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Using population genetic methods to identify the origin of an invasive population and to diagnose cryptic subspecies of *Telchin licus* (Lepidoptera: Castniidae)

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Abstract

Telchin licus, the giant sugarcane borer, is an important pest species of sugarcane in northeast Brazil. Four subspecies of *Telchin licus* are recognized in Brazil based on their geographic distribution and subtle differences in wing colour pattern. Some taxa are morphologically indistinguishable, and their accurate identification is key to their efficient control. Mitochondrial genes sequences (*cytochrome oxidase* I and subunit 6 of the *nicotinamide adenine dinucleotide dehydrogenase*) were applied to delimit taxonomic entities of *T. licus*, and to infer the origin of a newly established population in the state of São Paulo. The molecular data indicated that specimens sampled at different regions in Brazil are morphologically cryptic but genetically isolated entities, and at least three subspecies were assigned to the sampled localities. These data also suggested that the population collected from the state of São Paulo must have a common origin with populations from northeast Brazil, which corroborate the hypothesis that ornamental plants infested with larvae of *T. licus* might have been transported from the northeast to the southeast regions.

Keywords: cryptic taxa, giant sugarcane borer, mitochondrial DNA, taxonomic diagnosis

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Introduction

The correct identification of species or intraspecific entities must be the primary goal for the accomplishment of biodiversity surveys, biological control and pest management strategies (Rosen, 1986; Paterson, 1991; Mills & Kean, 2010). Correct taxonomy is also essential to prevent, detect and

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respond to the invasion of a species in a new area (Douglas *et al.*, 2009). Hence, species misidentification can hinder attempts toward their conservation or control. The inaccurate recognition of species complexes can lead to erroneous employment of pest control approaches as different species can cause variable degrees of damage (Rugman-Jones *et al.*, 2010), and can present divergent answers to control measures (Ríos-Díez & Saldamando-Benjumea, 2011) and patterns of resistance to pesticides (Bickford *et al.*, 2007).

Telchin licus (Drury), the giant sugarcane borer, is the main pest attacking sugarcane (*Saccharum officinarum* L.) in northeast Brazil. Primarily restricted to the north and northeast regions, the giant sugarcane borer was recently reported in southeast Brazil, in the state of São Paulo (Almeida *et al.*, 2007), the main sugarcane producer area in Brazil, raising concerns about its control. It has been suggested that *T. licus* was introduced into sugarcane-producing areas through the transport of ornamental plants (Almeida *et al.*, 2007), as already reported for the castniid *Paysandisia archon* (Burmeister) in Europe (Sarto i Monteys & Aguilar, 2005). However, the origin of the population of *T. licus* currently found in São Paulo is still an issue under investigation.

Telchin licus is part of a species complex that includes *Telchin syphax* (Fabricius) and *Telchin atymnius* (Dalman), new combinations after *Erythrocastnia* and *Castniomera* were synonymized with *Telchin* (Gonzalez & Cock, 2004; Moraes & Duarte, 2009). There is scarce morphological variation among species in the *T. licus* complex, and taxa are commonly identified based on their wing colour pattern, despite the fact that several Neotropical Castniinae are mimetics (Miller, 1986). *Telchin licus* and *T. atymnius* share some sympatry in their distribution range, and both are known to feed on *S. officinarum* and Musaceae (Moraes & Duarte, 2009). Yet, their accurate identification becomes even harder as 12 geographically differentiated subspecies are recognized for *T. licus*, four of them in Brazil: *Telchin licus licus* (Drury), *Telchin licus albomaculata* (Houlbert), *Telchin licus laura* (Druce) and *Telchin licus rubromaculata* (Houlbert) (Lamas, 1995). These subspecies were proposed mainly based on their geographical distribution and subtle differences in wing colour. Little is known either on their limits or on the phylogenetic relationships among them.

Therefore, considering the sparse knowledge of the group and the economic importance of *T. licus*, there is an urgent need for a reliable source of information that would allow the proper identification of species/subspecies belonging to the *T. licus* species complex. Molecular markers, such as mitochondrial DNA (mtDNA), are excellent candidates to solve the taxonomic issues faced in the identification of species of the *T. licus* complex, as they have been successfully used to solve taxonomic problems where morphological data is uninformative, aiding in the definition of species boundaries and in the identification of the origin of new populations (Sperling & Hickey, 1994; Peña *et al.*, 2006; Behere *et al.*, 2007; Porreta *et al.*, 2007; Norgate *et al.*, 2009). Indeed, a region of the animal mtDNA was proposed as a 'DNA barcode' for species diagnoses and delimitation (Hebert *et al.*, 2003). The benefit of using mtDNA lays on its intrinsic characteristics, such as the high rate of evolutionary change, mostly maternal inheritance, high genetic polymorphism among conspecifics and the availability of universal primers (Avise, 1986; Moritz *et al.*, 1987; Simon *et al.*, 1994).

