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Diversity of boll weevil populations in South America: a phylogeographic approach

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Abstract

A phylogeographic approach was conducted to assess the geographic structure and genetic variation in populations of the boll weevil *Anthonomus grandis*, which is the most harmful insect pest of cotton in the Americas. COI and COII mitochondrial gene sequences were analyzed to test a former hypothesis on the origin of the boll weevil in Argentina, Brazil and Paraguay, using samples from Mexico and USA as putative source populations. The analysis of variability suggests that populations from South American cotton fields and nearby disturbed areas form a phylogroup with a central haplotype herein called A, which is the most common and widespread in USA and South America. The population from Texas has the A haplotype as the most frequent and gathers in the same group as the South American populations associated with cotton. The sample from Tecomán (México) shows high values of within-nucleotide divergence, shares no haplotype in common with the South American samples, and forms a phylogroup separated by several mutational steps. The sample from Iguazú National Park (Misiones Province, Argentina) has similar characteristics, with highly divergent haplotypes forming a phylogroup closer to the samples from cotton fields, than to the Mexican group. We propose that in South America there are: populations with characteristics of recent invaders, which would be remnants of "bottlenecks" that occurred after single or multiple colonization events, probably from the United States, and ancient populations associated with native forests, partially isolated by events of historical fragmentation.

Introduction

The boll weevil *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) has been subject of research since early 1890s when it became a cotton pest in USA. The hypothesis that this weevil may have originated in southern Mexico and later spread to North America together with its wild host plants (Malvaceae of the tribe Gossypieae) during Pleistocene time, was supported by studies on its biology, behavior, host-plant associations, phylogenetic relationships with other *Anthonomus* species, and morphometric variation among populations (Burke et al., 1986; Jones & Burke, 1997; Jones, 2001). The rapid dispersal of *A. grandis* between 1889 and 1916 throughout the Cotton Belt of the United States resulted from the expansion of area under cultivation.

At present, the origin and relationships of boll weevil populations from South America have not been elucidated (Burke et al., 1986; Scataglini, Confalonieri & Lanteri, 2000) and there is little information on alternative host-plants (Lanteri, Loiácono & Marvaldi, 2002; Lanteri, Marvaldi & Suárez, 2002). In South America, the boll weevil was firstly recorded in Venezuela (1949) and Colombia (1951) from cultivated *Gossypium hirsutum* and wild *Cienfuegosia affinis* and

Thespesia populnea (Cross et al., 1975). This weevil species appeared in Brazil in 1983 infesting about 90% of the cotton fields, and subsequently invaded cultivated and non-cultivated areas of Paraguay (1991), Argentina (1993) and Bolivia (1997) (Manessi, 1997, Scataglini, Confalonieri & Lanteri, 2000). In Argentina, the boll weevil was initially recorded at Iguazú National Park, a protected subtropical forest area, and after one decade it became widely spread in the Provinces of Misiones, Corrientes, Formosa and Chaco, where some putative wild hosts are distributed (Lanteri, Loiácono & Marvaldi, 2002; Lanteri, Marvaldi & Suárez, 2002).

The introduction of *A. grandis* into South America is mainly suggested by the lack of infestation records before 1949 and a high dispersal rate associated with the expansion of cotton cultivation.

Burke et al. (1986) classified specimens from Venezuela, Colombia and Brazil as corresponding to the "Southeastern form" of the species, which ranges from Texas to Florida in USA, but stressed the necessity of clarifying the relationships between the Brazilian samples and other boll weevil populations.

Molecular techniques have become important tools to analyze infra- and inter-specific variation in insects, to estimate the gene flow level among populations, to test hypotheses on geographical origins and dispersal pathways of species, and to develop effective pest control strategies (Confalonieri et al., 1998, Confalonieri, Scataglini & Remis, 2002; Normark & Lanteri, 1998; Emerson, Oromí & Hewitt, 2000; Sequeira et al., 2000). Despite the importance of *A. grandis* as a pest, very little is known about the genetic structure of its populations (Roehrdanz & North, 1992; Scataglini, Confalonieri & Lanteri, 2000; Roehrdanz, 2001; Kim & Sappington, 2004), particularly in South America (Scataglini, Confalonieri & Lanteri, 2000). In North America, a recent analysis with molecular markers revealed a geographic genetic pattern that is consistent with previous hypotheses about boll weevil origin and its northward range expansion from Mexico (Kim & Sappington, 2004).

In a previous contribution we have applied the RAPD technique (Random Amplified Polymorphic DNA) to analyze genetic similarities among boll weevil populations from Argentina, Brazil and Paraguay, and to compare them with potential source populations from Mexico (Tecomán) and USA (Mississippi) (Scataglini, Confalonieri & Lanteri, 2000). The results were somewhat unexpected, since boll weevils from Iguazú National Park (Argentina) were genetically more similar to those from Tecomán, than to other specimens from geographically closer South American populations. In addition, populations from Tecomán and Iguazú registered the highest percentage of polymorphic loci and heterozygosity levels, typical of central populations (Scataglini, Confalonieri & Lanteri, 2000). These results led us to suspect the ancient condition of some South American boll weevil populations, which may have occurred in the region infesting native host plants prior to cotton cultivation.

The main goal of this paper is to test the hypothesis of natural occurrence of the boll weevil in South America prior to extensive cotton cultivation, through a phylogeographic study based on mitochondrial DNA sequence data from the Cytochrome Oxidase I and II genes (COI and COII). Phylogeography has introduced a phylogenetic-historical perspective to investigate the evolution of populations, and contributes to the drawing of conclusions regarding sequences of colonization, diversification, and extinction of genetic lineages in certain areas (Avise, 2000; Lanteri & Confalonieri, 2003). A phylogeographic approach is appropriate for evolutionary studies of the boll weevil, because the assessment of geographic structure and analysis of genetic variation within and among South American populations will reflect underlying spatial and/or temporal discontinuities in available habitats.

Materials and methods

Sampling

Most of the specimens studied were collected by specialists and technicians from different institutions (see acknowledgements) working on boll weevil population dynamics. Argentine samples were collected from traps with "glandure" pheromone (Manessi, 1997), used for monitoring populations near the borders with Brazil and Paraguay. All insects were preserved in 100%

ethanol, but only those being fixed soon after trapping were included in DNA analyses.

Seven populations of *A.grandis* from Argentina, Brazil, Paraguay, Mexico and USA, were analyzed (further details are given in Table 1). Samples were collected from cotton fields or nearby areas, except for the sample from Iguazú National Park (Misiones, Argentina), which was collected from an area of wild vegetation. The sample from USA was collected in western Texas (Lubbock), and that from Mexico (Tecomán) near the Southern Pacific coast. One specimen of the "Peruvian square weevil", *A. vestitus* Boheman (Burke & Cross, 1966), from San Clemente, Manabí Province, Ecuador, was included in the phylogenetic analysis as an outgroup.

