Independent evolving lineages within an asexual weevil: is *Naupactus cervinus* a complex of species?

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INTRODUCTION

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. The Paranaense forest (southern Brazil, eastern Paraguay and north-eastern Argentina) is considered the probable geographic area of origin of *N. cervinus*. There, both sexual and parthenogenetic forms have been found, although male records were absent since the 1950'. Parthenogens reproduces by apomictic parthenogenesis, 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 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).

Two divergent lineages within *N. cervinus* were identified on the basis of mitochondrial and nuclear loci, called "forest" and "grassland" clades after their geographic location (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. If this highly divergent lineage is an incipient species by natural selection, geographic isolation or the result of a strong population subdivision driven by the invasion of different *Wolbachia* strains 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.

RESULTS

1.- Phylogenetic Analysis

Mitochondrial genes retrieved three subclades: the previously identified as "forest" (clade Ia) and "grassland" (clade Ib), and another highly divergent from the previously mentioned (clade II) which included individuals from Cerro Azul and Chapeco.

The nuclear dataset only two highly supported clades. One of them (clade A) included all individuals from clade Ia and some individuals from clade Ib. The other clade (clade B) included most individuals from clade Ib and all individuals from Clade II.
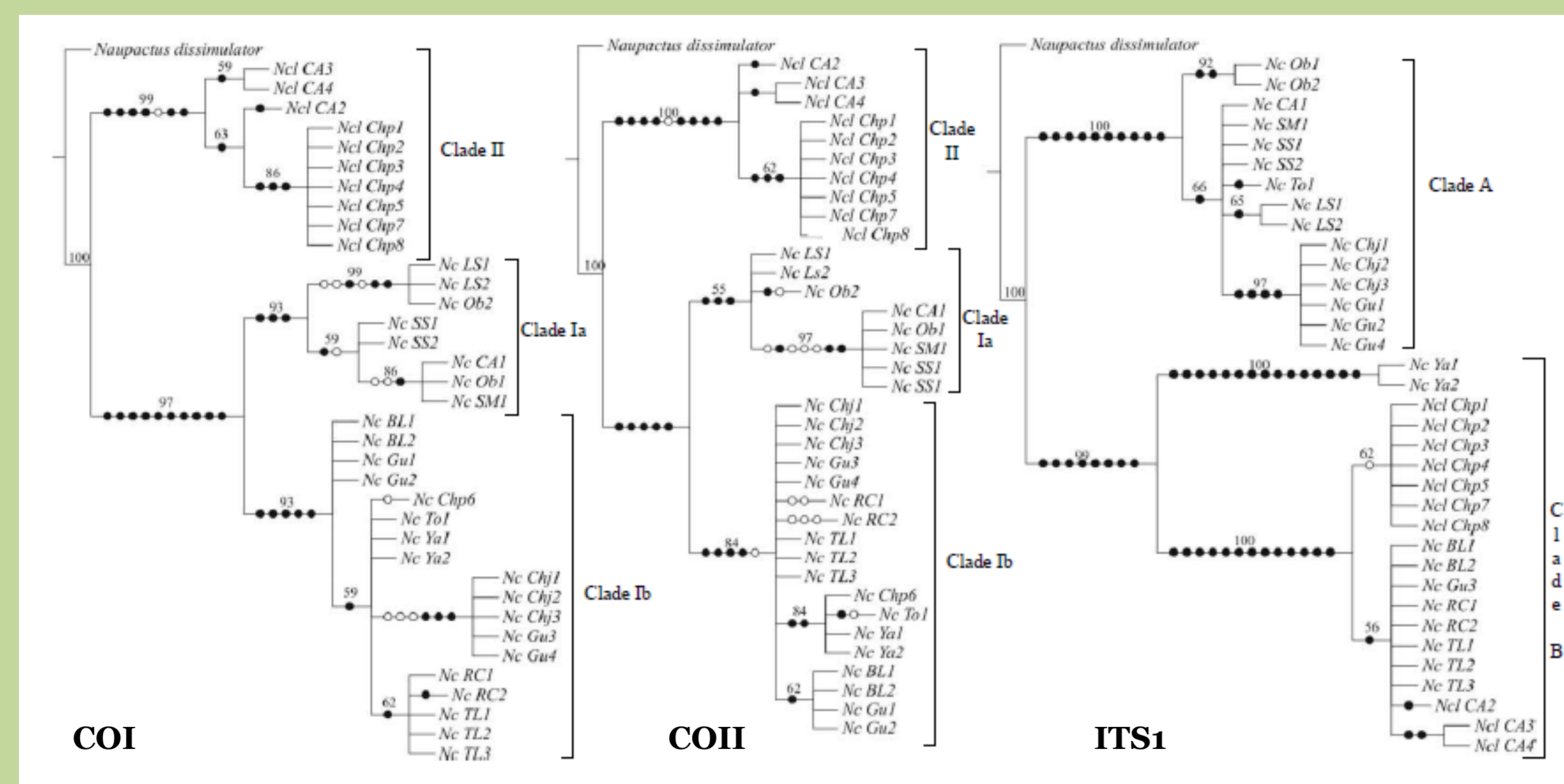