The objectives of this study were to use mtDNA to (i) investigate the origin of specimens of *T. licus* sampled from the state of São Paulo by using population genetics methods in order to understand the dissemination of this important pest throughout Brazil, and (ii) assess the effectiveness of mtDNA, and specifically of the 'barcode' region, in diagnosing *T. licus* taxonomic units and in recovering the relationships among them.

Fig. 1. Map of Brazil with *Telchin licus* populations' origin indicated.

Material and methods

Samples

Specimens of *T. licus* were sampled from five sites in northeast, southeast and central Brazil in 2009 and 2010. Larvae were collected from sugarcane plants from Coruripe, AL (CO_AL, $n=10$), São Miguel, AL (SM_AL, $n=10$), Limeira, SP (LI_SP, $n=10$), Tangará da Serra, MT (TS_MT, $n=11$) and Araporã, MG (AR_MG, $n=3$) (fig. 1) and were immediately fixed in 96% ethanol. Vouchers of adults from each sampling locality were obtained by rearing larvae until pupation and later adult emergence, and were maintained in the Centro de Tecnologia Canavieira (CTC) by L. C. Almeida. Additionally, two adults of *T. licus* were sampled from the Amazon Forest region in the margins of Madeira River, *ca.* of 150 km from Porto Velho, RO (PV_RO); vouchers were preserved in the Museu de Zoologia of Universidade de São Paulo (MZUSP) (Voucher codes: C1_Mol07 and C2_Mol08). Sample sets from each locality were treated as separated populations.

DNA extraction, amplification and sequencing

Genomic DNA was obtained according to the Invisorb Spin Tissue kit (STRATEC Molecular, Berlin, Germany) protocol from the last abdominal segment of each larva and from one leg of each adult. DNA extractions were stored in TE buffer at −20°C. The mitochondrial genes *cytochrome c oxidase* I (*cox1*, *ca.* 1480 bp) and the subunit 6 of the *nicotinamide adenine dinucleotide dehydrogenase* (*nad6*, *ca.* 500 bp) were amplified using the follow primers combinations: LCO (5′ GGTCAACAAATCATAAAGATATTGG) + HCO (5′ TAAACTTCAGGGTGACCAAAAAATCA) (Folmer *et al.*, 1994) and Jerry (5′ CAACATTTATTTTGATTTTTTGG

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3′) + PatII (5′ TCCATTACATATAATCTGCCATATTAG 3′) (Caterino *et al.*, 2001) for *cox1*, and tPro-J10090 (5′ ATCWATAATCTCCAAAATTAT 3′) + ND6-N10624 (5′ GGNCCATAAAAAATATTWGT 3′) (Silva-Brandão *et al.*, 2011) for *nad6*. Reactions were done in a 25 µl final volume using 1 µl of total DNA, 2.0 mM of $MgCl_2$, 40 µM of dNTPs, 0.2 µM of each primer, 1 U of GoTaq DNA Polymerase (Promega, Madison, WI, USA), and 10% of 10 × Taq buffer. The PCR program to amplify *cox1* included an initial denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 45–47°C for 30 s, and polymerization at 72°C for 1.5 min, followed by an extension step at 72°C for 10 min (Silva-Brandão *et al.*, 2005). The amplification protocol for *nad6* was as follows: an initial denaturation step at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 45°C for 45 s, and elongation at 60°C for 1.5 min, followed by an extension step at 60°C for 5 min (Silva-Brandão *et al.*, 2011). Amplicons were purified of primers and deoxynucleotides with ExoSAP-IT (GE Healthcare, Bucks, UK), and then sequenced by ABI Prism BigDye Kit protocol. Mitochondrial regions were sequenced in an ABI 3700 automated sequencer, with primers used for amplification. Sequences were analyzed with the program FinchTV v. 1.4.0 (Geospiza Inc., Seattle, WA, USA), and aligned manually using BioEdit v. 7.0.5.3 (Hall, 1999).

Genetic distances and clustering analyses

All analyzes were applied to concatenated data of the mitochondrial genes *cox1* and *nad6*, and genetic variation and DNA polymorphism were also estimated for each mitochondrial region separately. Standard parameters of DNA polymorphism were estimated in DnaSP v. 5.10 (Librado & Rozas, 2009) and in MEGA v. 5.0 (Tamura *et al.*, 2011). The DNA polymorphism using the sliding window method was estimated for the determination of the number of segregating sites (S) across each portion of the mtDNA sequences, set with a window length = 100 and a step size = 25 (Librado & Rozas, 2009).