Ninety-three boll weevils were suitable for DNA sequence determination of COI and COII genes from over 200 specimens assayed. These genes were analyzed on different individuals in order to evaluate the congruence of independent patterns of haplotype lineage sorting. The Mexican sample from Tecomán could only be assayed for COI (and a previous RAPD analysis by Scataglini, Confalonieri & Lanteri, 2000) because of the few specimens suitable for molecular studies, and the unavailability of additional samples by restrictions in shipping biological material.

DNA Extraction and Sequencing methods

DNA extraction from single individuals was performed following the method by Reiss, Schwert and Ashworth (1995), with slight modifications for small-sized specimens. The amount of DNA extracted depended on the body size and degree of preservation of each specimen. This DNA was used as template for PCR amplification of mtDNA with primers S1718 (5′GGA GGA TTT GGA AAT TGA TTA GTT CC 3′) - A2442 (5′GCT AAT CAT CTA AAA ATT TTA ATT CCT GTT GG 3′) for COI fragments (Normark, 1996); and COII S (5′GGT CAA ACA ATT GAG TCT ATT TGA AC 3′) – COII A (5′CCA CAA ATT TCT GAA CAT TGA CCA 3′) for COII fragments (Litzenberger & Chapco, 2001).

Amplification reactions were performed in 2 mM Tris–HCl (PH = 8); 10 mM KCl; 0.01 mM EDTA; 0.1 mM DTT; 5% glycerol; 3 mM $MgCl_2$; 200 μM of each DNTP; 0.5 μM of each primer and 2.5 units of *Taq* polymerase (Promega) in final volume of 50 μl. DNA amplification was carried out in a thermal cycler (Techne) under the following conditions: the first period of denaturation was 94 °C for 6 min., followed by 35 cycles of denaturation at 94 °C for 1 min., annealing at 46 °C for 1.30 min. and extension at 72 °C for 1.5 min. Final extension at 72 °C for 5 min. terminated the reactions. A negative control with no template was included for each series of amplifications to eliminate the possibility of contamination.

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 QIAquik Gel Extraction Kit (QIAGEN Inc.).

Nucleotide sequences were obtained with a PE Biosystems automated 377 DNA sequencer. These have been deposited in the EMBL, GenBank under accession numbers AY266610-31 and AY267841-56

Data analyses

Sequences were translated to check for the presence of stop codons or frame shifts that might indicate the amplification of pseudogenes (Sorenson & Fleischer, 1996; Zhang & Hewitt, 1996; Bensansson, Zhang & Hewitt, 2000). Aminoacid sequences were inferred according to the invertebrate mitochondrial code. Alignment was done using CLUSTAL W (Thompson, Higgins & Gibson, 1994).

COI and COII haplotypes were analyzed separately, using maximum parsimony (MP) and Bayesian methods. The parsimony analysis, including all different haplotypes as "terminal taxa", was performed with NONA (Goloboff, 1994). Exact searches (number of taxa < 18) were carried out using "whennig" to obtain a Wagner tree, followed by the command "mswap+". Heuristic searches were done by stepwise-addition using ten series of random addition sequences, followed by tree bisection-reconnection (TBR) branch rearrangement, by the "mult* 10" command. Branch support was estimated by bootstrapping (1000 replicates). The trees obtained were represented as networks or phylograms.

The average numbers of nucleotide differences per site between sequences (either within or between populations) were estimated according to Nei (1987), and Nei and Kumar (2000), using the MEGA v2.1 program (Kumar et al., 2001). Models of nucleotide substitution used to estimate distance measures between populations and Bayesian searches, were chosen according to distance analyses performed by the log likelihood function of MODELTEST 3.0 (Posada & Crandall, 1998). MODELTEST compares 56 different nested models of DNA substitution in a hierarchical hypothesis-testing framework and uses log likelihood scores to establish the model of DNA evolution that best fits the data. Log likelihood scores for the different models were estimated using PAUP* (Swofford, 1998).

All Bayesian searches were performed by Mr. Bayes 2.03 (Huelsenbeck & Ronquist, 2001), including the models of nucleotid substitution selected by MODELTEST 3.0. They were run with four simultaneous chains for 1.500.000 generations, sampling every 100 generations and applying temperatures of 1, 0.5, and 0.3, which influence the rate of switching between chains. In order to assess if searches were effectively sampling from optimal trees and not from local optima, posterior probability values for the pairs of repeated runs were plotted against each other and correlation indexes were recorded. The burning or stationarity generation was determined by plotting generations versus ln likelihood values; all trees below the stationarity level were discarded. The Bayesian trees obtained were rooted with *A. vestitus* Boheman.

Results

COI variation

A 279 bp segment of the mtDNA COI gene, corresponding to positions 296 to 574 of *Drosophila yacuba* (Clary & Wolstenholme, 1985), was sequenced and aligned from 57 boll weevil individuals and the outgroup. To eliminate the possibility of pseudogene amplification, sequences were aligned with those obtained for several insect orders (Lunt et al., 1996). Within-gene heterogeneity and evolutionary rates of insect COI sequences are associated with functional constraints of the proteins they encoded (Lunt et al., 1996). Consequently, the comparison of these sequences allows the identification of regions of conserved and variable aminoacid residues. The alignments of translated weevil sequences showed the same distributional pattern of variation and the same aminoacid sequence within the conserved regions. This result, along with the absence of stop codons or frame shifts, excludes the possibility of pseudogene amplification.

A total of 102 sites were variable, resulting in 19 different haplotypes (Table 1), with 42 parsimony-informative characters. Nucleotide frequencies over the 279 bp segment showed a strong A + T bias as in other Curculionidae (Langor & Sperling, 1997; Normark & Lanteri, 1998; Sequeira et al., 2000; Scataglini, Lanteri & Confalonieri, 2005), averaging 65.8% among individuals. These biases were much stronger for third-base positions (84.9%) than for first- (59.5%) or second-base positions (53.1%). Overall nucleotide proportions were 33.6% T, 19 4%C, 32.2%A and 14.8%G. Estimates of nucleotide diversity were obtained using the substitution model of Tajima and Nei (1982) and Felsenstein (1981), which is the model that best fits our data. Corrected pairwise divergences among all haplotypes ranged from 0 (no substitution) to 0.147 ± 0.025, with the highest nucleotide diversity corresponding to haplotypes from Iguazú. The overall intraspecific uncorrected divergence is high (0.025 ± 0.0038) if compared to other species of Coleoptera (Langor & Sperling, 1997; Lunt, Ibrahim & Hewitt, 1998), although it is far below the level of overall mean divergence of *A. grandis* haplotypes with respect to *A. vestitus* (0.2290 ± 0.0357).

