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Population structure and genetic diversity in *Gynaikothrips uzeli* (Thysanoptera: Phlaeothripidae): is there a correlation between genetic and geographic proximity?

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ABSTRACT. *Gynaikothrips uzeli* (Thysanoptera: Phlaeothripidae) is a minuscule insect species, which forms galls, is subsocial, and parthenogenetic. It is associated with *Ficus benjamina* L. (Moraceae) and has a pantropical occurrence. The paucity of genetic studies on the order Thysanoptera led us to use inter-simple sequence repeat molecular marker to assess intra- and inter-gall, as well as intra- and inter-site, genetic variability and population structure of *G. uzeli*. Analyses indicated low genetic variability, probably related to haplodiploidy, genetic drift, the galling habit, and the low dispersal ability of *G. uzeli*. Populations were highly structured, with higher variation within populations than among them. Geographic distance does not appear to affect structure and genetic diversity, the latter being influenced by *G. uzeli*'s bioecological traits, by numerous

introductions during a short period, and by a possible recent, common ancestry.

Key words: *Ficus benjamina*; Genetic variability; Haplodiploidy; Thrips; Heterozygosity; Inter-simple sequence repeat

INTRODUCTION

Gynaikothrips uzeli Zimmerman (1900) is a minuscule insect (2.5 to 3.6 mm), which is phytophagous, forms galls (Retana-Salazar and Sánchez-Chacón, 2009), and shows subsocial behavior and parthenogenetic reproduction (Crespi, 1993; Kumm and Moritz, 2008). The species was originally described from southwestern Asia, but is distributed across the tropics, with records in the three Americas (Held et al., 2005; Cambero-Campos et al., 2010; Cavalleri et al., 2011). Its broad distribution can be explained, at least partly, by its very small size and cryptic behavior, which hamper its detection during international transport of agricultural products (Brunner et al., 2002).

While feeding on leaves of its host plant *Ficus benjamina* (Rosales: Moraceae), this species injects toxins that cause the leaves to curl and dry (Wolfenbarger, 1946). Such galls, when formed, serve not only as a food source, but also as shelter and protection against predators (Gonçalves-Alvim and Fernandes, 2001). The damage caused by *G. uzeli* on *F. benjamina* promotes the loss of its ornamental value, and determines the status of *G. uzeli* as a plague. The association between galling thrips and the host plant is a highly species-specific trophic strategy (Ananthakrishnan, 1993), such that the species *G. uzeli* and *Gynaikothrips ficorum*, which are morphologically distinguished only by the length of the posteroangular pair of setae on the pronotum (Priesner, 1939; Del Cãnizo, 1945; Retana-Salazar, 2006; Cambero-Campos et al., 2010), can also be distinguished by their host plants, *F. benjamina* and *Ficus microcarpa*, respectively (Mound et al., 1995; Dobbs and Boyd Jr., 2006). *G. uzeli*'s life cycle is approximately 30 days long, with eight generations per year, on average, in humid subtropical climates (Farong et al., 1995). These features make this species important in the study of the evolution of social behavior in haplodiploid species, not only in terms of its ecological role, but also in terms of its genetics (Kranz et al., 2001).

Genetic markers are useful for the identification, characterization, and evaluation of genetic diversity. Among them, inter-simple sequence repeat (ISSR) molecular markers are remarkable for their use in the genetic characterization of plants, fungi, vertebrates, and insects (Wolfe, 2005). This method does not require previous isolates or specific DNA sequencing and allows the detection of polymorphism in DNA regions flanked by microsatellites (Zietkiewicz et al., 1994).

Studies on Thysanoptera have grown in number in recent years. However, only one study has been conducted that aimed to assess the genetic and population parameters of *G. uzeli*. Brito et al. (2012) evaluated the population structure and genetic diversity of six populations of *G. uzeli* from two states in Brazil, and found reduced genetic diversity and strong population structure, with higher genetic variation among populations than within them. However, some questions, such as the variability at lower levels (intra- and inter-galls) and the effect of distance, were not assessed. In this light, we used ISSR molecular markers in the current study to assess the genetic diversity and population structure of *G. uzeli* among individuals from the same gall (intra-gall variation), among individuals from different galls in the same tree (inter-gall variation), among individuals from different trees from the same site (intra-site variation),

and among populations from different sites (inter-site variation). Then, we assessed the influence of geographic distance and behavioral aspects on the generation of genetic variability.