A minimum spanning network representing the genealogical relationships of haplotypes was constructed using the method of Statistical Parsimony (Templeton *et al.*, 1992) as implemented in TCS v. 2.1 (Clement *et al.*, 2000), using 95% confidence.

Sequences divergence among individuals of *T. licus* were quantified by using the *p*-distance model of nucleotide substitution (Nei & Kumar, 2000), implemented in the program MEGA v. 5.0 (Tamura *et al.*, 2011). The neighbor-joining (NJ) clustering algorithm (Saitou & Nei, 1987) was applied to graphically obtain the phenetic distance among individuals of *T. licus* estimated by using the *p*-distance model. Robustness of each branch was determined by using 1000 replicates of the non-parametric bootstrapping procedure (Felsenstein, 1985).

Concatenated data of *cox1* and *nad6* was used to estimate a Bayesian inference (BI) tree. The dataset was partitioned in two regions, and the program MEGA v. 5.0 (Tamura *et al.*, 2011) was used to determine the available substitution model with the best fit to each mitochondrial region. The best fit models suggested for *cox1* and *nad6* sequences were HKY + G and HKY (Hasegawa *et al.*, 1985), respectively. The tree was estimated by using the program Beast v. 1.6.1 (Drummond & Rambaut, 2007), with two Markov Chain Monte Carlo runs for 40,000,000 generations, each MCMC starting from a random tree, using a relaxed clock and Yule speciation model, and sampling trees every 5000 generations. The posterior distribution of trees was summarized with TreeAnnotator v. 1.4.7 (Drummond & Rambaut, 2007), using a 20% burn-in and computing the maximum clade credibility tree with average branch lengths.

The effectiveness of the 5′ extremity of *cox1*, the proposed 'barcode' region (Hebert *et al.*, 2003), for diagnosing taxonomic units of *T. licus*, was accessed by constructing a NJ tree with sequences of this fragment (*ca.* 640 bp), by using the nucleotide substitution model Kimura-2-Parameters (K2P) (Kimura, 1980) and 1000 replicates of bootstrap (Felsenstein, 1985). A minimum spanning network was also constructed using this mitochondrial region in TCS v. 2.1 (Clement *et al.*, 2000) with 95% confidence.

Population structure

Within and among population divergences were compared by using standard AMOVA (Excoffier *et al.*, 1992), as implemented in the program Arlequin v. 3.5 (Excoffier & Lischer, 2010). Sequences of adults from Porto Velho (RO) were not analyzed due to the low number of sampled specimens. Two analyses were conducted to quantify the distribution of the molecular variation attributed to the presence of genetic structure: (i) among individuals from all populations (non-hierarchic) and (ii) among clusters recovered with NJ analysis of concatenated datasets. The degree of structure was interpreted by the Φ statistics associated with the different hierarchical levels in which the variation is distributed (Excoffier *et al.*, 1992). The parameters applied were: 10,000 permutations to determine significance, computed distance matrix using pairwise difference, and gamma *a* value equal 0. Pairwise linearized F_{ST} (Slatkin, 1995) was estimated by using Arlequin v. 3.5. The hypothesis of genetic isolation by geographical distance was assessed with the Mantel test (Mantel, 1967), with 10,000 permutations, using Slatkin's linearized F_{ST} (Slatkin, 1995) and linear geographic distances.

Results

The analyzed *cox1* and *nad6* fragments were 1476 bp and 501 bp long, respectively (GenBank accession numbers: *cox1*, JQ685038–JQ685083 and *nad6*, JQ685084–JQ685129). The mean genetic distance among *cox1* sequences was 0.012 (0–0.026). For *nad6,* this value was a little higher, 0.016 (0–0.036). Overall genetic distance was 0.013 (0–0.028) for concatenated datasets (table 1).

The DNA polymorphism of combined datasets using the sliding window method showed sequences of the 3′ extremity of *cox1* (*ca.* 840 bp) as the most variable, followed by sequences of *nad6* and the 5′ end of *cox1* (fig. 2).

