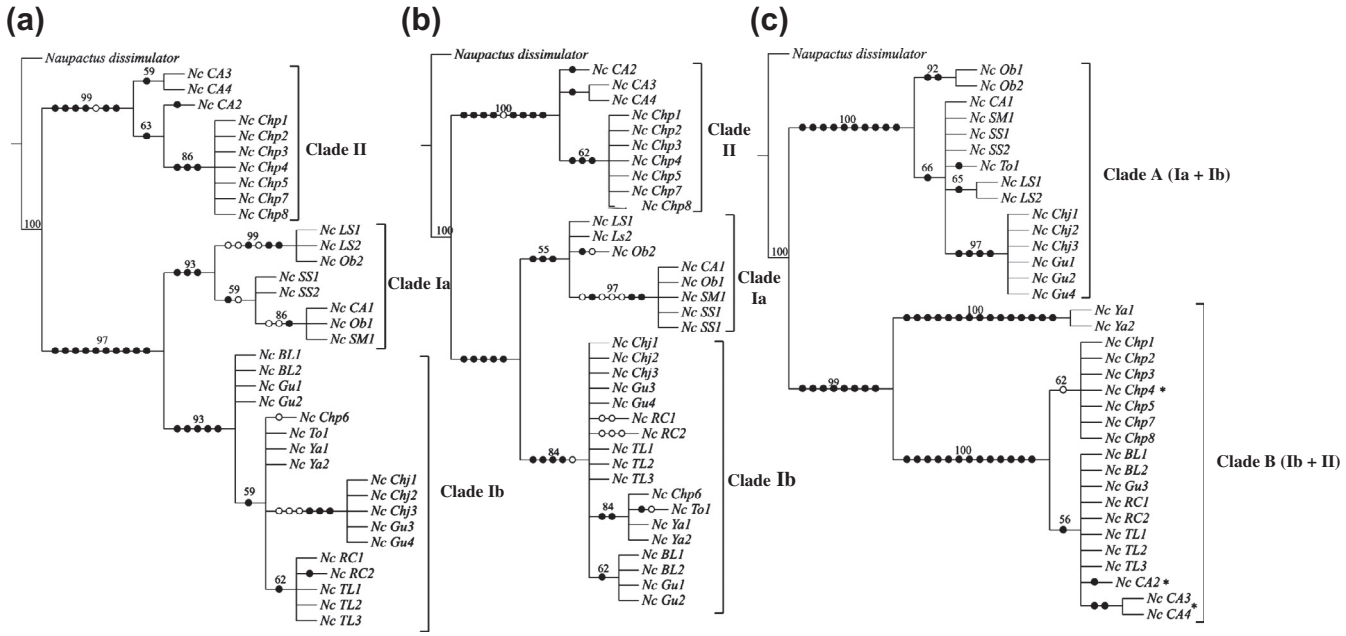

Fig. 2. (a) Most parsimoniuos tree for COI haplotypes of *N. cervinus*, with *N. dissimulator* as outgroup. Numbers above the branches are bootstrap values equal or higher than 50%. Black circles are homologous character states and white circles are homoplastic ones. See text for explanation of clades Ia, Ib and II. (b) Strict consensus of two most parsimoniuos trees for COII haplotypes of *N. cervinus*, with *N. dissimulator* as outgroup. Numbers above the branches are bootstrap values equal or above 50%. Black circles are homologous character states and white circles are homoplastic ones. See text for explanation of clades Ia, Ib and II. (c) Most parsimoniuos trees of ITS1 alleles of *N. cervinus*, with *N. dissimulator* as outgroup. Numbers above the branches are bootstrap values equal or higher than 50%. Black circles are homologous character states, and white circles are homoplastic ones. See text for explanation of clades A and B. Asterisks indicate individuals of the mitochondrial clade II.

Rambaut, 2007). We used the tree with maximum clade credibility as the target tree and kept median node heights, with the posterior probability limit set to 0.5. It was visualized with Figtree v.1.3.1 (Rambaut, 2009). A posterior probability value $\geqslant$0.95 was considered as strong support for a speciation event (Leaché and Fujita, 2010). To better visualize the species trees and divergence times (node heights) and to give further support for the preferred hypothesis (see Husemann et al., 2013) we used DENSITREE v.1.37 (Bouckaert, 2010).

We estimated the divergence time between the most highly divergent clades of *N. cervinus* using rates of 0.006 and 0.014 substitutions/site/Mya for the COI gene as the lower and upper limits of divergence. These rates correspond to the standard pairwise distances of 1.2% and 2.8% per Mya estimated for insects by Caccone and Sbordoni (2001) and Buckley et al. (2001) respectively. COII and ITS1 rates were calculated as a fraction of the COI rate.

Primetv (Sennblad et al., 2007) was used to reconcile gene trees which were incongruent with the species trees by accessing the web service at http://prime.sbc.su.se/cgi-bin/primetv.cgi.

2.3.2.2. Species delimitation II: single locus coalescent-based approach using the GMYC method. We applied the Generalized Mixed Yule Coalescent approach (GMYC) (Pons et al., 2006) to delimit mtDNA clusters on the COI + COII tree and identify putative independently evolving entities. We combined both datasets because mitochondrial genes are in linkage disequilibrium. This method optimizes a threshold age that corresponds to the shift from coalescent to species diversification (Yule) branching processes and calculates the number of the resulting independent entities. The likelihood of the null model that all samples belong to a single species is compared to that of the alternative model that separate coalescent groups nested within the species tree through a likelihood ratio test. Confidence limits are provided which correspond to threshold values ±2 log*L* units around the ML estimate. The point of highest likelihood of this mixed model (threshold) can be interpreted as the species boundary (Pons et al., 2006) and is the most likely position in which a shift between the two processes has occurred.