2.- Species delimitation analyses using Beast

We applied a multilocus coalescent-based approach using *Beast (Heled & Drummond 2008) to estimate the species tree from multilocus data. A posterior probability value P ≥ 0.95 was considered as strong support for a speciation event (Leache and Fujita, 2010).We tested the following hypotheses:

H1: *N. cervinus* is a complex of two species, one including forest and grassland clades and another one including the highly divergent individuals from Cerro Azul and Chapeco;

H2: *N. cervinus* comprises three species, one including the forest clade, another one including the grassland clade and the last one including the highly divergent individuals sampled at Cerro Azul and Chapeco;

H3: *N. cervinus* comprises two species, one including forest and the highly divergent individuals sampled at Cerro Azul and Chapeco, 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 Chapeco, and another one including forest weevils.

According to H1, all split events hypothesized had posterior probability values P ≥ 0.95. DensiTree analysis gave additional support for these systematic relationships because most trees in the whole set of "species trees" overlap with no 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. This result was supported by DensiTree

analysis showing large uncertainties in the topologies within *N. cervinus* (denoted by blue and red colors). 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 the *N. cervinus* species complex and its sister species *N. dissimulator* had a probability < 0.95, leading us to reject the whole hypothesis. Indeed, this was supported by the uncertainty in the topologies revealed by DensiTree analysis. Similar results were obtained for H4. Therefore, the preferred hypothesis is H1, which assumes that individuals from Chapeco and Cerro Azul belonging to the mitochondrial clade II would represent a separate evolutionary unit.

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.
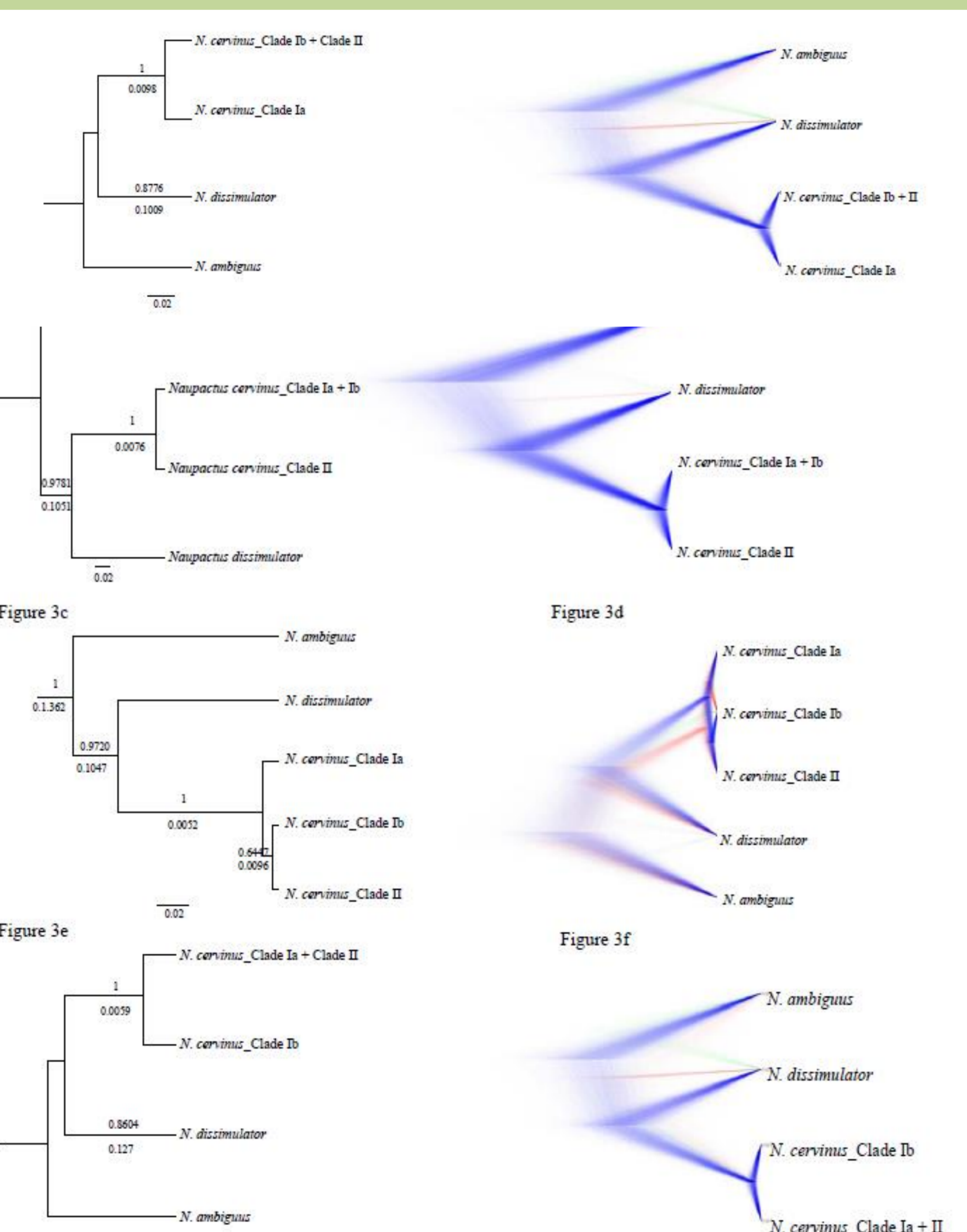
3.- Reconcilitation Analysis