The 46 combined mtDNA sequences recovered two disconnected networks with 13 haplotypes each. Fixing the connection limit at 45 steps resulted in a joined network (data not shown). The first network (fig. 3 IA) is composed of the three sample sets from Limeira (LI_SP), Coruripe (CO_AL) and São Miguel (SM_AL). This network shows three haplotypes for samples from LI_SP (6, 7, 8), two of them exclusive occurrences for this locality, six for samples from CO_AL (1, 2, 3, 4, 5, 6) and seven for samples from SM_AL (1, 6, 9, 10, 11, 12, 13). Only haplotypes 1 and 6 are shared among sampled localities. The second network is formed by

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Table 1. Statistical summary of nucleotide sequences of *T. licus*.

	cox1	*nad6*	*cox1*+*nad6*
Total base pairs (bp)	1,476	501	1,977
Number of haplotypes	19	13	21
Haplotype diversity (Hd)	0.830	0.772	0.914
Variable sites	64	24	88
Average number of nucleotide differences (k)	17.529	7.911	25.441
N° of polymorphic amino acids/total	8/492	7/167	15/659
Mean pairwise divergence (%)			
All nucleotide positions	1.2	1.6	1.3
1st/2nd/3rd codon position	0.5/0/3.0	1.3/0.1/3.4	0.7/0/3.1

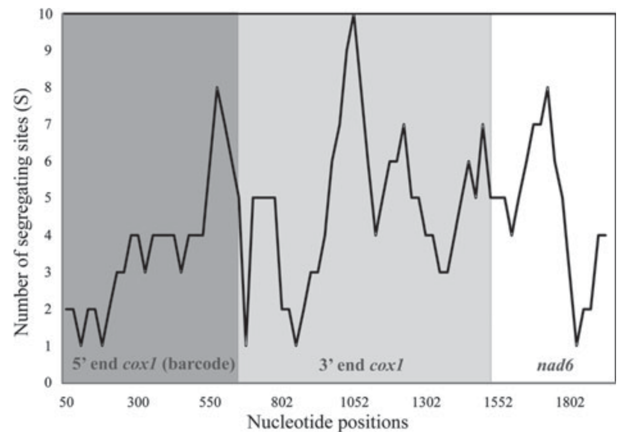

Fig. 2. Number of segregating sites (S) across mitochondrial regions.

samples from Araporã (AR_MG), Tangará da Serra (TS_MT) and Porto Velho (PV_RO) (fig. 3 IB). This network shows nine exclusive haplotypes for samples from TS_MT (from 14 to 22), and two for samples from PV_RO (23, 24) and AR_MG (25, 26).

The NJ and the BI trees clustered the samples in two main clades (fig. 3 II). The clade A is supported by high bootstrap and posterior probability (PP) values, and is formed by samples from SM_AL, LI_SP and CO_AL, with no geographical distinction. Clade B comprises two main clusters: the highly supported clade B1 is formed by samples from AR_MG, and the moderately supported clade B2 is composed by specimens from TS_MT, sampled from sugarcane fields, and PV_RO, sampled from a region of Amazon Forest. Genetic *p*-distance within clade A was 0.1%, 0 within B1 and 0.6% within B2. The genetic *p*-distance was 2.3% between clades A and B1, 2.6% between A and B2 and 1.1% between B1 and B2.

Using only sequences of the 5′ extremity of *cox1* resulted in a weakly supported clade B and a paraphyletic clade B2. Hence, B2 was considered to be two subclades named C1 (with moderate bootstrap support) and C2 (showing weak bootstrap support) (fig. 4 I). There were two segregating sites restricted to samples from TS_MT, one exclusively found in samples from AR_MG, and three shared between the two localities (TS_MT and AR_MG). Sequences of this mitochondrial region alone were not sufficient to recover the clades recuperated by using the concatenated datasets of *cox1* and *nad6*.

Two disconnected networks were recovered with the 5′ end of *cox1*. The first network, composed by samples from LI_SP, CO_AL and SM_AL, only had two haplotypes (data not shown). The other network, composed by samples from AR_MG, TS_MT and PV_RO, had eight haplotypes distributed in a structure similar to the clades found with NJ analysis (fig. 4).

The estimated value of Φ_{ST} for *T. licus* populations was 0.86 ($P<0.001$), and most of the variation was found among populations (table 2). The hierarchical AMOVA among clusters recovered in the NJ tree of combined datasets showed that 89.95% of variation is among groups (table 2). The higher pairwise Slatkin's value was found between populations from AR_MG and LI_SP, while the lowest genetic variation was found between populations from SM_AL and LI_SP (table 3).

The correlation analysis revealed the lack of a significant association between genetic and geographic distances (Mantel test, $P=0.79$).

Discussion

Inaccurate identification, and definition of species boundaries for cryptic pest species or intraspecific taxa have important implications for handling biological invasions (Douglas *et al.*, 2009; Guillemaud *et al.*, 2011) and can result in inadequate application of control measures (Rosen, 1986; Bickford *et al.*, 2007). The taxonomic limits of the Neotropical castniids are frequently based on wing colour pattern and geographical distribution (Moraes & Duarte, 2009; Moraes *et al.*, 2010), even though these traits are not always sufficient to provide a clear picture of the boundaries of these entities, or yet to produce a coherent classification in some cases.