For GMYC we obtained an ultrametric tree including only the non-identical haplotypes of concatenated COI + COII sequences under a strict molecular clock using BEAST v. 1.6.1 (Drummond and Rambaut, 2007). Tree prior was set to coalescent constant size. A Markov chain Monte Carlo run with 100 million generations and sampling of every 1000 generations have been performed. Burn-in was determined with Tracer 1.5.0 (Rambaut and Drummond, 2007). The maximum clade credibility tree was found using TreeAnnotator (Drummond and Rambaut, 2007) with all options set to default and imported into the statistics software R 2.15.1 (http://cran.r-project.org). We optimized both single and multiple-threshold GMYC models to our dataset. The last allows for different switching times from speciation to coalescence adjusted to different evolutionary lineages across the tree (Monaghan et al., 2009). We used the script available within the SPLITS package for R (http://r-forge.r-project.org/projects/splits/).

We carried out this test including: (i) the whole set of *N. cervinus* sequences; (ii) *N. cervinus* + *N. dissimulator* sequences (to test the performance of the test when assayed with two good species).

2.3.2.3. Species delimitation III: single locus coalescent-based approach using the K/θ method. The *K*/θ method, formerly called the "four times rule" ("4X rule") (Birky et al., 2005, 2010; Birky and Barraclough, 2009; Schön et al., 2012), was applied to check if the observed *N. cervinus* clades could be considered as independently evolving lineages. The *K*/θ method is an operational criterion designed for asexual organisms to identify species based on the idea that a (monophyletic) lineage achieves species status when the molecular differences with its sister lineage are too great to be attributed to random drift alone, i.e. when the mean sequence divergence between specimens belonging to these lineages is greater than the depth of clades formed by random drift with a 95% confidence (Birky et al., 2005). According to this rule, clades

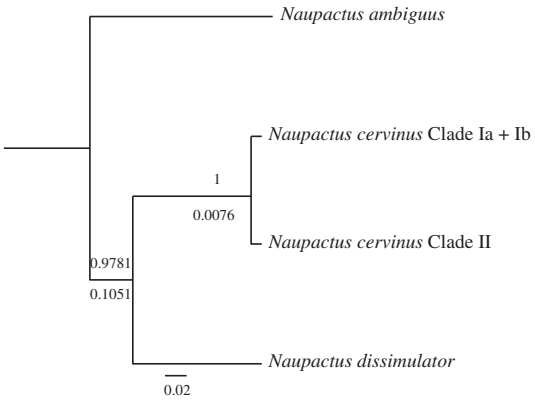

Fig. 3a. Consensus species tree based on the co-estimation of three gene trees for *N. cervinus* and *N. dissimulator* under H1 (see text for details). Two mitochondrial (COI and COII), and one nuclear (ITS1) loci were included in the analysis using *Beast. Posterior probabilities (=speciation probability) of each node are above, and accumulated divergences (used to estimate divergence dates) below the branches. *N. ambiguus* was used as outgroup.

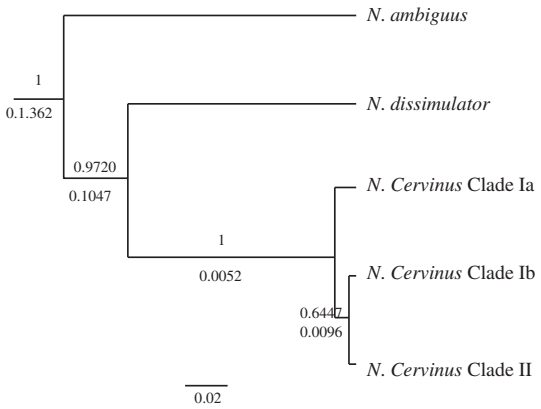

Fig. 3c. Consensus species tree based on the co-estimation of three gene trees for *N. cervinus* and *N. dissimulator* under H2 (see text for details). Two mitochondrial (COI and COII), and one nuclear (ITS1) loci were included in the analysis using *Beast. Posterior probabilities (=speciation probability) of each node are above, and accumulated divergences (used to estimate divergence dates) below the branches *N. ambiguus* was used as outgroup.

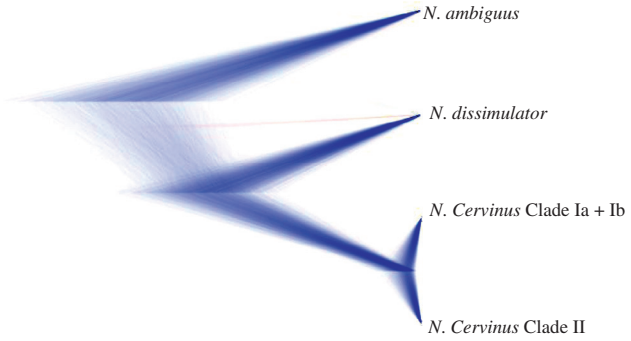

Fig. 3b. Densitree analysis under H1 (see text for details) displaying all trees of the Markov chain Monte Carlo with a burn-in of 1000 trees. Higher levels of uncertainty are represented by lower densities.