Populations from Iguazú (Ig) and Tecomán (Te) registered the highest level of haplotype diversity (Table 1). The remaining samples have a reduced number of different haplotypes and share one haplotype (A1) that is the most frequent among them, and is absent in Ig and Te (Table 1). The sample from the locality of Tecomán, in relatively close proximity to the putative central area of boll weevil distribution, shows a population nucleotide diversity (0.015 ± 0.003) lower than that of Iguazú (0.071 + 0.012) (Table 1), located in a supposedly marginal area. In addition, according to estimates of net corrected distances among all populations, the samples associated with cotton fields from South America are much closer to the Lubbbock (Tx) sample from USA, than to the Iguazú sample (Table 2).

Table 1. Details on collection sites, sample sizes (N) and haplotypes

Localities	Acronyms	States/Countries	Vegetation	N	Frequencies of haplotypes	Mean nucleotide diversity
Lubbock	Tx	Texas, USA	Cotton fields	17	4 A1, 4 Tx1, 1 Tx2. (COI) 6 A2, 1 Tx3, 1 Tx4. (COII)	0.009 ± 0.004 (COI) 0.003 ± 0.002 (COII)
Tecomán	Te	Colima, México	Cotton fields	10	4 Te1, 1 Te2, 1 Te3, 1 Te4, 1 Te5, 1 Te6, 1 Te7 (COI)	0.015 ± 0.003 (COI)
Londrina	Lo	Paraná, Brazil	Cotton fields	17	7 A1, 1 Lo1, 1 Lo2, 1 Lo3.(COI) 6 A2, 1 Lo4 (COII)	0.006 ± 0.002 (COI) 0.005 ± 0.003 (COII)
Caacupé	Ca	Cordillera, Paraguay	Cotton fields	17	10 A1 (COI) 7 A2 (COII)	0 (COI) 0 (COII)
Laguna Naick Neck	Nn	Formosa, Argentina	Cotton fields	12	7 A1 (COI) 3 A2, 2 Pe2 (COII)	0 (COI) 0.003 ± 0.004 (COII)
Puerto Península	Pe	Misiones, Argentina	Close to cotton fields	11	5 A1, 1 Pe1 (COI) 3 A2, 2 Pe2 (COII)	0.006 ± 0.003 (COI) 0.003 ± 0.003 (COII)
Iguazú National Park	Ig	Misiones, Argentina	Wild vegetation	9	1 Ig1, 1 Ig2, 1 Ig6, 1 Ig7, 1 Ig8 (COI). 1 Ig9, 1 Ig10, 1 Ig 11, 1 Ig12 (COII)	0.071 ± 0.012 (COI) 0.093 ± 0.021 (COII)

Mean nucleotide diversity: Tajima-Nei (COI) and Tamura–Nei (COII) corrected net distance measures and standard errors within populations for mitochondrial sequences.

Table 2. Corrected net distance measures between populations for mitochondrial sequences

	A.v.	Lo	Tx	Pe	Nn	Ca	Te	Ig
A.v.		0.3478 (0.0409)	0.3449 (0.0407)	0.3474 (0.0409)	0.3475 (0.0947)	0.3452 (0.0408)	–	0.3872 (0.022)
Lo	0.225 (0.032)		0.2×10^{-4} (3.2×10^{-5})	0.0006 (0.0006)	0.0006 (0.0007)	0	–	0.022 (0.010)
Tx	0.224 (0.032)	0.004 (0.002)		0.0006 (0.0005)	0.0006 (0.0006)	1.5×10^{-6} (0.3×10^{-5})	–	0.023 (0.010)
Pe	0.226 (0.032)	3×10^{-5} (0.0001)	0.004 (0.002)		0.0007 (0.0007)	0.0006 (0.0006)	–	0.0240 (0.010)
Nn	0.225 (0.032)	7.8×10^{-5} (0.0001)	0.004 (0.002)	0		0.0005 (0.0006)	–	0.0240 (0.010)
Ca	0.225 (0.032)	7.8×10^{-5} (0.0001)	0.004 (0.002)	0	0		–	0.0227 (0.010)
Te	0.212 (0.032)	0.037 (0.012)	0.037 (0.011)	0.036 (0.011)	0.037 (0.012)	0.0337 (0.012)		–
Ig	0.227 (0.032)	0.021 (0.007)	0.024 (0.007)	0.018 (0.006)	0.021 (0.007)	0.021 (0.007)	0.055 (0.013)	

Estimations based on Tajima-Nei (COI, below the diagonal) and Tamura–Nei (COII, above the diagonal). Acronyms of populations according to Table 1. A.v = *Anthonomus vestitus*. Standard errors are given between brackets.

COII variation

The analysis of 327 bp corresponding to position 256 to 582 of *Drosophila yacuba*, was sequenced and aligned for 32 boll weevil individuals and the outgroup. Because COII sequences of *A. grandis* include one open reading frame without any stop codon, and the same conserved aminoacidic residues as in other insect species (Liu & Beckenbach, 1992), we interpret that they represent the functional COII gene rather than nuclear pseudogenes.

A total of 103 sites were variable, resulting in nine different haplotypes (Table 1), with 27 parsimony-informative characters. Variability shows the same pattern of distribution as in COI. There is a haplotype called A2, that is the most frequent throughout all samples, but it is absent in Iguazú (Ig) (Table 1).

Nucleotide frequencies over the 327 bp segment show a strong A + T bias, averaging 65.3% among individuals. The biases were much stronger for third-base positions (75.3%) than for first- (55.6 %) or second-base positions (64.9%). Overall nucleotide proportions were 30.8% T, 20% C, 34.5% A and 14.7% G. These results are also similar to those obtained from COI analysis.

The model of nucleotide substitution that best fits the data is Tamura and Nei (1993). Corrected pairwise divergences among all haplotypes ranged from 0 (no substitution) to 0.105 ± 0.04, with higher nucleotide differences corresponding to haplotypes from Iguazú. The Iguazú sample shows the highest value of within-population divergence (0.093 ± 0.004), same as in COI gene (Table 1). The overall intraspecific uncorrected divergence is again high (0.025 ± 0.0038) in comparison with other insect species (Liu & Beckenbach, 1992), and the divergence from the outgroup is 0.2329 ± 0.06948. Estimates of net distance measures show a similar pattern as in COI gene, with Iguazú more differentiated from the remaining samples (Table 2).