Our analyses of mitochondrial markers indicated a high genetic variability among populations of *T. licus* from Brazil. Indeed, overall Φ_{ST} values, paired F_{ST} and the genealogical network analyzes pointed out to a high genetic structure among populations. This structuring is in agreement with the occurrence of at least four subspecies of *T. licus* in Brazil (Lamas, 1995). Considering Lamas' (1995) checklist and pictures of type specimens, samples from LI_SP, CO_AL and SM_AL (clade A in fig. 3) were identified as *Telchin licus licus* (picture presented in Houlbert (1918) as *Castnia licoides*, Pl. CDXLIV), although its type specimen had been originally sampled from the state of Santa Catarina (Lamas, 1995). We considered the sample set from TS_MT and PV_RO (clade B2 in fig. 3) as *Telchin licus albomaculata* (picture presented in Houlbert (1918) as *Castnia albomaculata*, Pl. CDXLIV). The type specimen of *T. licus albomaculata* is from Amazônia, Brazil, nearby to Peru (Lamas, 1995). Lamas' (1995) checklist presents the name *Telchin licus laura* for Mato Grosso (MT), but this name is currently used for samples from Minas Gerais (MG), and samples from AR_MG were named as such (clade B1 in fig. 3) (photo of the type specimen is available online at http://www2.nrm.se/en/lep_nrm/l/castnia_laura.html). There are indeed two possible hypotheses concerning the identification of this subspecies: (i) the geographical range of *T. licus laura* can be broader than initially inferred from the original type

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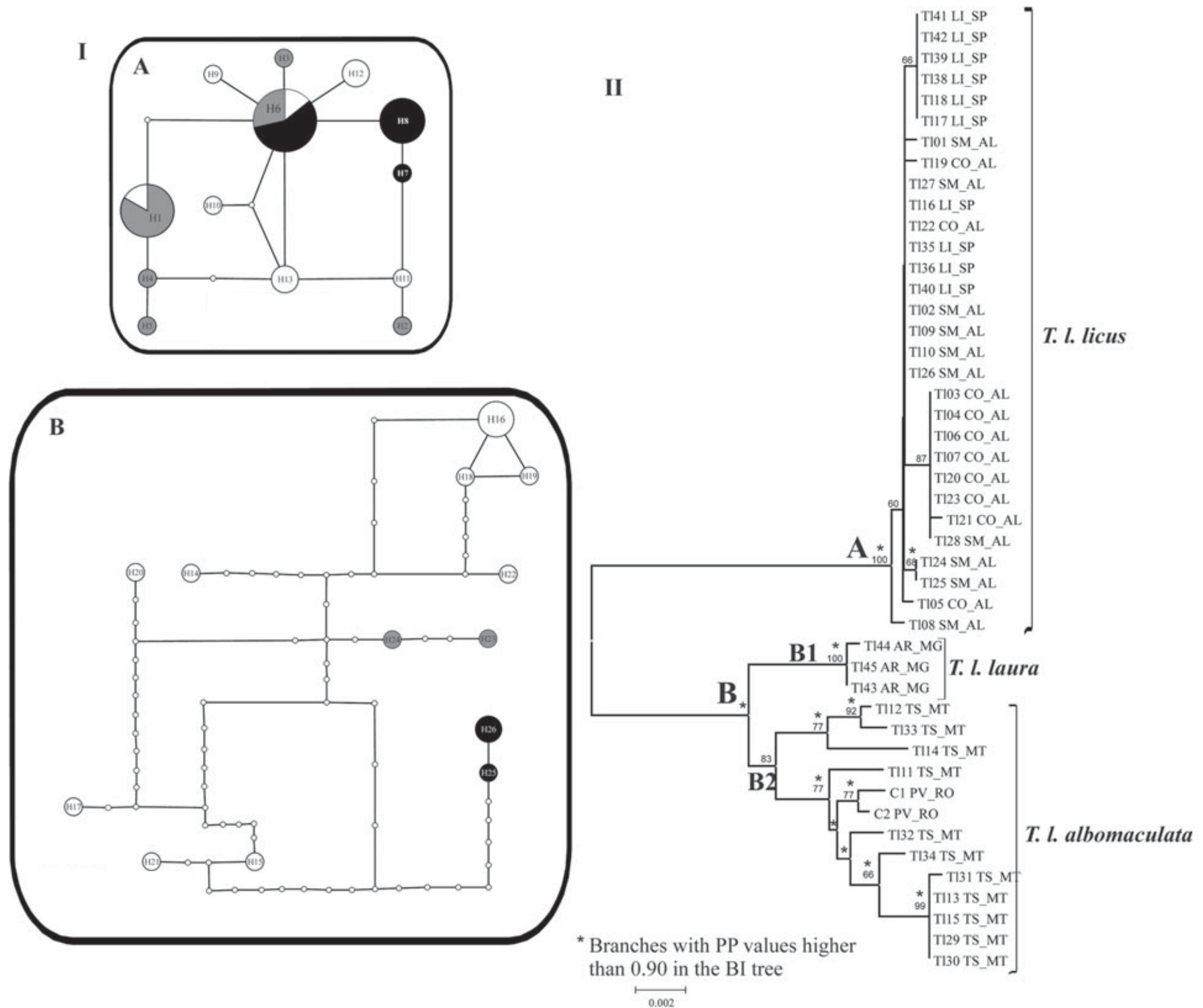