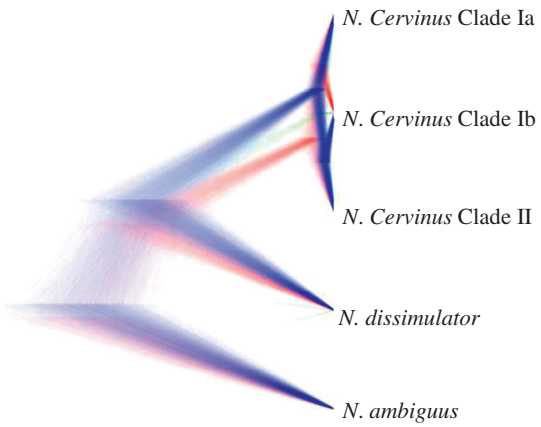

Fig. 3d. Densitree analysis under H2 (see text for details) displaying all trees of the Markov chain Monte Carlo with a burn-in of 1000 trees. Higher levels of uncertainty are represented by lower densities.

are truly independent lineages worthy of species status when sequence differences between individuals in different clades (interclade divergence) exceed four times the mean pairwise sequence differences within the clades (intraclade divergence).

We estimated interclade distances for COI + COII dataset between *N. cervinus* clades using uncorrected *p*-distances (K) with Mega v. 5 (Tamura et al., 2011). Intraclade divergences were quantified through the nucleotide diversity π (Nei and Li, 1979) using DnaSP v.5.10.00 (Librado and Rozas, 2009).

Then, we estimated $\theta \approx 2N_e\mu$ by $\pi/(1-4\pi/3)$ (Nei, 1987). Finally, we tested each pair of clades to see if $K/\theta > 4$. When clades had different values of θ, we used the larger one (Birky et al., 2010).

Additionally, we followed Schön et al. (2012) and estimated coalescence time (τ) through K/θ to be used together with the sample sizes of a clade (n_1) and its sister clade (n_2) to find the probability that the two clades are samples from populations that have been evolving independently long enough to become reciprocally monophyletic (see Rosenberg, 2003) entering these values in a lookup table kindly provided by Noah Rosenberg. Indeed, a $K/\theta > 4$ is a mean reference value for this test, but lower values of this ratio can also indicate a significant ($\geqslant 0.95$) pattern of reciprocal monophyly, depending on the sample size of both sister clades.

We carried out this test hypothesizing Clade II as an independent evolutionary unit of (i) Clade Ia (Forest clade); (ii) Clade Ib (Grassland clade); (iii) The whole nominal species *N. cervinus* (for-

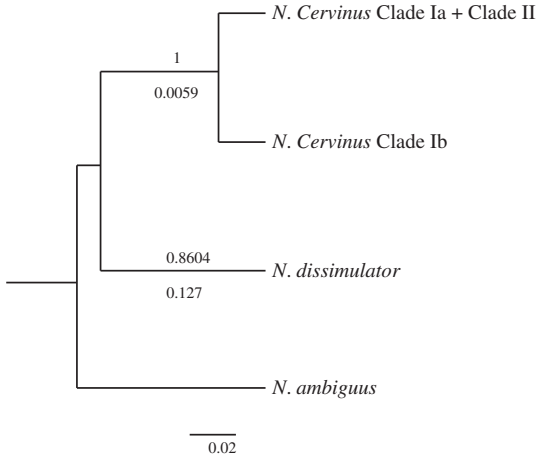

Fig. 3e. Consensus species tree based on the co-estimation of three gene trees for *N. cervinus* and *N. dissimulator* under H3 (see text for details). Two mitochondrial (COI and COII), and one nuclear (ITS1) loci were included in this analysis using *Beast. Posterior probability (=speciation probability) of each node are above, and accumulated divergences (used to estimate divergence dates) below the branches. *N. ambiguus* was used as outgroup.

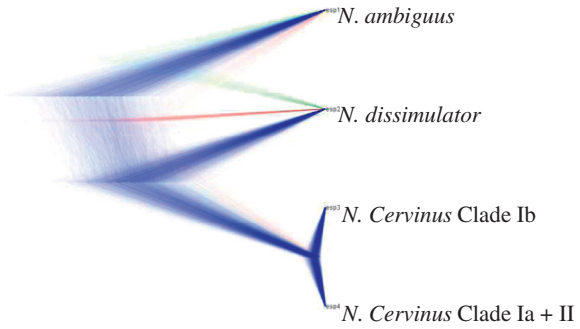

Fig. 3f. Densitree analysis under H3 (see text for details) displaying all trees of the Markov chain Monte Carlo with a burn-in of 1000 trees. Higher levels of uncertainty are represented by lower densities.

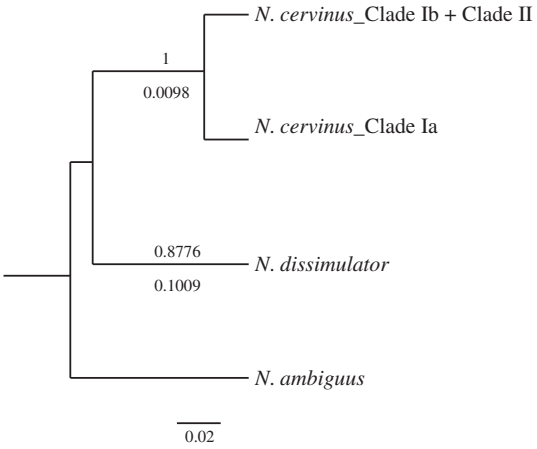

Fig. 3g. Consensus species tree based on the co-estimation of three gene trees for *N. cervinus* and *N. dissimulator* under H4 (see text for details). Two mitochondrial (COI and COII), and one nuclear (ITS1) loci were included in this analysis using *Beast. Posterior probability (=speciation probability) of each node are above, and accumulated divergences (used to estimate divergence dates) below the branches. *N. ambiguus* was used as outgroup.