Phylograms

The MP analysis of 19 COI haplotypes yielded six most parsimonious trees of 137 steps, each with a Consistency Index (CI) of 0.81 and a Retention Index (RI) of 0.80. The consensus tree is represented in the network of Figure 1(a), where branch lengths are proportional to the mutational steps that differentiate haplotypes. The MP analysis of COII haplotypes yielded a single most parsimonious tree of 128 steps, CI = 0.88 and RI = 0.55, represented in the network of Figure 1(b).

The COI phylogram (Figure 1(a)) shows the Tecomán haplotypes clustered together, over the right side of the net, far from the remaining haplotypes. A star topology is recovered for this group, with the most frequent haplotype (Te5) occupying a central position. Haplotypes from cotton fields of South America and USA (Texas) are also grouped together. Again, there is one most frequent haplotype (A1) occupying a central position, from which five closely related haplotypes are derived, two of Lubbock (Texas) and three of Londrina (Brazil). Samples from Caacupé (Paraguay) and Laguna Naick Neck (Formosa, Argentina) are monomorphic for the A haplotype. Finally, all haplotypes from Iguazú, and one from Puerto Península (Misiones Province, Argentina) are grouped together on the left side of the network, relatively far from each other, and closer to the Texas-South American samples from cotton, than to the "Mexican group".

The COII phylogram (Figure 2(b)) shows a similar pattern of relationships between genotypes, except that Mexican haplotypes are lacking. There is a group formed by one A2 central haplotype, related to samples from Texas (Tx), Londrina (Lo) and Puerto Península (Pe), and a second group including Iguazú haplotypes (Ig). In this network, one haplotype from the Pe sample is closer to those from cotton fields than to that from Iguazú.

Bayesian trees

Bayesian searches were performed using the Felsestein (1981) and Tamura and Nei (1993) models of nucleotide substitution for COI and COII sequences, respectively. The topologies of the Bayesian trees (Figure 2(a) and (b)) suggest similar results as the MP phylograms. The COI tree (Figure 2(a)) shows two groups clearly differentiated: the "Mexican group" and a second cluster including the Texas-South American cotton samples and the Misiones samples from wild vegetation areas. Individuals from Iguazú form a disctinct clade separated from the samples associated to cotton fields. The COII tree (Figure 2(b)) shows similar pattern as the COI tree, with a single