Fig. 3. (I) Minimum spanning networks for *T. licus* populations based on combined datasets of *cox1* and *nad6*. A and B networks are composed of samples from clades A and B in the neighbor-joining tree. Circle areas are directly proportional to the number of individuals showing such haplotype. Small white circles indicate unsampled haplotypes. Each branch is equivalent to one base pair change. (II) Neighbor-joining tree obtained with *p*-distance model of nucleotide substitution of concatenated data of *cox1* and *nad6* sequences. Numbers on the branches indicate bootstrap values of 1000 replicates (when exceed 50%). Branches recovered by Bayesian inference (BI) with posterior probability (PP) values higher than 0.90 are indicated by an *. Sampled sites: CO_AL, Coruripe, AL; LI_SP, Limeira, SP; SM_AL, São Miguel, AL; TS_MT, Tangará da Serra, MT; AR_MG, Araporã, MG; PV_RO, Porto Velho, RO. (I (A) ●, LI_SP = Limeira, SP; ◉, CO_AL = Coruripe, AL; ○, SM_AL = São Miguel, AL; I (B) ●, AR_MG = Araporã, MG; ◉, PV_RO = Porto Velho, RO; ○, TS_MT = Tangará da Serra, MT)

description and extends the limits beyond Mato Grosso, reaching the state of Minas Gerais, or (ii) populations of *T. licus laura* from Mato Grosso and Minas Gerais are indeed distinct entities. In this case, the name could not be applied, and a new name should be proposed for the population of Minas Gerais. This second possibility should only be considered after evaluation of samples of *T. licus laura* from Mato Grosso. Nevertheless, the first hypothesis is more likely as there are three specimens identified as *T. licus laura* in the collection of the Department of Zoology of Universidade Federal do Paraná (OM): one specimen 20-ii-1967 from Uberlândia (MG), one 02-i-1984 from Chapada dos Guimarães (MG) and one 24-i-1968 from Brasília (DF). Additionally, a specimen of *T. licus laura* was recently reported in Paraguay (Rios & Gonzalez, 2011).

Adults of *T. licus laura* are easily differentiated from adults of *T. licus licus* and *T. licus albomaculata* by the presence of an additional submarginal orange patch in the tornus of dorsal and ventral forewings. On the other hand, adults of *T. licus licus* and *T. licus albomaculata* are morphologically indistinguishable; however, our data indicate they are genetically isolated entities. These two subspecies belong to a special category of cryptic taxa; and, in these cases, even the most careful morphological examination does not allow for their differentiation, although they can be recognized as 'genetical species' (Paterson, 1991). Our data suggests a close phylogenetic relationship between *T. licus laura* and *T. licus albomaculata*, but only the inclusion of the unsampled subspecies *T. licus rubromaculata* will permit a broader understanding of the relationships among these subspecies.