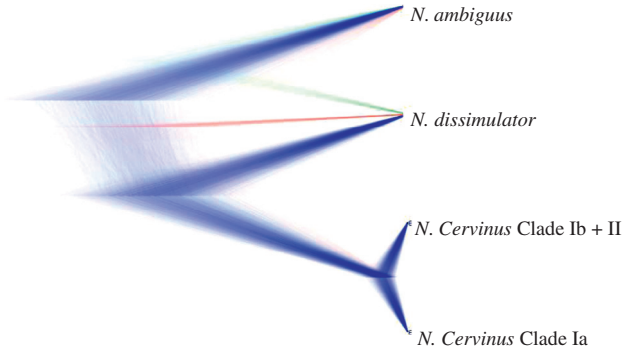

Fig. 3h. Densitree analysis under H4 (see text for details) displaying all trees of the Markov chain Monte Carlo chain with a burn-in of 1000 trees. Higher levels of uncertainty are represented by lower densities.

est + grassland clades). We also applied this analysis to see if both forest and grassland clades are independently evolving lineages.

We made these calculations only for COI + COII sequences, as they seem to have reached reciprocal monophyly (Birky, 2009; Birky et al., 2010).

2.3.3. Comparison of molecular divergence with other asexual Naupactini species

Molecular divergence accumulated between lineages of *N. cervinus* was also compared with that accumulated between *Aramigus uruguayensis* and the species complex *A. tessellatus* (Normark, 1996; Normark and Lanteri, 1996). These species also belong to Naupactini and show geographic parthenogenesis, huge intraspecific variation and highly divergent lineages.

We retrieved the COI sequences deposited by Normark (1996) and Normark and Lanteri (1996) in GenBank (Accession numbers U25287-U25534 and U25216-U25511) and estimated a phylogeny using Beast v. 1.6.1 (Drummond and Rambaut, 2007) to date the node between the *A. tessellatus* species complex and *A. uruguayensis*. We used the same priors, settings and evolutionary rates as before, and ran the program for 100,000,000 generations, sampling trees every 10,000 generations. Convergence was assessed as previously described, and the tree was visualized with FigTree v. 1.3.1 (Rambaut, 2009) after summarizing the information using TreeAnnotator (Drummond and Rambaut, 2007).

2.4. Morphological analysis

Specimens were analyzed for six morphometric rates, in order to asses if their molecular divergence is congruent with the morphological divergence. The rates are as follows: (1) maximum width of frons between the anterior margin of eyes, over minimum width of rostrum across the apex ($wF/w-R$); (2) maximum length of rostrum over minimum width of rostrum ($l+R/w-R$); (3) maximum length of funicular article 2 over maximum length of funicular article 1 (lant2/lant1); (4) maximum width of pronotum over maximum length of pronotum ($w+P/l+P$); (5) maximum length of elytra along midline over maximum width of elytra across disc; ($l+E/w+E$); and (6) maximum length of elytra over maximum length of pronotum ($l+E/l+P$). The media of each rate was calculated for six specimens of each of the three main groups recovered based on the molecular analyses. We also analyzed characters of the vestiture, especially color pattern of the elytral scales; and length, density and slope of the elytral setae; and characters of the female genitalia such as the length of the spermathecal duct.

3. Results

3.1. Estimation of genetic variability

COI dataset was 725 bp in length and had 33 segregating sites, of which only two were singletons. For *N. cervinus* we identified 10 out of the 17 haplotypes reported by Rodriguero et al. (2010b) (A–C, F, E, M, P–R; Table 1) and three new haplotypes from Cerro Azul and Chapecó (arbitrarily named S, T and U) (Fig. 1, Table 1). For *N. dissimulator* we report eight different haplotypes from nine sampling sites (arbitrarily named A–G) (Table 1), and one for *N. ambiguus* (haplotype A, Table 1).

COII sequences were 647 bp in length and included 23 segregating sites and two singletons. In this sample we identified 13 haplotypes (arbitrarily named 1–13; Table 1). For *N. dissimulator* we report eight different haplotypes from nine sampling sites (arbitrarily named 1–8) (Table 1) and one for *N. ambiguus* (arbitrarily named 1, Table 1). The absence of stop codons and mutations that alter the reading frame of both translated mitochondrial sequences excluded numt (Nuclear Mitochondrial DNA) amplifications.

Sequencing of ITS1 yielded sequences similar in length to those obtained by Rodriguero et al. (2010b), and had 61 segregating sites (only two singletons). Seven haplotypes out of the eight reported in our previous contributions were identified in this sample (I–IV and VI–VIII) plus three new ones from the locations of Cerro Azul and