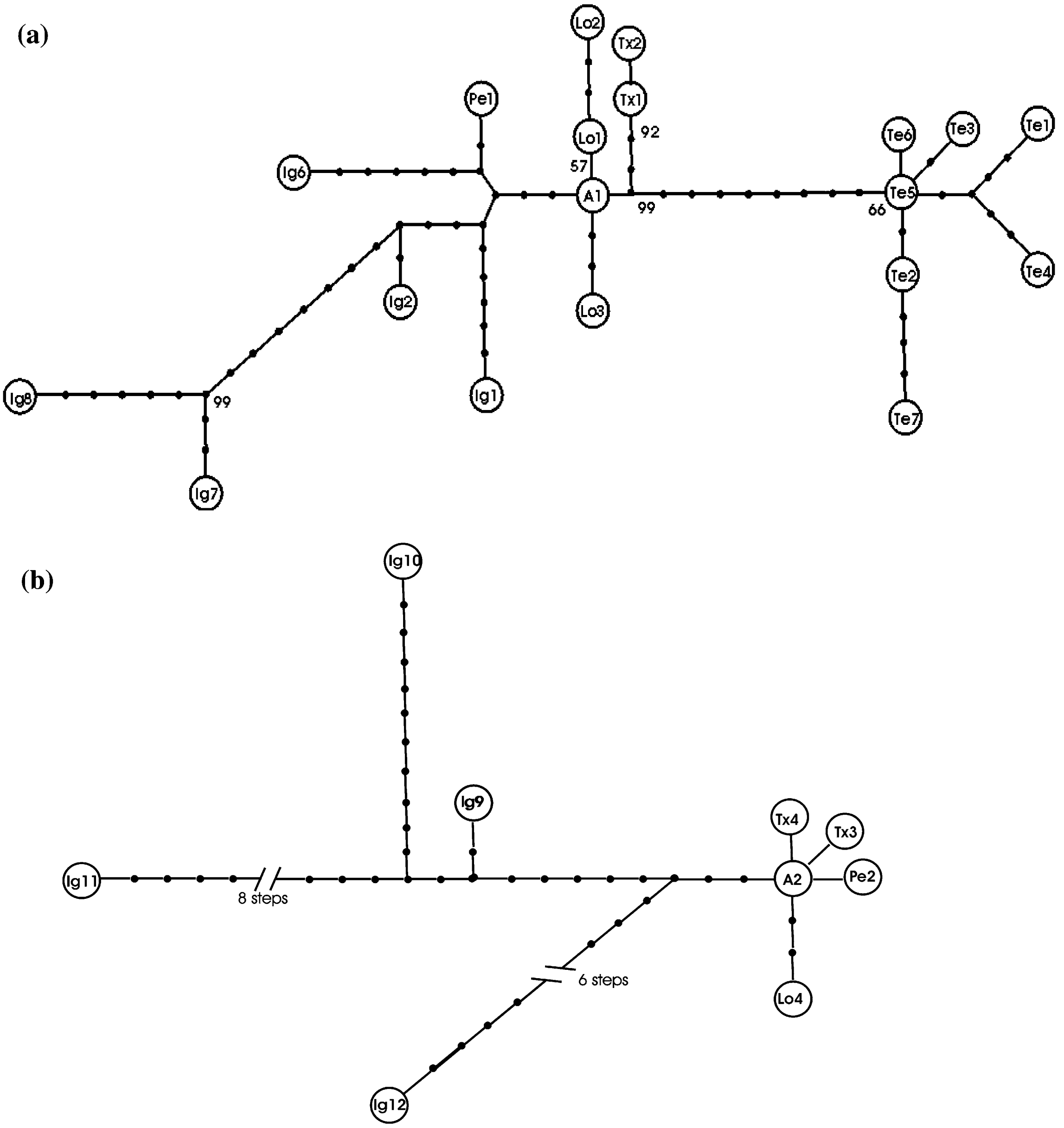


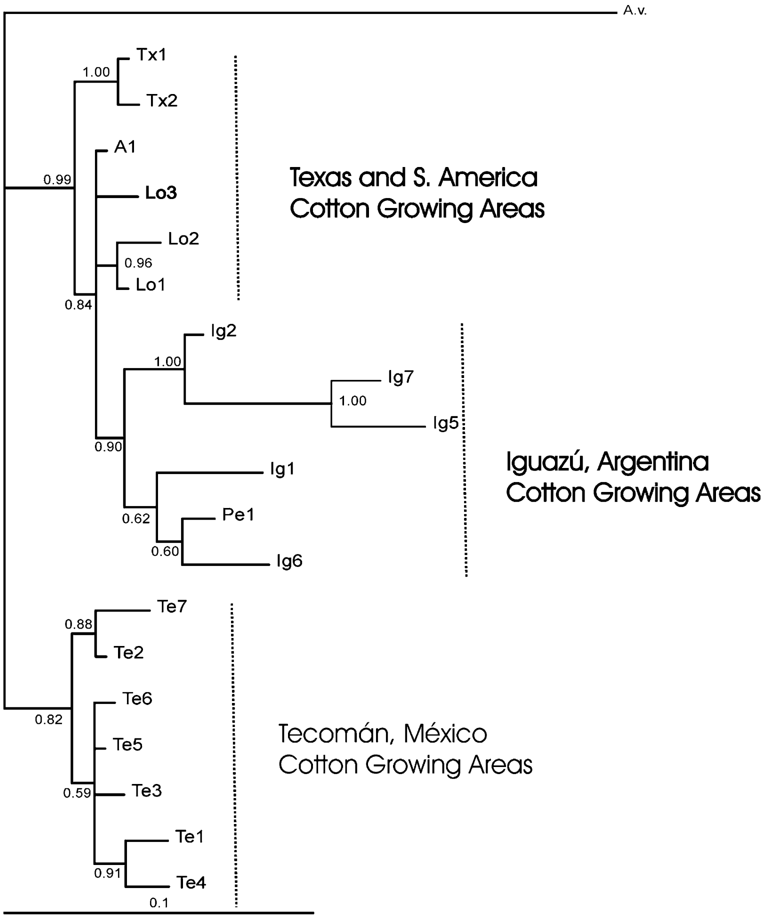
Figure 1. Unrooted COI (a) and COII (b) networks based on Maximum Parsimony analyses. Bars represent connections between haplotypes and correspond to one mutational step. Intermediate haplotypes missing in the sample are represented by "0". Acronyms of haplotypes according to Table 1. Numbers on nodes correspond to Bootstrap values.

well-supported group corresponding to the Iguazú sample.

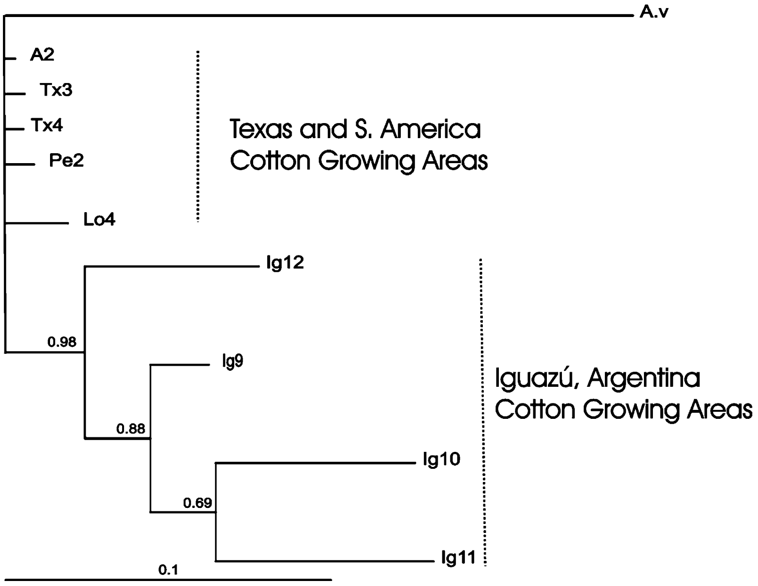
Discussion

Phylogeographic approaches are particularly useful for the analysis of population structure at different levels and for testing hypotheses on related historical events (Avise et al., 1987). The phylograms based on COI and COII haplotypes reveal that *A. grandis* has a strong population structure, as indicated by the distinct phylogroups (= star-like groups) in both networks. Each of these phylogroups shows a central, most frequent and widespread haplotype, from which derive

(a)


A.v.
Tx1
1.00
Tx2
A1
0.99
Lo3
Lo2
0.96
Lo1
0.84
Texas and S. America
Cotton Growing Areas
Ig2
1.00
Ig7
1.00
Ig5
0.90
Ig1
0.62
Pe1
0.60
Ig6
Iguazú, Argentina
Cotton Growing Areas
Te7
0.88
Te2
Te6
0.82
Te5
0.59
Te3
Te1
0.91
Te4
0.1
Tecomán, México
Cotton Growing Areas

(b)


A.v
A2
Tx3
Tx4
Pe2
Lo4
Texas and S. America
Cotton Growing Areas
Ig12
0.98
Ig9
0.88
Ig10
0.69
Ig11
0.1
Iguazú, Argentina
Cotton Growing Areas

◄

Figure 2. Bayesian phylogenetic analyses of COI (a) and COII (b) haplotypes. Acronyms of haplotypes according to Table 1. Numbers on nodes correspond to Bayesian probabilities. The geographic distribution and associated vegetation of the main groups are indicated. A.v = *Anthonomus vestitus*.

other related haplotypes. These central haplotypes are interpreted as ancestral or more ancient than the rest within the group. For example, A1 and A2 haplotypes occupy a central position for the phylogroups recovered in the COI and COII networks, being the most common in samples taken from cotton fields or nearby disturbed areas, both in USA and South America; and Te5 is the central haplotype for the "Mexican group" in the COI phylogram.

The "Mexican group" is separated from the remaining haplotypes of the COI phylogram by several mutational steps. This is consistent with the long geographical distance between this phylogroup and those from South America, and with its high degree of morphological differentiation. From a morphological viewpoint, the "Mexican form" of *A. grandis* has been distinguished from the "Southeastern form", which is typical of southeastern USA from Texas to the Atlantic, and from the "Thurberia form", characteristic of the Sonoran desert in northwestern Mexico and southwestern USA (Burke, 1968; Burke et al., 1986). These "informal forms" were previously treated as subspecies, but the use of scientific names was avoided after the finding of numerous intermediates in natural hybrid zones and agricultural areas.

On the other hand, the close genetic relationship among the Texas and most of the South American samples from cotton fields is not correlated with geographical distance. This is in agreement with Burke et al. (1986), who stated that boll weevils from Brazilian cotton fields and the "Southeastern form" seem to have a similar morphology. In addition, this result strongly supports the traditional hypothesis proposing that this pest has been introduced from USA to South America as a result of cotton trade.