MATERIAL AND METHODS

Collection and extraction of genomic DNA

For the genomic analyses, we used adult *G. uzeli* found in the galls of *F. benjamina*. Collections were carried out in different ways according to each analysis: a) intra-galls: four trees were randomly selected (01, 03, 04, and 06; Figure 1) and from each of them, a single gall was collected, from which ten individuals were sampled; b) inter-galls: two trees were randomly selected (03 and 09; Figure 1), from which eight galls were collected per tree, with two individuals collected per gall, for a total of 16 individuals per tree; c) intra-site: eleven trees in the city of Jequié (Bahia, Brazil) were sampled and, in each of them, three thrips, each from a different gall, were collected (Figure 1); and d) inter-sites: ten trees in each of six different cities (Figure 2) were sampled following the method described in c, for a total of 30 individuals per site.

Figure 1. Collection points in the city of Jequié (Bahia, Brazil) of *Gynaikothrips uzeli* used for genomic analyses.

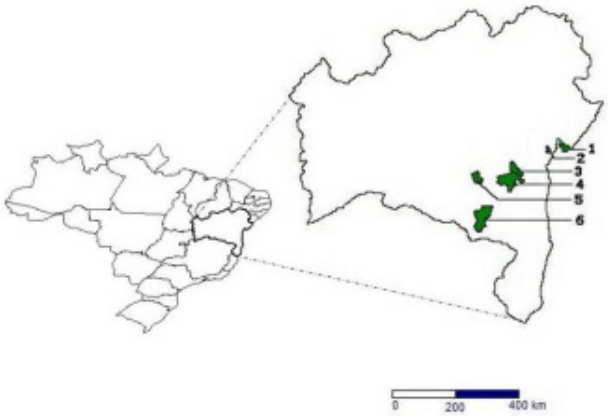

Figure 2. Collection sites of *Gynaikothrips uzeli* in Bahia, Brazil. 1: Salvador (SSA) - 12°59'06.1"S, 038°26'10.2"W; 2: Nazaré (NAZ) - 13°02'04.4"S, 039°00'34.0"W; 3: Jaguaquara (JAG) - 13°31'43.0"S, 039°58'25.1"W; 4: Jequié (JEQ) - 13°52'06.4"S, 040°04'23.9"W; 5: Contendas do Sincorá (CSI) - 13°45'44.4"S, 041°02'31.7"W; and 6: Vitória da Conquista (VCQ) - 14°51'39.2"S, 040°49'26.0"W.

DNA extraction was carried on an individual basis and followed the protocol by Roberts (1998), with modifications (substitution of potassium acetate 8 M with sodium acetate 8 M; longer centrifugation at 14,000 rpm instead of 16,000 rpm). The DNA was quantified in an L-Quant spectrophotometer.

ISSR-PCR

Initially, 39 ISSR primers (UBC series, University of British Columbia) were tested, and eleven of these were selected based on their definition, reproducibility, and number of generated bands (Table 1). Amplifications were carried out in a PTC-100 thermocycler (MJ Research Inc., Ramsey, MN, USA). Each reaction consisted of a total volume of 25 µL containing 1 µL 10 ng genomic DNA, 2.0 µL (200 µM each) dNTPs, 0.5 U Taq polymerase (Biotools), 1 µL 50 pmol primer, 2.5 µL 10X buffer with $MgCl_2$, and 18.4 µL milli-Q water. The thermocycler was programmed for an initial denaturation step of 3 min at 94°C, 40 cycles of 1 min at 92°C, 2 min at 53°C for the pairing of the primer, 2 min at 72°C for the elongation of fragments, and a final step of 7 min at 72°C. Reactions included a negative control containing all the components except the genomic DNA, and a positive control containing already amplified DNA, in order to assess the reproducibility of the results.