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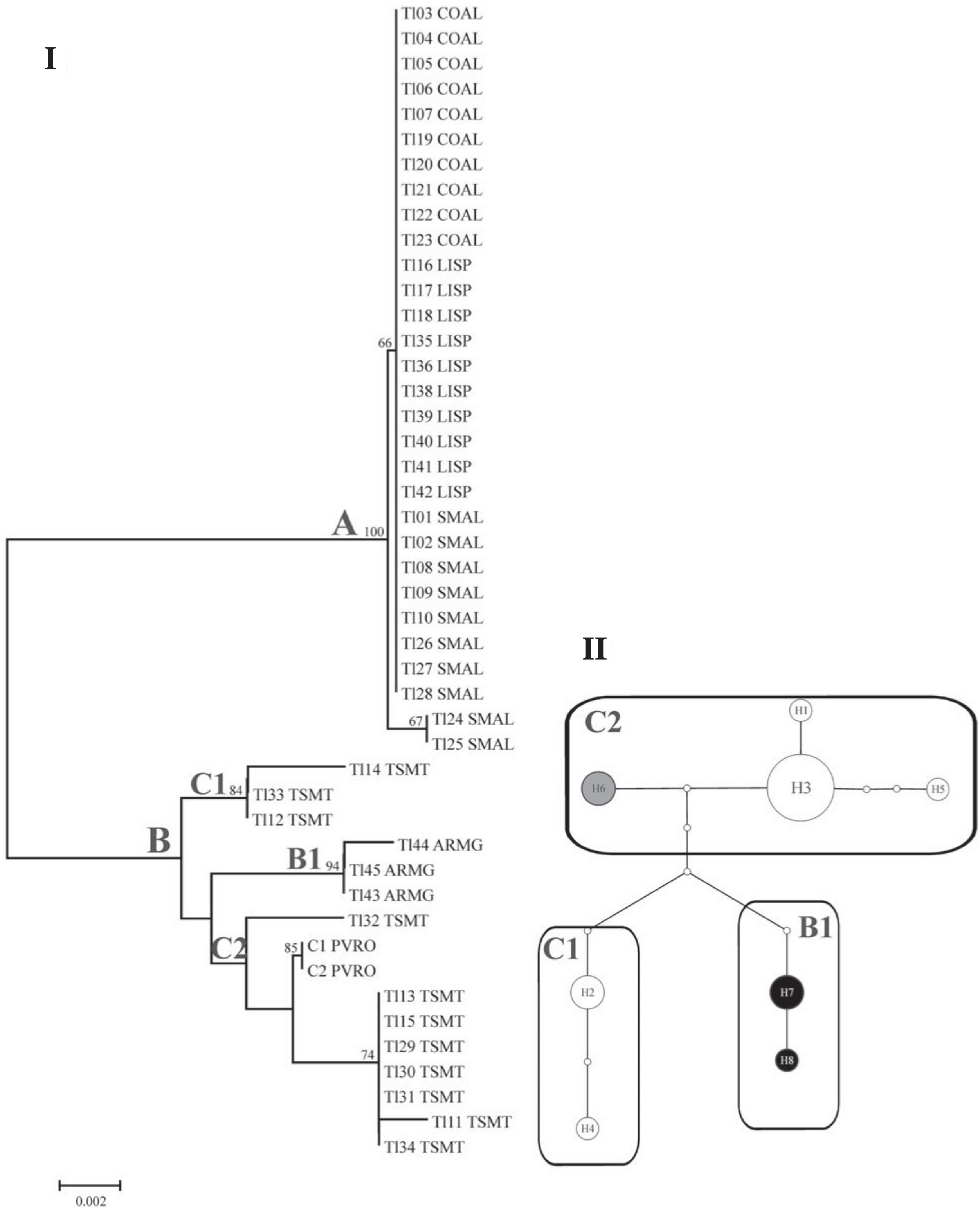

Fig. 4. (I) Neighbor-joining tree obtained with Kimura-2-Parameters model of nucleotide substitution of sequences of the 5′ extremity of *cox1*. Numbers on the branches indicate bootstrap values of 1000 replicates (when exceed 50%). (II) Minimum spanning network for *T. licus* populations based on sequences of the 5′ extremity of *cox1*. (II ●, ARMG = Araporã, MG; ●, PVRO = Porto Velho, RO; ○, TSMT = Tangará da Serra, MT)

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Table 2. Analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) for *T. licus* populations based on combined datasets of *cox1* and *nad6*.

Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation Indices
(a) among all populations					
Among populations	4	449.38	12.88 Va	85.78	$\Phi_{ST}=0.86$; $P<0.001$
Within populations	39	83.23	2.15 Vb	14.22	
Total	43	532.66	15.02		
(b) among clusters					
Among groups	2	440.78	21.05 Va	89.95	$\Phi_{CT}=0.90$; $P=0.103$
Among populations within groups	2	8.60	0.22 Vb	0.93	$\Phi_{ST}=0.91$; $P<0.001$
Within populations	39	83.28	2.14 Vc	9.12	$\Phi_{SC}=0.09$; $P<0.001$
Total	43	532.66	23.40		

Table 3. Slatkin's linearized F_{ST} values (Slatkin, 1995) among populations of *T. licus*. Sampled localities: CO_AL, Coruripe, AL; LI_SP, Limeira, SP; SM_AL, São Miguel, AL; TS_MT, Tangará da Serra, MT; AR_MG, Araporã, MG. All values are significant at significance level 0.05.