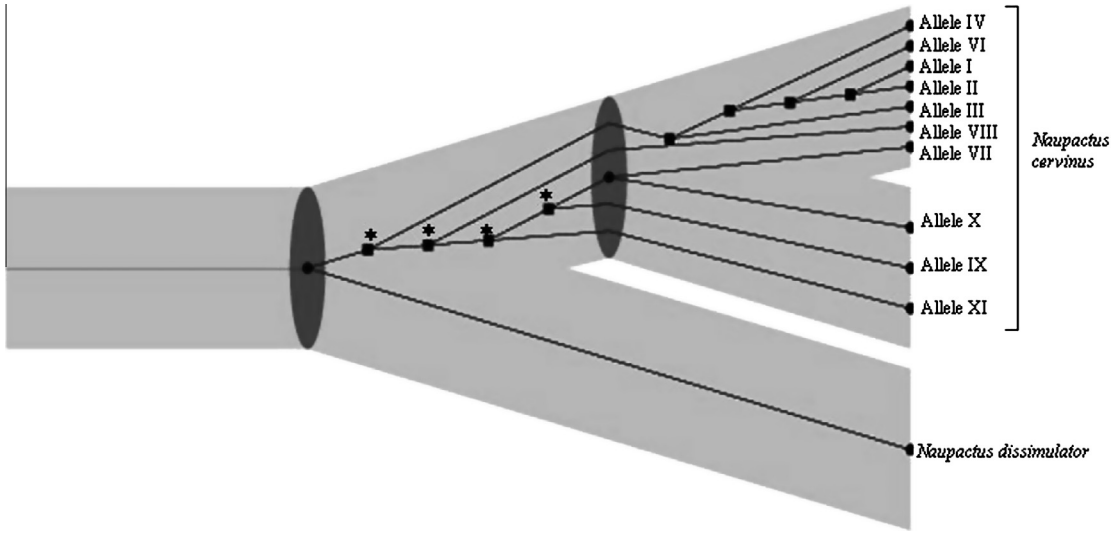

Fig. 4. Reconciliation of species tree (after H1) (thick grey branches) and ITS1 gene tree (thin black branches) using Primetv. Speciation events are indicated with ovals and shared ancestral polymorphisms with asterisks.

Chapecó (arbitrarily named IX, X and XI; Table 1).These new genotypes were found in the same individuals bearing the new COI haplotypes S, T and U. For *N. dissimulator* we report six different haplotypes from nine sampling sites (arbitrarily named I–VI) (Table 1), and one for *N. ambiguus* (arbitrarily named I, Table 1).

3.2. Phylogenetic relationships

Maximum parsimony search of the COI dataset yielded three most parsimonious tree 133 steps long. Two main clades with high bootstrap support values were recovered (Fig. 2a). One of them included subclades previously identified as "forest" (clade Ia) and "grassland" (clade Ib) (Rodriguero et al., 2010b). The other one (clade II) is highly divergent from the previously mentioned (see the number of mutational steps separating them) and included individuals from Cerro Azul and Chapecó (Fig. 1, and Fig. 2a). A similar topology was retrieved by Bayesian inference, although some clusters within each main clade differed slightly (See Supplementary material, Fig. S1).

Phylogenetic analysis of the COII dataset retrieved four trees 116 steps long. A strict consensus tree showed the same two clades and subclades as for the COI dataset (Fig. 2b), supported by moderate to high bootstrap support. A similar topology was retrieved by Bayesian inference (See supplementary material, Fig. S2). As before, some clusters within each main clade had slight differences with parsimony tree.

From the ITS1 dataset, we retrieved one most parsimonious tree 270 steps long (Fig. 2c) with two highly supported clades. One of them (clade A, Fig. 2c) included all individuals from clade Ia and some individuals from clade Ib (Fig. 2a and b). The other clade (clade B, Fig. 2c) included most individuals from clade Ib and all individuals from Clade II (Fig. 2a and b). A similar general topology was retrieved by Bayesian inference (See supplementary material, Fig. S3).

3.3. Species delimitation analyses

3.3.1. Species delimitation analyses using *Beast

In order to test if the highly divergent clade recovered with both COI and COII genes can be considered as a separate species, we performed a multilocus coalescent-based analysis for species delimitation, including individuals of *N. dissimulator* and *N. ambiguus* as outgroup species.

According to H1, all split events hypothesized had posterior probability values $\geqslant 0.95$ (Fig. 3a). DensiTree analysis gave additional support for these systematic relationships (Fig. 3b) because most trees in the whole set of "species trees" overlap with no major conflicting topologies, node ages and/or heights. Conversely, H2 provided a small probability for the occurrence of a split event between clades Ib and II (Fig. 3c). This result was supported by DensiTree analysis showing large uncertainties in the topologies within *N. cervinus* (denoted by blue[1] and red colors in Fig. 3d).

H3 assigned high speciation probability to the split event between clade Ib and the group composed of clade Ia and clade II. However, a split between *N. dissimulator* and its sister clade *N. cervinus* species complex had a probability of less than 0.95 (Fig. 3e), leading us to reject the whole hypothesis. Indeed, this was supported by the uncertainty in the topologies revealed by DensiTree analysis (Fig. 3f). Similar results were obtained for H4 (Figs. 3g and 3h). Therefore, the preferred hypothesis is H1, which assumes that individuals from Chapecó and Cerro Azul belonging to the mitochondrial clade II would represent a separate evolutionary unit.

We compared the degree of molecular divergence achieved by *N. cervinus* lineages to that observed between asexual lineages of a closely related weevil which is in fact a species complex (*Aramigus tessellatus*). Genetic divergence accumulated in both groups of weevils is fairly similar (Clade I–Clade II = 0.0076 (see Fig. 3a) and *A. tessellatus* – *A. uruguayensis* = 0.00602).

The analysis of divergence times using *BEAST under the preferred hypothesis (H1, Fig. 3a) indicated that that both *N. cervinus* lineages (I vs II) would have split from each other between 540,000 and 1,270,000 ybp. The species pair *A. tessellatus* – *A. uruguayensis* would have diverged ca. 430,000–1,000,000 ybp, using the same rates as above (phylogeny not shown).

Reconciliation of the ITS1 gene tree with the H1 species tree suggested shared ancestral polymorphisms between Clade I and Clade II individuals. In fact, some nuclear alleles might have diverged before the split of these two lineages (Fig. 4), making incomplete lineage sorting of ITS1 alleles a plausible explanation for such incongruence.

[1] For interpretation of color in Fig. 3, the reader is referred to the web version of this article.

Table 2
Summary of results of K/θ tests for mitochondrial sequences of *N. cervinus*. Quantities K and θ are explained in the text, n_1 and n_2 are sample sizes, and $P_{rec.monophyl}$ is the inferred probability of reciprocal monophyly between two sister clades.