Table 1. Selected inter-simple sequence repeat (ISSR) molecular markers, their respective sequences, and the analysis in which they were used: 1, intra-gall; 2, inter-galls; 3, intra-site; and 4; inter-sites.

Primers	Sequence (5'→3')	Analysis
UBC 812	GAG AGA GAG AGA GAG AA	3 and 4
UBC 825	ACA CAC ACA CAC ACA CC	1, 2, 3, and 4
UBC 825b	ACA CAC ACA CAC ACA CT	3 and 4
UBC 827	ACA CAC ACA CAC ACA CG	1, 3, and 4
UBC 836	AGA GAG AGA GAG AGA GYA	1 and 4
UBC 846	CAC ACA CAC ACA CAC ART	4
UBC 855	ACA CAC ACA CAC ACA CYT	3 and 4
UBC 856	ACA CAC ACA CAC ACA CYA	1, 2, 3, and 4
UBC 884	HBH AGA GAG AGA GAG AG	2
UBC 888	GAT CAA GCT TNN NNN NAT GTG G	3 and 4
UBC 889	CAT GGT GTT GGT CAT TGT TCC A	1, 2, 3, and 4

The DNA fragments resulting from the amplification were separated by electrophoresis on a 1.2% agarose gel and stained with Gelred (Biotium) diluted to 1:500. The amplified fragments were visualized under ultraviolet light and photographed under the L-Pix EX system (Loccus Biotecnologia, Cotia, SP, Brazil).

Data analysis

The products of amplification were coded according to the presence (1) or absence (0) of bands. The TFPGA v1.3 program (Miller, 1997) was used to estimate genetic diversity (H_E), the percentage of polymorphic loci, and Nei's (1978) genetic distance, as well as to construct a dendrogram using the unweighted pair group method with arithmetic mean (UPGMA). Analysis of molecular variance (AMOVA) (Excoffier et al., 1992) and an estimate of the index of structure among sites (F_{ST}) were carried out using the Arlequin v3.5.1.2 program (Excoffier

et al., 2005). The statistical significance of the estimated structure index was tested with 1000 permutations.

Using a Bayesian approach, we further estimated genetic diversity and population structure using the HICKORY 1.1 software (Holsinger and Lewis, 2005), with the indices H_B and θ_B being analogous to H_E and F_{ST}, respectively. The analyses were carried out using four models chosen *a priori* (full model, f = 0 model, θ = 0 model, and f free model), with the best model being defined according to the lowest deviance information criterion value. A Markov Chain Monte Carlo was run for a total of 100,000 generations, with a burn-in of 20%.

To test for a correlation between geographic and genetic distances among samples, we used a Mantel test based on Nei's genetic distance. This analysis was performed using the Arlequin v3.5.1.2 program (Excoffier et al., 2005), with 1000 permutations.

Bayesian inference was also used to assess population structure using STRUCTURE version 2.3.1 (Falush et al., 2007). The most probable number of populations (k) was estimated with an admixture model with correlated allele frequencies, without previous information on the origin of the population. The program performed 10,000 iterations, after a burn-in of 10,000 iterations, to assess population subdivision under k = 1 to 10. Twenty replicates were carried out for each k, in order to evaluate whether different runs could produce different likelihood values and to quantify variation in the probability. Individual proportions and admixture average (Q) for each population in each genetic cluster found by the program were recorded for the model. Results generated by STRUCTURE were later analyzed with STRUCTURE HARVESTER version 0.6.7 (Earl and vonHoldt, 2012), according to the method by Evanno et al. (2005).

Except for the analyses within galls, in which we only assessed genetic diversity and allelic clustering as generated by STRUCTURE HARVESTER, the remaining analyses involved all the above mentioned programs.

RESULTS

The data on genetic diversity (H_E and H_B), population structure (F_{ST} and θ_B), and AMOVA are summarized in Table 2.

Table 2. Number of primers utilized and bands generated, genetic diversity values (H_E and H_B), population structure (F_{ST} and θ_B), and analysis of molecular variance (AMOVA) results for intra-gall, inter-gall, intra-site, and inter-site analyses of *Gynaikothrips uzeli*.