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, making incomplete lineage sorting of ITS1 alleles a plausible explanation for such incongruence.
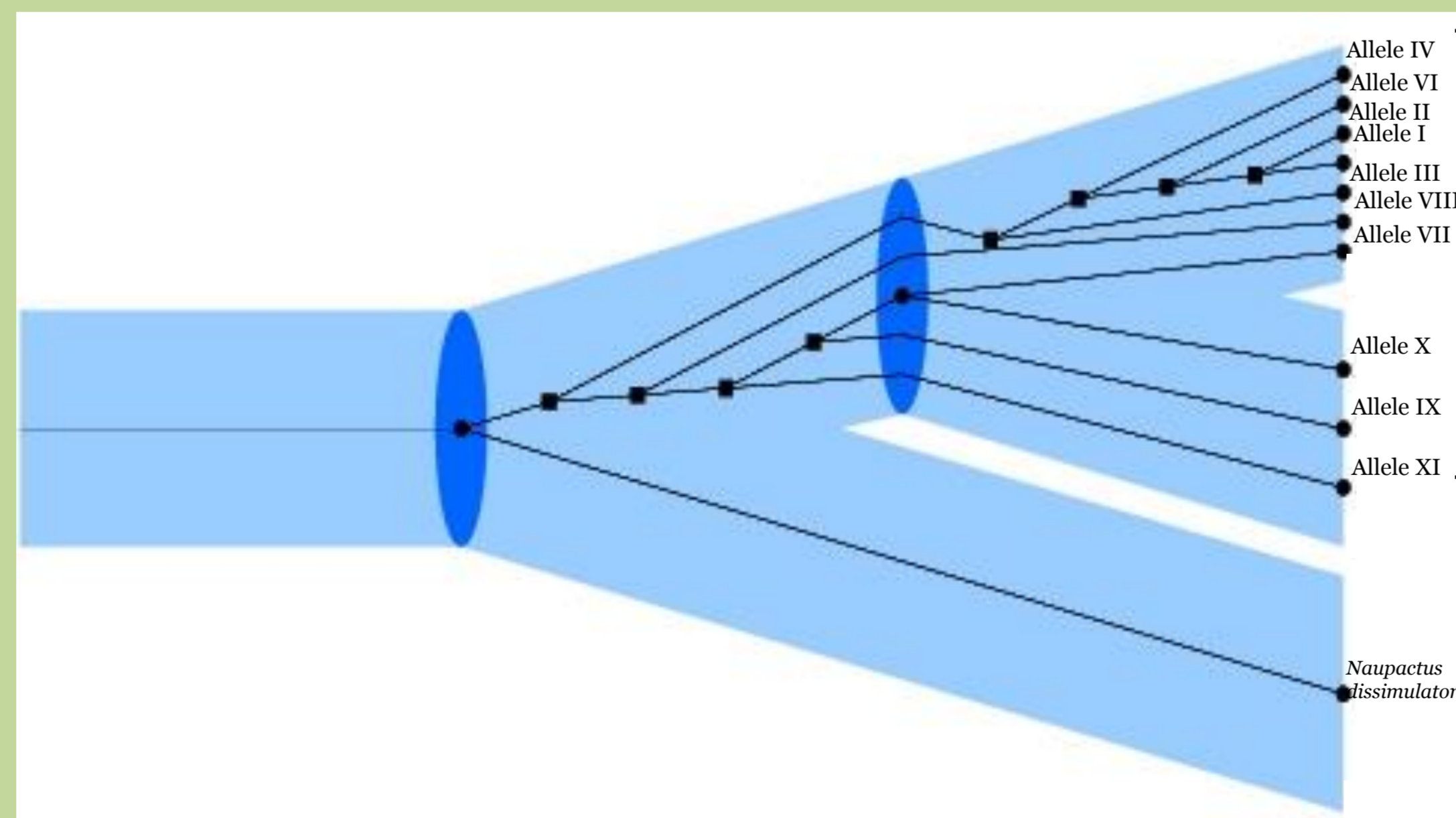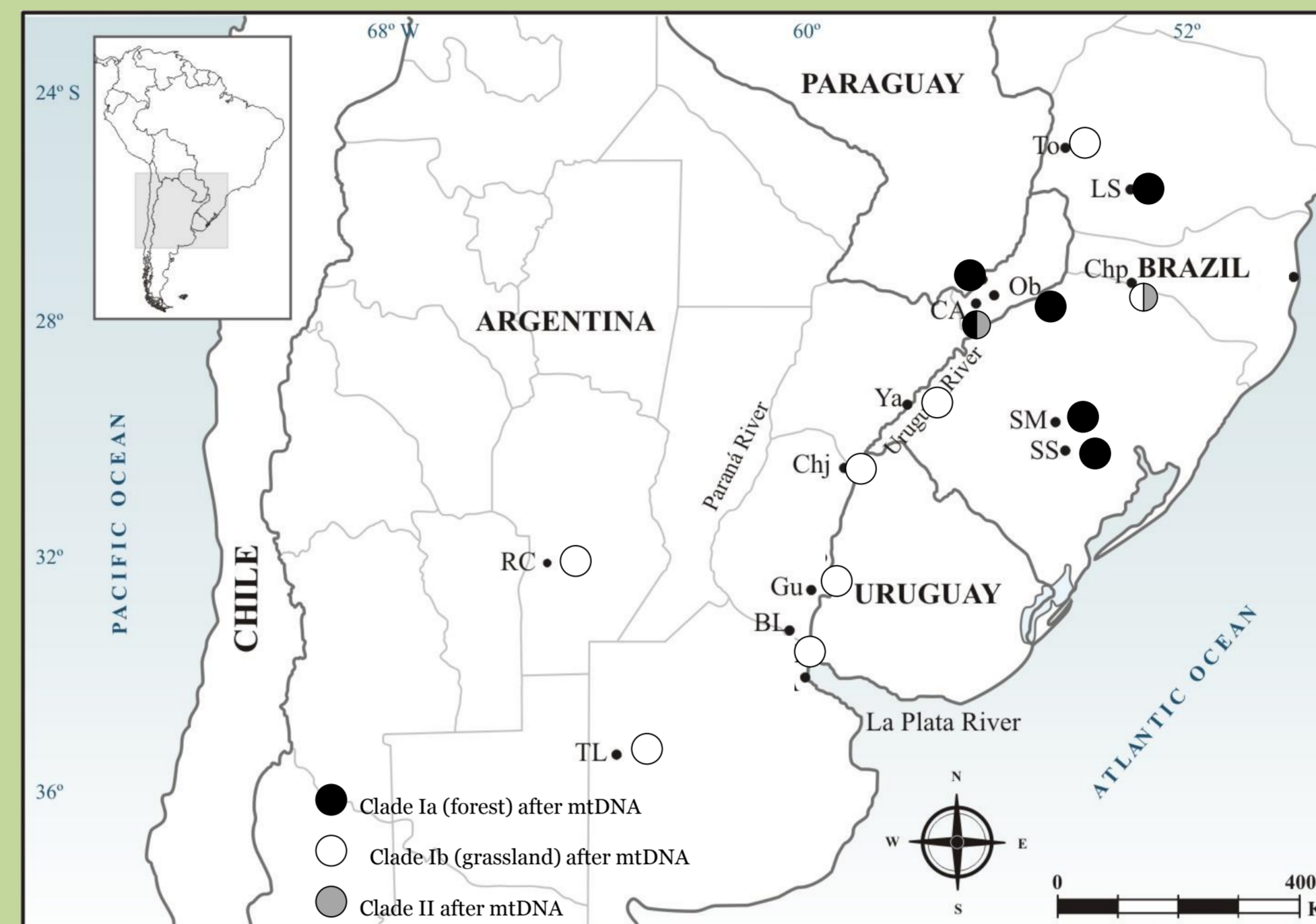