Comparison	K	θ	K/θ	n_1, n_2	$P_{rec.monophyl}$
Ia vs. II	0.04500	0.00755	5.96410	8, 10	1.0
Ib vs. II	0.04700	0.00274	17.15345	18, 10	1.0
Ia vs. Ib	0.02200	0.00755	2.91578	18, 8	0.96406
I vs. II	0.04600	0.01195	3.85023	26, 10	0.98880

3.3.2. Species delimitation analysis using GMYC method

Neither single ($LL_{Null\ Model}$ = 105.141, $LL_{GMYC\ Model}$ = 107.601, LTR = 4.927, df = 3, p = 0.177) nor multiple ($LL_{Null\ Model}$ = 105.141, $LL_{GMYC\ Model}$ = 107.983, LTR = 5.684, df = 3, p = 0.338) GMYC models provided a significantly better fit to the data than the null model's hypothesis of the entire sample being derived from a single species with uniform branching.

When included *N. dissimulator* in GMYC analysis, we obtained a similar result. Neither single ($LL_{Null\ Model}$ = 156.838, $LL_{GMYC\ Model}$ = 159.554, LTR = 5.433, df = 3, p = 0.143) nor multiple ($LL_{Null\ Model}$ = 156.838, $LL_{GMYC\ Model}$ = 159.899, LTR = 6.123, df = 3, p = 0.190) GMYC models provided a significantly better fit to the data than the null model, even when a different species of *N. cervinus* was included.

To summarize, this test could not identify any independent evolutionary unit, neither within *N. cervinus* complex, nor when the sample also included the sister species *N. dissimulator*.

3.3.3. Species delimitation analyses using K/θ method

The K/θ method recognized two independent evolutionary units when clade II was compared to both Ia (forest) and Ib (grassland) clades (Table 2). These two sister clades have a K/θ ratio <4 (Table 2); however, the probability of reciprocal monophyly of both clades given reciprocal monophyly of their samples is $\geqslant$0.95. Therefore, according to this criterion, Ia and Ib can be considered as two independent evolutionary unit. When the whole clade I (Ia + Ib) is compared to clade II, K/θ is nearly 4 (Table 2), and the probability of reciprocal monophyly is highly significant (Table 2). Summarizing, according to this test, the three lineages (Ia, Ib and II) can be considered independent evolutionary units.

3.4. Wolbachia genetic variation

The alignment of the sequences *coxA*, *fbpA*, *gatB*, *hcpA* and *wsp* obtained from three different Clade II individuals (IDs NcCA2, NcChp2 and NcChp6; clade II in Fig. 2a and b) and two *N. cervinus* individuals (IDs NcCA1 and NcChp1; clades Ia and Ib, respectively, in Fig. 2a and b), with those from *Naupactus cervinus* by Rodriguero et al. (2010a,b) (GenBank accession numbers: *coxA*: GU079631; *fbpA*: GU0796312; *gatB*: GU079633; *hcpA*: GU079634; *wsp*: GQ402145) revealed the same nucleotide sequence for every gene and individual assayed. Therefore, we determined that both subsamples were infected with the same *Wolbachia* strain (strain *wNau5*, see Rodriguero et al., 2010a,b).

3.5. Morphological analysis

The specimens assigned to clade II (Fig. 5a) show conical rostrum, with sides more strongly convergent towards apex ($w + R/w - R \geqslant 1.55$, X 1.58) than those of clade Ib ($w + R/w - R \leqslant 1.54$, X 1.45); wider pronotum with more curved sides ($w + P/l + P \geqslant 1.25$, X 1.27) than those of clade Ib ($w + P/l + P \leqslant 1.24$, X 1.17); and elytra more slender and elongate ($l + E/w + E \geqslant 1.45$ X 1.50; $l + E/l + P \geqslant 2.55$ X 2.63) than those of clade Ib ($l + E/w + E \leqslant 1.44$ X 1.40; $l + E/l + P \geqslant 2.55$ X 2.43). The most important variation is seen in the rate maximum length of the elytra/maximum length of the pronotum. Specimens of clade Ia show intermediate rate, although closer to those of clade II.

Moreover, in the specimens of clade II (Fig. 5a) the color of the scaly vestiture is not uniformly pale brown but brown intermix with whitish scales, the elytral setae are slightly longer, more dense and also more erect regarding the elytral surface; and the spermathecal duct is longer than that of the specimens of clade Ib (Fig. 5b). Specimens of clade Ia show characters of the vestiture intermediate between those of clades Ib and II, although closer to clade Ib.

Slight morphometric differences and vestiture features are typical of closely related or incipient species of large species complex (Lanteri 1984, 1992; Lanteri et al., 1987; Lanteri and Díaz, 1994).

4. Discussion

Cryptic species constitute an important proportion of Earth biodiversity. Revealing this hidden diversity is not only important for a better understanding of the evolutionary processes and patterns of ecosystem functioning, but also relevant for conservation purposes (Dincă et al., 2011).

The results obtained in our study using the multilocus approach, the K/θ method and the morphometric analysis suggest an ongoing process of incipient speciation within *N. cervinus*, although GMYC analysis gave opposite results. Different levels of divergence within *N. cervinus* would reflect a case of incomplete speciation, rather than a single species. Thus, *N. cervinus* might be a species complex, or a "cluster of species in *statu nascendi*" (Dobzhansky and Spassky, 1959).

4.1. Mitochondrial vs. nuclear genomes: incongruence obscures the status of N. cervinus lineages

The topologies recovered from single-locus analyses showed controversial relationships between Clade I and Clade II datasets. At first glance, mitochondrial DNA suggested one highly divergent lineage (II) in addition to the less divergent "forest" (Ia) and "grassland" (Ib) clades reported in our previous contribution within the nominal species *N. cervinus* (Rodriguero et al., 2010b).