Analysis	Sample	No. of primers	No. of bands	H_E	H_B	F_{ST}	θ_B	Variance	
								Within	Among
Intra-gall	Gall A	5	30	0.1776	-	-	-	-	-
	Gall B	5	53	0.1673	-	-	-	-	-
	Gall C	5	39	0.168	-	-	-	-	-
	Gall D	5	41	0.1315	-	-	-	-	-
Inter-gall	Tree A	4	34	0.1614	0.1841	0.6613	0.5923	33.87%	66.13%
	Tree B	4	30	0.1614	0.2254	0.5532	0.5644	44.68%	55.32%
Intra-site	11 trees	8	78	0.2057	0.2733	0.3206	0.4398	67.94%	32.06%
Inter-site	6 cities	10	112	0.2621	0.2349	0.3630	0.4797	63.69%	36.31%

Intra- and inter-gall analyses

The plots generated with STRUCTURE for intra- and inter-gall analyses (Figure 3) revealed that all sets of alleles were shared among individuals in similar proportions.

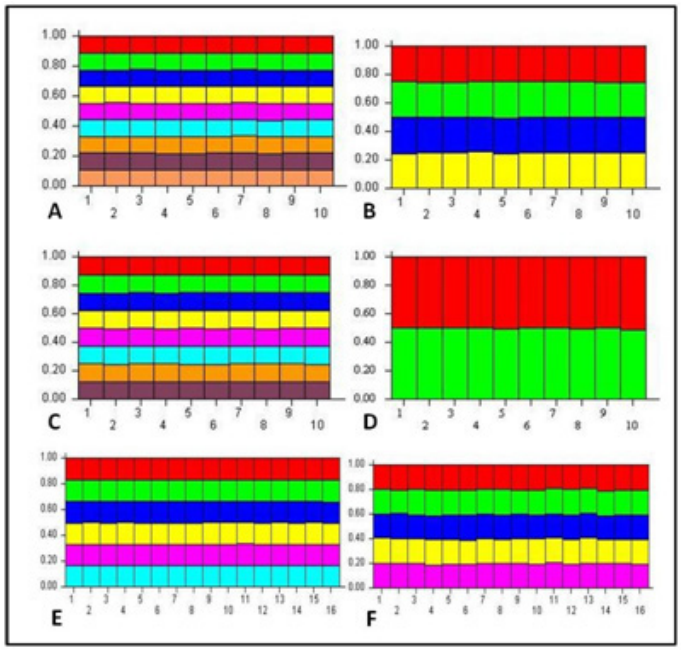

Figure 3. STRUCTURE plots showing allelic clustering in the intra- and inter-gall analyses. Intra-gall: **A.** gall A (tree 1); **B.** gall B (tree 3); **C.** gall C (tree 4); **D.** gall D (tree 6). Inter-gall: **E.** tree A (tree 3); **F.** tree B (tree 9).

Intra-site analysis

In the intra-site analysis (Figure 4A), the UPGMA dendrogram clustered the eleven *F. benjamina* trees into two groups, irrespective of the proximity between them. The plot generated by STRUCTURE revealed the formation of two groups of alleles shared by all individuals analyzed, although in different proportions (Figure 4B).

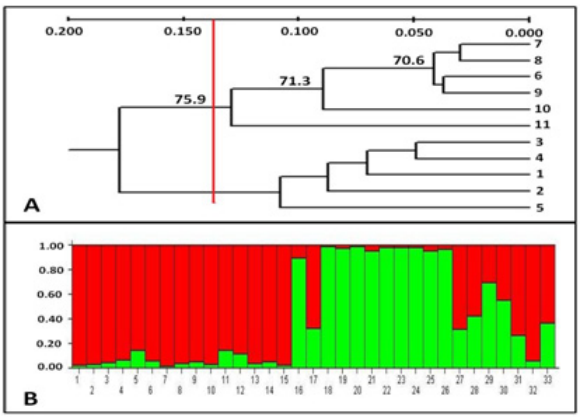

Figure 4. A. Dendrogram generated by the UPGMA method for analysis of intra-site population structure. The cut-off point was set at the median of the distances (Nei, 1978). Other bootstrap values lower than 55% are not shown. **B.** STRUCTURE HARVESTER plot showing grouping of alleles of the same 11 trees studied.