	1	2	3	4
1. CO_AL	–			
2. LI_SP	0.80682	–		
3. SM_AL	0.30773	0.21983	–	
4. TS_MT	6.05616	6.56139	5.97958	–
5. AR_MG	24.56071	58.50156	26.88000	1.34971

Barcode diagnosing T. licus *entities*

The proposal of applying a fragment of mtDNA as animal 'barcode' presents two different approaches: the identification or diagnosis of species, and the description or delimitation of new species (Hebert *et al.*, 2003). Most of the current criticism is driven to the second approach (DeSalle *et al.*, 2005). Despite pitfalls of the 'DNA barcode' proposal, the 5′ end of *cox1* has been successfully used for species diagnose in well-studied groups of Lepidoptera (Hebert *et al.*, 2004; Silva-Brandão *et al.*, 2009).

Therefore, the nomenclature proposed above to identify the genetic groups recovered in the populations of *T. licus* must consider the sample set from TS_MT that was recovered as paraphyletic when only the 5′ extremity of *cox1* was used as input. Several reasons may result in lack of monophyly of particular clades, and it was suggested that 23% of animal species are polyphyletic when relying in their mtDNA data (Meyer & Paulay, 2005; Meier *et al.*, 2006). It was also pointed out that one of the major flaws of the 'barcode' proposal is the use of a single molecular region, the 5′ extremity of *cox1*, and the question is whether this fragment is sufficient to achieve the purpose of diagnosing animal species (DeSalle *et al.*, 2005). Alternately, other mitochondrial regions were shown to be as, or more, informative than the proposed 'barcode' region (Roe & Sperling, 2007; Wahlberg & Wheat, 2008). By our own results on *T. licus* DNA polymorphism, it was possible to infer that the 5′ end of *cox1* was the less variable of the mtDNA regions analyzed (fig. 2).

By using this region, part of the samples from TS_TM (clade C2 in fig. 4) was more closely related to *T. l. laura* (clade B1 in fig. 4) than to other individuals from TS_TM (clade C1 in fig. 4). Both adults from PV_RO are also within clade C2, and we confidently know they are morphologically compatible with the name *T. licus albomaculata*, as earlier discussed. In this way, we could alternatively name only the sample set in clade C2 in fig. 4 as *T. licus albomaculata*. The remaining samples from TS_MT might be considered as a different taxonomic unit from both *T. l. laura* and *T. l. albomaculata*. Considering just the less informative region of the 5′ end of *cox1*, we can conceive that there are two taxonomic entities in Mato Grosso, as presented by Lamas (1995). For now, there is no data to fully discuss this issue, and additional sampling is undoubtedly necessary. However, it is possible to envisage that cryptic variation indeed exists within sympatric populations of *T. licus* subspecies, since morphologically divergent forms of adults of *T. licus* were never described in Tangará da Serra, MT (L.C. Almeida, personal communication). For other groups, such as thrips, it already has been shown that cryptic species can be collected even from the same individual host plant (Rugman-Jones *et al.*, 2010), reinforcing that morphological similarity is not indicative of genetic unity.

The origin of the population from São Paulo

The genetic distance and paired F_{ST} data (table 3), coupled with the position of the samples from LI_SP grouped with samples from CO_AL and SM_AL in the genealogical network (fig. 3 I) and in the NJ topology (fig. 3 II), suggested that the population collected from the state of São Paulo must have a common origin with these populations from northeast Brazil. These data corroborate the hypothesis that ornamental plants infested with larvae of *T. licus licus* might have been transported from the northeast region of Brazil to the state of São Paulo (Almeida *et al.*, 2007), giving origin to founders of the population currently attacking sugarcane plants. As this episode was recently reported (Almeida *et al.*, 2007), there was not enough time to establish a marked genetic differentiation among these populations.

Conclusions

Castniidae is a very interesting moth family for several reasons: the day-flying or crepuscular habit, the resemblance to butterflies, and the economic importance of some of its species. Despite these, the group is poorly represented in collections (Miller, 1986) and nearly nothing is known on its molecular systematic. The *T. licus* species complex is the classic example of a group where traditional morphological traits are not sufficient to disentangle complicated taxonomic relationships and where molecular data can be successfully employed (as found for other groups of butterflies (Silva-Brandão *et al.*, 2009)). Speciation is not always accompanied by

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morphological change (Bickford *et al.*, 2007), and the recognition of cryptic entities must be made using alternative data as it is of utmost importance when pest/disease control is under discussion, as a failure in the correct identification of biological important species can be disastrous (Ellis *et al.*, 2009; Paredes-Esquivel *et al.*, 2009; de León & Nadler, 2010).

Our study on the genetic variability of *T. licus* populations from Brazil was the first attempt to relate the geographic range of this group to its genetic variation in order to determine the origin of a population and to delimit the taxonomy of the known subspecies. Sampling from other localities within Brazil, and abroad, would help to reveal the limits of the subspecies proposed for *T. licus* and to explore the mechanisms of speciation for this and other Neotropical species of castniids.

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