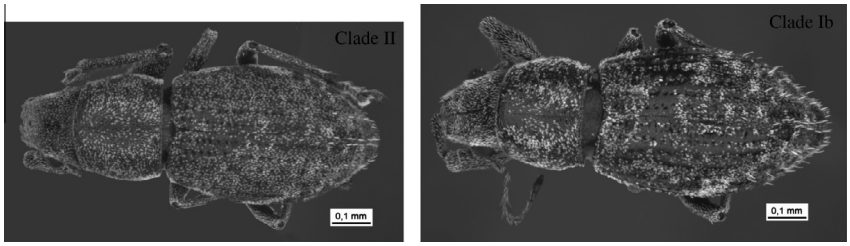

Fig. 5. Habitus photographs of most divergent clades of the *N. cervinus* species complex:a. clade II; b. clade Ib (grassland clade).

In contrast, nuclear markers indicated only two well supported clades, with individuals from clade Ib mixing with both clade Ia and II. However, these incongruent relationships between genomes do not necessarily contradict the hypothesis of different evolutionary units, because they can be explained by incomplete lineage sorting of nuclear alleles and different evolutionary rates (Schön et al., 2012). Discordances between gene and species trees are expected to arise because more than one copy "survives" when allelic lineages do not pass through population bottlenecks prior to speciation (Avise, 2004). It is known that sister taxa will exhibit a polyphyletic gene tree (as observed for the ITS1 gene tree in the present study) shortly after separation; however, the probability of becoming reciprocally monophyletic will increase as time passes by, and after about 4 N generations gene trees will be good estimations of species relationships (Avise, 2004). Times to monophyly are extended for nuclear loci, mainly because of their expected fourfold larger effective population sizes, and/or balancing selection in sexual species, and at least twofold larger expected effective population sizes in apomictic parthenogenetic species. Then, the phylogenetic status of species is a function of both the pattern of population splitting and the historical demography of the populations involved (Avise et al., 1984).

The lower evolutionary rate in nuclear genes, as was reported by Rodriguero et al. (2010b) for *N. cervinus*, could also account for discrepancy in the results. Schön et al. (2012) ascribed incongruence between the number of independent evolutionary units between nuclear and mitochondrial sequences to this phenomenon for the ancient asexual darwinulid genera *Penthesilenula* and *Darwinula*. As soon as mutations accumulate, signs of divergence will be evident.

In an attempt to resolve these disparities in results from different sources of data, we applied a multilocus Bayesian phylogenetic analysis, which takes into account sources of incongruence between different datasets (e.g. incomplete lineage sorting or lateral transfer). Nowadays, this is considered a better approach than the concatenation method (Degnan and Rosenberg, 2006; Kubatko and Degnan, 2007). The branch leading to both clade I (clade Ia + Ib) and clade II had a high posterior probability (speciation probability $\geqslant 0.95$), supporting their identity as different evolutionary units (Leaché and Fujita, 2010). The K/θ method rule provides extra support for this idea, since this ratio is approximately equal to $4N_e$ generations and statistically significant for the whole clade I of *N. cervinus* compared to clade II individuals. This method also defines the two lineages within the nominal species as different evolutionary units, although with a low but significant value of K/θ ratio. This result can be interpreted as the consequence of an incipient but still incomplete process of speciation. On the other hand, GMYC analysis concluded that the three lineages under consideration belong to a single species. However, it also failed to identify the independent evolutionary units that undoubtedly constitute both sister species *N. cervinus* and *N. dissimulator*, so the performance of this methodology for the case under study (i.e. an asexual species) is put into question. Indeed, efficiency of GMYC varies across biologically relevant values of N_e and N_e fluctuations (Essestyn et al., 2012), the rate of speciation (Essestyn et al., 2012) and the occurrence of population structure (Lohse, 2009). Either an increase in N_e and/or SR, as well as variations in N_e decreased both accuracy and precision of the method with simulated data (Essestyn et al., 2012). Thus, as the number of deep coalescences increases, the estimated number of species decreases, eventually resulting in underestimates of species number (Essestyn et al., 2012). The intermingling of speciation and coalescent processes in gene genealogies resulting from large population sizes and/or high speciation rates is detrimental for the shape of the inflection point (i.e. the time threshold), which becomes less pronounced, potentially causing an underestimation of biodiversity by GMYC analysis.

To conclude, both multilocus coalescent-based analysis and the K/θ method -specially designed for asexual species- were able to detect at least two evolutionary units, one of them corresponding to clade I individuals, and the other one to clade II individuals. The third methodology, based on a single locus, failed to support this conclusion, although its efficiency depends on several factors that in many cases may not be accomplished. We then propose that *N. cervinus* is a complex of species formed by at least two evolutionary units.

Morphological analysis of *N. cervinus* individuals revealed slight morphological divergence between clades I and II. They - mainly differentiate in external morphometric features, in contrast with species and morphotypes of the *A. tessellatus* species complex, which show distinct characters in the shape of the spermatheca and spermathecal duct (Lanteri and Díaz, 1994; Normark and Lanteri, 1996). However, the divergence time is almost the same for both lineage pairs (*N. cervinus* clade I vs. clade II and *A. tessellatus* species complex vs. *A. uruguayensis*). These results indicate that both asexual complexes of the Naupactini tribe would have achieved similar levels of genetic differentiation over a comparable period of time, giving rise to independent evolutionary units but with different degrees of morphological variation.

4.2. Mitochondrial genetic divergence: geographic isolation, divergent selection or Wolbachia infection?

The accumulation of genetic differences in *N. cervinus* species complex can be explained as a consequence of diverse factors: *Wolbachia* infection, past geographic isolation and divergent selection.