The highly differentiated phylogroup with strongly divergent haplotypes found in the sample from Iguazú National Park, an undisturbed natural area of South America, suggests that this is an ancestral boll weevil lineage, as previously proposed by Scataglini, Cofalonieri and Lanteri (2000). In this case, geographical distance cannot explain genetic differentiation because Iguazú is very close to the localities of Caacupé (Paraguay) and Laguna Naick Neck (Argentina), but their haplotypes do not gather in the same phylogroup as expected, except in the case that a past fragmentation event had occurred. On the contrary, populations from Caacupé and Laguna Naick Neck are monomorphic for the A haplotype, which is absent in the Iguazú sample. From a morphological point of view, in South America it is possible to distinguish two boll weevil forms, although with several intermediates. Specimens from cotton fields (Figure 3(a) and (b)) are usually robust, with very convex elytra, strongly curved rostrum (Figure 3(a)), and the teeth of the front femora are wide and short (Figure 3(b)); those from the "Iguazú group" are usually smaller, less robust, with moderately convex elytra, slightly curved rostrum (Figure 3(c)), and the teeth of the front femora are more slender, pointed and longer (Figure 3(d)). Although the distinction between these two forms is congruent with the differentiation recovered by mtDNA phylogenetic analysis, we consider it is premature to propose that these lineages are cryptic species.

Recent invaders

Populations of an insect pest that is a recent invader are expected to have very few haplotypes or a single haplotype remnant of a "bottleneck" appearing after colonization. This is probably the case for South and North American samples from cotton fields showing low or null haplotypic variability.

It is interesting that the Puerto Península (Pe) sample, which is mostly composed by A, has one haplotype in the COI phylogram (Figure 1(a)) and in the Bayesian tree (Figure 2(a)), which is in the same group of Iguazú; however, another haplotype of the COII phylogram (Figure 1(b)), and also of the Bayesian tree (Figure 2(b)), is included in the A group outside the Iguazú group. Puerto Península is located in a disturbed area right outside the Iguazú National Park, next to the cotton fields of Paraguay, the neighboring country. Hence, the different composition of the samples through time is likely to be explained by weevils coming from two different sources: a protected area (Iguazú) and nearby cultivated fields (Para-

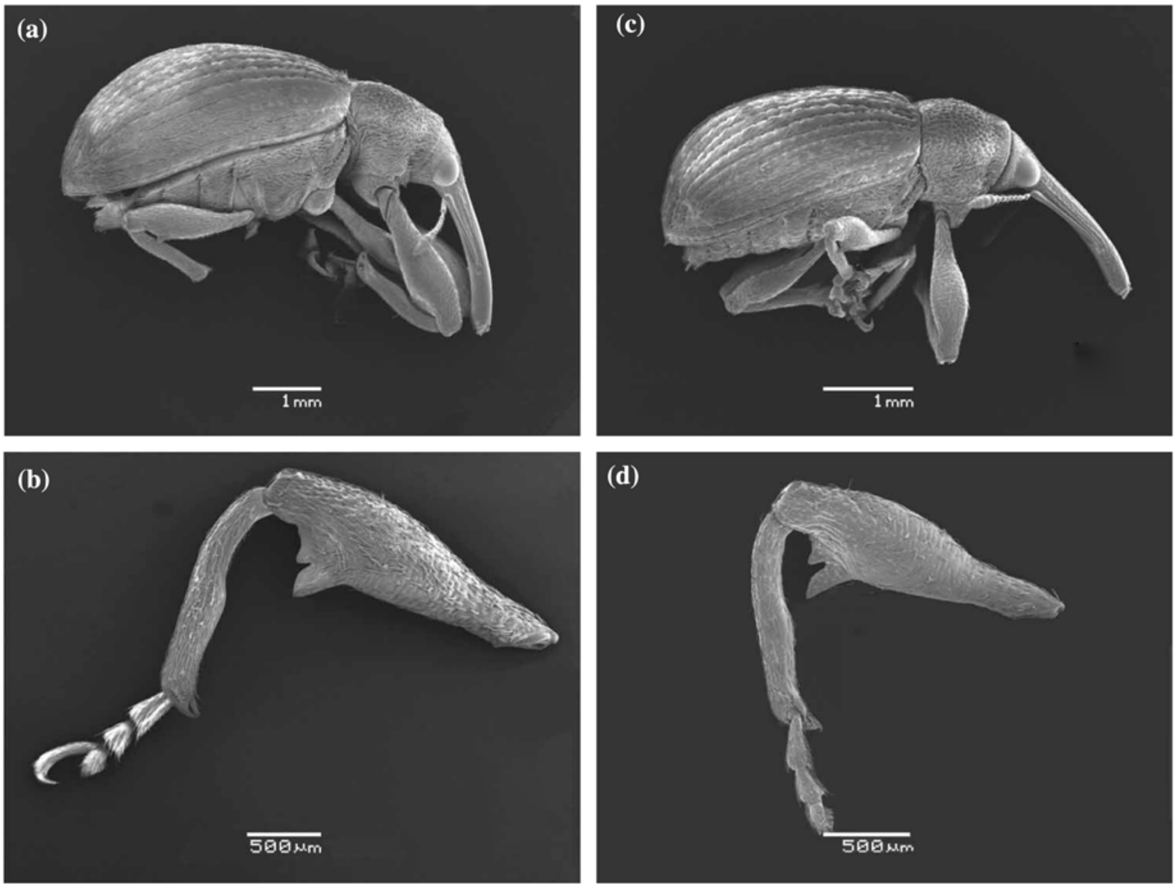


Figure 3. Scanning electron microscope photographs of the two most distinct morphs of South American boll weevils. (a–c): lateral habitus. (b–d): front femora and tibiae. (a,b): specimen associated to cotton growing areas (Laguna Naick Neck, Formosa, Argentina). (c,d): specimen associated to wild vegetation area (Iguazú National Park, Misiones, Argentina).

guay). In this context, boll weevils that were collected in traps on the border of Argentina and Paraguay could have dispersed from harvested cotton fields, under the effect of predominant winds (Manessi, 1997). A similar explanation has been proposed for boll weevils infesting cotton fields in Arizona (USA), which were found to belong to the ''Thuberia form'' from wild vegetation (*Gossypium thurberi*), and to the ''Southeastern form'' from cotton fields (Burke et al., 1986). This highlights the importance of sizes and distances from source populations on wild hosts when making predictions about the characteristics of weevils invading cultivated areas.

The fact that Caacupé and Laguna Naick Neck populations are monomorphic for the A haplotype is consistent with the ''bottleneck'' assumption, related to the hypothesis of introduction of boll weevils from USA. This hypothesis would be supported by historical records and information provided by agricultural services according to which the boll weevil irrupted in cotton fields of Brazil during the 80's and invaded Paraguay at the beginning of the 90's. The pest may have reached Argentina via Paraguay, mainly across the border with the Formosa Province.