4.- Species delimitation analysis using GMYC method

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. Thus, 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*.

MATERIAL AND METHODS

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 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/h method, Birky et al., 2010). Third, 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. Fourth, we analyzed six morphometric rates, in order to asses if their molecular divergence is congruent with the morphological divergence.

Location	Acronym	N	COI	COII	ITS1	Clade
BL, ER, Entre Ríos	BL	1	A	1	I	---
AR, ER, Brazo Largo	BL	2	M	7	VII	Ib
AR, MI, Cerro Azul	CA	2	Q	3	IV	Ia
			S	11	IX	II
			T	14	X	II
AR, ER, Chajarí	Chj	3	F	6	VI	Ib
AR, ER, Gualeguaychú	Gu	4	F	7	VI	Ib
			M	6	VI	Ib
			M	6	VII	Ib
AR, MI, Oberá	Ob	2	Q	2	III	Ia
			R	3	III	Ia
AR, CB, Río Cuarto	RC	2	A	4	VII	Ib
			B	5	VII	Ib
AR, BA, Tres Lomas	TL	3	B	6	VII	Ib
AR, CO, Yapeyú	Ya	2	C	8	VIII	Ib
BR, SC, Chapecó	Chp	8	E	10	---	II
			U	12	XI	II
BR, PR, Laranjeiras do Sul	LS	2	R	1	I	Ia
BR, RS, Santa María	SM	1	Q	3	IV	Ia
BR, RS, São Sepé	SS	2	P	3	IV	Ia
BR, PR, Toledo	To	1	C	9	II	Ib

5.- 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. These two sister clades have a K/θ ratio <4; however, the probability of reciprocal monophyly of both clades given reciprocal monophyly of their samples is $P ≥ 0.95$. Therefore, according to this criterion, Ia and Ib can be considered as two independent evolutionary units. When the whole clade I (Ia + Ib) is compared to clade II, K/θ is nearly 4, and the probability of reciprocal monophyly is highly significant. According to this test, the three lineages can be considered independent evolutionary units.

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

6.- Wolbachia genetic variation

The alignment of the sequences coxA, fbpA, gatB, hcpA and wsp obtained from three different clade II individuals and two clade I individuals with those from *N. cervinus* by Rodriguero et al. (2010a,b) revealed the same nucleotide sequence for every gene and individual assayed. Therefore, both subsamples were infected with the same *Wolbachia* strain.

Photo Credit: Merijn Salverda and Richard Stouthamer

7.- Morphological analysis

The specimens assigned to clade II show conical rostrum, with sides more strongly convergent towards apex than those of clade Ib; wider pronotum with more curved sides than those of clade Ib; and elytra more slender and elongate than those of clade Ib. 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 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. 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.

Conclusions

- 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".

- Discrepancies among molecular markers 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.

- GMYC results could be a consequence of fluctuations in Ne and/or speciation rate and population structure, since these factors affect the efficiency of this method.

- 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.

- *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.

- However, this is not the case for *N. cervinus* because both lineages are infected with the same strain of *Wolbachia*. High rates of horizontal transfer between populations of the same host species could explain *Wolbachia*'s lack of variation in this weevil.

- Clade I and clade II individuals were sampled simultaneously from a variety of native and cultivated plants in Argentina and orange trees in Brazil. 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.