The first process -*Wolbachia* infection- could explain the divergence of mitochondrial DNA because mitochondrial genealogy with deep internal and short terminal branches will be recovered if different bacterial strains are maintained by natural selection in the same host species, due to the genetic linkage among uniparentally inherited genomes (Rodriguero et al., 2010b). Something like that was observed in the fig-pollinating wasp *Eupristina verticillata*, a complex of three cryptic species, where each clade harbours a different *Wolbachia* strain (Sun et al., 2011). In these situations, the tree topology could easily be mistaken as evidence for population structure and admixture (Hurst and Jiggins, 2005). However, this is not the case for *N. cervinus* because both lineages are infected with the same strain of *Wolbachia*, as well as occurs in the parthenogenetic collembolan *Folsomia candida* (Frati et al., 2004) and in the fig-pollinating wasp *Pleistodontes imperialis* (Haine et al., 2006). Baldo et al. (2008) proposed high rates of horizontal transfer between populations of the same host species to explain *Wolbachia*'s lack of variation in some hosts (e.g. Benlarbi and Ready, 2003; Parvizi et al., 2003; Ahrens and Shoemaker, 2005; Narita et al., 2006), what could explain a single strain infecting the *N. cervinus* species complex. In spite of this, we still can rule out *Wolbachia* as the speciation motor within this weevil, because no variation was observed for this endosymbiont among the different host lineages.

In asexual taxa, the second process (i.e. allopatry) may, at least theoretically, lead to two distinct clusters (Barraclough and Herniou, 2003). After secondary contact, these clusters formed in geographic isolation will be able to coexist only if their functional differences prevent them from coalescing back into a single cluster. Otherwise, assuming that all individuals have an equal chance of contributing to the next generation, the expected gene tree will return gradually to a single coalescent with common ancestor at *2N* generations (Barraclough et al., 2003). The third process (i.e. ecological divergence by natural selection) can occur in response to lo-

cal environmental differences or ecological interactions, ultimately leading to sympatric speciation (Rundle and Nosil, 2005). Therefore, regardless of the geographic scale, natural selection is to be invoked as a cause of accumulation (if sympatry)/maintenance (if allopatry) of genetic divergence.

A. tessellatus occurs in hilly areas of central Argentina (Córdoba, Tandilia and Ventania), and in the vicinity of the banks of the La Plata River, where it displays high morphological and molecular diversity (Lanteri and Díaz, 1994; Normark and Lanteri, 1998). During the dry cycles of the Pleistocene, both areas would have served as shelter for some species not adapted to dry climates and open areas, protecting them from extinction and favoring incipient speciation processes (Ringuelet, 1955, 1961; Simpson Vuilleumier, 1971; Ab'Sáber, 1977; Haffer, 1982). The highest level of molecular divergence for *N. cervinus* seems to have occurred in the Paranaense forest (Rodriguero, 2009; Rodriguero et al., 2010b), which was also affected by the wet-dry cycles of the Pleistocene, coinciding with the time inferred for *N. cervinus* lineages to have diverged. Since then, the barriers between such refuges would have disappeared allowing some incipient groups to exist in sympatry, as has been pointed out for several animal groups (de Vivo and Carmignotto, 2004; Ledru et al., 2005; Saia et al., 2008). This fact would explain the coexistence of different forest-adapted cryptic species of *N. cervinus* in subtropical forests of Brazil and Argentina.

In the arena of ecological speciation, selection pressures arise from the interaction between individuals and their environment during resource acquisition, for example, from an individual's quest to obtain food and other nutrients, or avoid predators (Rundle and Nosil, 2005). The shape of the trophi (hard jaws) in Bdelloidea (so-called 'ancient asexual scandals') has been viewed as the target of selection, with differences reflecting adaptations to different niches (Fontaneto et al., 2007b). For *N. cervinus*, however, a character related to differential exploitation of resources is unlikely to be under strong selection pressure because of its highly polyphagous habits (Chadwick, 1965). This is confirmed by the fact that our specimens were found on a wide variety of plants, such as eucalyptus, orange tree, rosebush, strawberry and diverse native species (Rodriguero, 2009).

Clade I and clade II individuals were sampled simultaneously from a variety of native and cultivated plants in Argentina and orange trees in Brazil (e.g. NcCA1, NcCA2, NcCA3 AND NcCA4 in Cerro Azul; NcChp1 and NcChp2 in Chapecó, Fig. 1). If these lineages resulted from ecological divergence in sympatry, that are maintained by natural selection, or are the consequence of secondary contact upon allopatric divergence is at this point a conundrum. Identifying genes of the *N. cervinus* genome targeted by adaptive selection could be useful to explore both scenarios.

5. Final conclusion: through a glass, darkly...

Population genetic theory predicts that asexual organisms can diversify into species, which are, in fact, independent evolutionary units (Birky and Barraclough, 2009). This means that every population whose individuals are joined by the same evolutionary processes of mutation, selection, and random drift behaves as independent arenas and evolves separately. As a result, they form genotypic clusters separated by deep gaps due to geographic isolation and/or divergent selection.

N. cervinus is a species complex or a cluster of species in *statu nascendi* (Dobzhansky and Spassky, 1959), as is the case for the *Aramigus tessellatus* species complex. What is seen in these weevil species could be paralleled by other species of this South American group scarcely studied, reflecting the complex changes occurred in this area during the Ice age. The phylogenetic relationships of the *A. tessellatus* complex have been studied in detail, while much less is known for *N. cervinus* and other members of the highly diverse genus *Naupactus*. Approaching this system through additional markers as microsatellites and modern techniques like comparative genomics will probably shed additional light on the complex evolutionary history of the "Fuller's rose weevil", which we are now looking through a glass, darkly...

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.04.011.

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