Even though the hypothesis of one or more introductions from single or multiple foci sounds as the most plausible, it may not be the only explanation for the presence of a widespread A haplotype. Vicariance could also account for the disjunct distribution of the A haplotype in Texas and agricultural areas of South America. Accordingly, the former geographical range of *A. grandis* was probably broad and continuous from Texas to Argentina, and a posterior fragmentation of an ancient biota might have caused the extinction of the A haplotype in intermediate areas, if it is confirmed that is not present throughout them. Several vascular plants and phytophagous insects exhibit this biogeographical pattern (Raven, 1963; Lía et al., 2001), and there is no consensus about the historical reasons (long-distance dispersal or vicariance) for the disjunctions. For example, *Cienfuegosia drumondii*, a wild host plant of the boll weevil in southern Texas (Burke & Clark, 1976), also occurs in xerophytic vegetation areas of Argentina and Paraguay (Chacoan domain or subregion sensu Cabrera and Willink (1980), or Morrone, (2000), (2002), respectively), being absent in intermediate areas (Fryxell, 1979). Consequently, the current distributions of the A haplotype of *A. grandis* and *C. drumondii* could be interpreted as relicts of a broad area of semiarid vegetation dated as Plio-Pleistocene (Axelrod, 1950, 1958).

Warmer-humid and cool-dry cycles in the Plio-Pleistocene may have determined successive expansions and retractions of the South American forests, and divergence, secondary contacts, and extinctions of several groups of organisms (Simpson Vuilleumier, 1971; Haffer, 1977, 1982; Prance, 1982). Indeed, infraspecific phylogroups shown by mitochondrial DNA analyses have been used to interpret these historical events of alternate cycles of vicariance (genetic divergence) and dispersal (secondary contractions) at subspecies and population levels, such as those of the Glacial period in South America (Avise & Walker, 1998; Avise, 2000).

Wisconsin-Würm glaciation would have caused a major contraction of tropical forests and a continuous environment of xerophytic biogeographical units (Ab'Sáber, 1977). The Caatinga, Cerrado and Chaco provinces of the Chacoan subregion, might have been connected by a Pleistocene "savanna corridor", also called "diagonal of open formations" or "diagonal of dry vegetation" (Prado & Gibbs, 1993). The highest species diversity of the xerophytic genus *Cienfuegosia*, which comprises several boll weevil hosts, is registered along this "savanna corridor", and the allopoliploid *Gossypium mustelinum* is endemic to northeastern Brazil (driest Caatinga province). In this regard, it is worthy to mention that the first two infestation foci of the boll weevil in cotton fields were detected in these areas (Manessi, 1997), which in turn suffered the greatest impact due to deforestation and agricultural practices since the 80's, particularly at the Brazilian Cerrado province.

In brief, although the presence of a widespread A haplotype with disjunct distribution in Texas and agricultural areas of Brazil, Paraguay and Argentina, is consistent with the hypothesis of introduction, alternative explanations should not be discarded or ignored, especially because intermediate areas have not been investigated yet.

Ancient populations

The high haplotypic diversity of the Mexican and the Iguazú groups, confirms previous results based on RAPD analyses, according to which these samples show the highest percentage of polymorphic loci and levels of heterocigozity (Scataglini, Confalonieri & Lanteri, 2000).

In the case of the "Mexican group", this evidence strongly supports the hypothesis that *A. grandis* originated in one area of southern México (forests of Chiapas) and Guatemala (Burke et al., 1986; Jones, 2001), which has the highest species diversity among *Hampea*, the putative ancestral host of the *A. grandis* group (Fryxell & Lukefahr, 1967; Jones & Burke, 1997; Jones, Fryxell & Baro, 1997). However, this hypothesis is mainly based on the theoretical concept of "center of origin", critized by most modern historical biogeographers because it favors dispersal over vicariance (Croizat, Nelson & Rosen, 1974; Nelson & Platnick, 1981). From a modern viewpoint, ancestral biotas expanded, contracted and fragmented giving rise to new evolutionary units (species, subspecies, metapopulations), which enlarged or reduced their geographical ranges, re-establishing contacts among previously isolated populations, or leading them to extinction.

Information on fossil records, including one undescribed *Anthonomus* from the Dominican amber, would indicate that *A. grandis* occurred in Mexico by at least late Pliocene (Burke et al., 1986). At this geological time, the Panamá bridge was probably already elevated, and the South American biota became re-connected with that of Central and North America after a long period of geographical isolation (Halffter, 1964, 1974; Raven & Axelrod, 1975; Rzedowski, 1993).

According to the results of our phylogeographic analyses, the Mexican sample represents an ancient group in comparison with those from xerophytic areas of North and South America associated with cotton, but the original area of distribution of *A. grandis* cannot be assessed with certainty, because the Iguazú sample also exhibits features of an ancient population.

The Iguazú National Park is located in Argentina and Brazil, and is part of the Paranaense subtropical forest within the Amazonian domain or subregion (Cabrera & Willink, 1980, or Morrone, 2001, respectively). The boll weevil population at this site probably evolved in isolation during a long period, as a consequence of events of habitat fragmentation (*e.g.* expansions and retractions of the subtropical forests during the Plio-Plesitocene cycles). This may account for its high variability and the absence of haplotypes in common with geographically close populations. At present, however, this population would be undergoing secondary contact with populations coming from more xerophitic and/or severely disturbed neighboring areas, either by agricultural practices or other anthropic factors. Apparently, a similar situation is taking place in protected areas of subtropical forests of Misiones and Brazil. For example, molecular studies on boll weevil populations from Misiones using RAPD techniques of high resolution (Guzmán et al., 2004), show that genetic variability is much higher at the natural reserve of Urugua-í than in surrounding areas under the influence of cotton cultivation.

The finding of an ancient population of *A. grandis* in South America opens some questions concerning previous ideas on the phylogenetic relationships between the boll weevil and its ancestral host plants. These relationships are still uncertain because of the little information available on the over 500 Neotropical species included in *Anthonomus* and related genera (Lanteri, Marvaldi & Suárez, 2002). The boll weevil was assigned to the *A. grandis* group, which includes other four species from Mexico and Costa Rica, presumably related to the Neartic subgenus *Anthonomorphus* Dietz, associated to Malvae (Burke, Clark & Cross, 1984; Clark & Burke, 1986). On the other hand, *A. grandis* has been suggested to be a derived species of *Loncophorus* Chevrolat, a Neotropical genus associated to Bombacaceae and Tiliaceae (Clark 1988, 1995; Jones, 2001). Indeed, the lack of navicular elytra has led to consider *A. grandis* as a *Loncophorus* species (Clark, 1995), probably related to *L. costalimai* Clark found in Matto Grosso, Brazil (Clark, 1988).

Changes in the taxonomy of the host plants have also contributed to the uncertainty about the relationships among *A. grandis* and the *A. grandis* group. In this sense, Bombacaceae was previously assigned to tribe Bombae of Malvaceae, *Hampea* was transferred from Bombacaceae to Malvaceae, and Gossypieae was previously placed in Bombacaceae or within or near Hibisceae. The secondary compounds that usually limit the host range of phytophagous insects (Becerra, 1997), are probably similar in these plant taxa.

Anthonomus grandis is a oligophagous weevil (Burke, 1976; Anderson, 1993), with larvae developing in three species of *Hampea*, several *Gossypium* and *Cienfuegosia*, the Pantropical species *Thespesia populnea*, and occasionally, in other Gossypieae and *Hibiscus* (Cross et al., 1975; Burke et al., 1986). The remaining four members of the *A. grandis* group are usually monophagous, have a single species of *Hampea* as host, and show narrower geographical ranges: *A. hunteri* occurs in the Yucatán Península, and *A. mallyi, A. townsendi*, and *A. palmeri*, in small areas of southern Mexico and Costa Rica (Jones & Burke, 1997; Jones, 2001).

Hampea is a relatively young genus of Malvaceae, with 21 species distributed over Mexico, Central America and Colombia. Some of these inhabit rainforests, while others are adapted to montane habitats or to regions subject to rainy-season-dry-season cycles (Jones, Fryxell & Baro, 1997). *Hampea* has been proposed as the ancestral host for both *A. grandis* and the whole *A. grandis* group (Burke & Cate, 1979; Burke et al., 1986; Jones & Burke, 1997; Jones, 2001), despite the deficient knowledge of_boll weevil populations and their host plants in South America.

On the contrary, *Gossypium* and *Cienfuegosia* are ancient genera that probably started their differentiation in the Americas during the Cretaceous-Tertiary, and continued their diversification at lower taxonomic levels, during the climatic cycles of the Plio-Pleistocene (Fryxell 1965, 1979). In addition, Burke et al. (1986) proposed that the northward range expansions of the boll weevils along the Pacific and Gulf coasts from southern Mexico to the United States, accompanied the diversification of *Gossypium* and *Cienfuegosia*. These range expansions would have been favored by the simultaneous occurrence of salt-tolerant species adapted to marine habitats (Fryxell 1965, 1979), and broader coastal areas resulting from the drop of the sea level (Donn, Farrand & Ewing, 1962).

Gossypium is regarded as a "key genus" to interpret the past and present distribution of *A. grandis* because it develops on several wild and semi-wild species and varieties of the subgenera *Houzingenia* and *Karpas*, which also occur in houseyard gardens (Cross et al., 1975). The geographical range of the boll weevil is coincident with that of *G. barbadense* (from Central America to Argentina and Paraguay, with its highest variability in the Cauca Valley, Colombia) (Hutchinson, Silow & Stephens, 1947); G. *hirsutum* (native to Central America); *G. thurberi* (southwestern North America) and *G. mustelinum* (northeastern Brazil) (Fryxell, 1979). The importance of *G. barbadense* as alternative host plant for the dispersal of the boll weevil in South America was documented by Argentine agricultural authorities, who led a campaign to remove this *Gossypium* species from subtropical and western areas of Salta and Formosa Provinces, respectively. This measure aimed to prevent potential invasions from Bolivia (Gutiérrez et al., 1960), where the presence of the insect was not detected until 1997.

Cienfuegosia shows the highest species diversity in Paraguay and Argentina, which are inhabited by 11 of its 23 species and four of the five sections belonging to *Cienfuegosia* subgenus (Krapovickas, 2000). Its distribution throughout the Americas is pretty much coincident with the current geographical range of *A. grandis* from Texas to Argentina, including Cuba and Caribbean islands near Venezuela. At present, four species of *Ciefuegosia* are known as boll weevil hosts: *C. rosei* (Southern Mexico, Pacific coast), *C. affinis* (Bolivia, Brazil, Paguay and Venezuela), *C. argentina* (Argentina, Bolivia and Paraguay) and *C. drumondii* (Argentina, Paraguay and Texas), but future studies will probably lead to the discovery of new hosts within this plant genus.

In conclusion, *Hampea* is unlikely to be the ancestral host of *A. grandis* if boll weevil populations were ancient in South America, and this host plant would have spread widely throughout the continent since Plio-Pleistocene times. Consequently, we propose *Gossypium* and *Cienfuegosia* as the ancestral hosts because of their ancient origin and wider distribution in America, and *Hampea* as the secondary host. This hypothesis fits better with a modern viewpoint on host-plant selection by phytophagous insects and on the relevance of host biogeography to insect-host associations (Bernays & Chapman, 1994; Schoonhoven, Jermy & Van Loon, 1998; Becerra & Venable, 1999).

Unfortunately, although recent molecular analyses provided new insights on the evolution of *Gossypium* (Wendel, Schnabel & Seelanan, 1995; Zhao et al., 1998; Small & Wendel, 2000), the long lasting and complex history of cotton cultivation and hibridization in America has blurred some phylogenetic signals useful to reconstruct boll weevil history. The evolution of sibling species (or varieties) of *Gossypium* since the Pleistocene, was followed by earliest stages of domestication of cotton, about 9000–7000 years ago (Phillips, 1963; Fryxell, 1965; Brubaker & Wendel, 1994). At that time, wild and primitively cultivated cotton were in close geographical proximity. After Columbian colonization, the agricultural practices and commercial exchange increased and expanded, and several *Gossypium* and other Gossypieae are being used as ornamentals (*e.g. Thespesia populnea*) or houseyard plants (*e.g. G. barbadense*) (Fryxell, 1979; Manessi, 1997).

Although historical biogeographical factors prior to cotton cultivation determined the distribution of boll weevil haplotypes in Argentina, Brazil and Paraguay, the explosive increase in population densities of the insect during the last two decades, was probably the result of massive destruction of native environments and their replacement by cotton (Scataglini Confalonieri & Lanteri, 2000). This recent historical event that took place mainly along the "savanna corridor" of South America, resembles that of the Cotton Belt of the United States, between 1898 and 1916, but the environmental conditions (temperature, humidity and host plant diversity) in Argentina, Brazil and Paraguay are probably more favorable for the development of the insect. In both cases, cultivated cotton favored the rapid dispersal of the pest, thus causing important damages and economic losses.

Without any doubt, phylogeography using mitochondrial genes provides an excellent tool to investigate the evolution of insect pests. However, the former hypothesis on *A. grandis* history could only be tested after the analysis of samples from several sites within its distribution range, especially between Mexico and Argentina (*e.g.* Central America, Colombia, northeastern Brazil and Piedmont areas of Bolivia), since it is possible that other ancient lineages occur along this range. The only way to clarify the evolutionary scenario for *A. grandis* is through studies including the phylogeny of all putative boll weevil relatives, and detailed information on the phylogeny and phylogeography of their host plants.

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