Inter-site analysis

Pairwise genetic distance among populations (Table 3) indicated a smaller distance between the Salvador and Jaguaquara populations (0.0516) and a higher distance between the Salvador and Vitória da Conquista populations (0.1535).

Table 3. Nei's (1978) genetic distance among the different sites in the State of Bahia, Brazil, sampled for *Gynaikothrips uzeli*.

	CSI	SSA	JAG	NAZ	JEQ	VCQ
CSI	*****					
SSA	0.0537	*****				
JAG	0.0752	0.0516	*****			
NAZ	0.0864	0.0964	0.1039	*****		
JEQ	0.0921	0.1023	0.1052	0.0578	*****	
VCQ	0.1498	0.1535	0.1338	0.1518	0.0869	*****

Contendas do Sincorá (CSI), Salvador (SSA), Jaguaquara (JAG), Nazaré (NAZ), Jequié (JEQ), and Vitória da Conquista (VCQ).

The dendrogram generated by the UPGMA method and the plot generated by STRUCTURE grouped the populations into three distinct clusters (Figure 5).

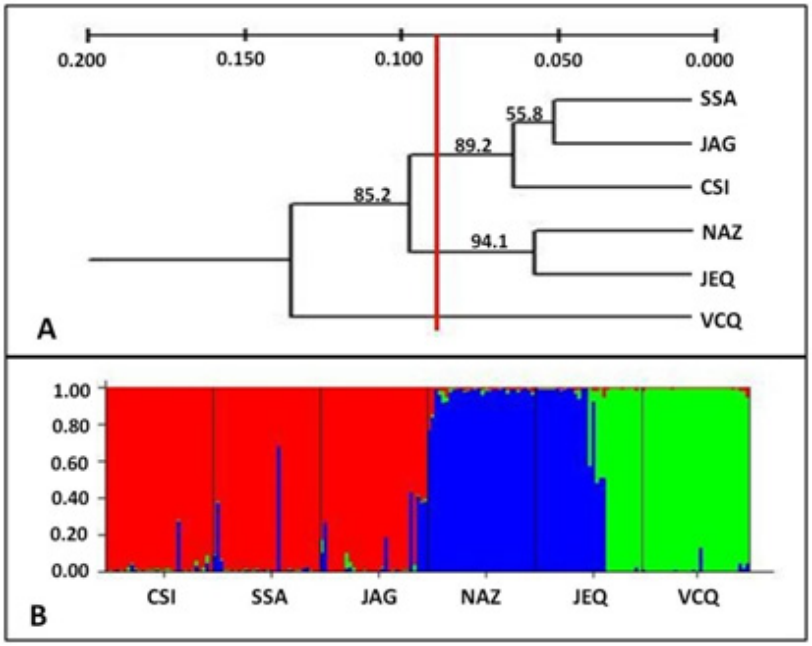

Figure 5. A. Dendrogram generated by UPGMA for the six populations of *Gynaikothrips uzeli* studied. The cut-off point was set at the median of the distances (Nei, 1978). **B.** STRUCTURE HARVESTER plot showing the same grouping of the six populations studied.

AMOVA carried out under two hierarchical levels showed that 63.69% of the genetic variation is within populations, whereas 36.31% is among populations (Table 4).

Table 4. Analysis of molecular variance (AMOVA) for the six populations of *Gynaikothrips uzeli* considering two hierarchical levels.

Source of variation	d.f	SQ	Component of variance	Variance (%)	P value
Among populations	5	601.222	3.80679	36.31	<0.001
Within populations	173	1155.421	6.67873	63.69	<0.001
Total	178	1756.642	10.48552		
Fixation index (F_{ST})	0.3630				

d.f. = degrees of freedom; SQ = sum of squares.

The Mantel test was not significant (r = 0.3579; P = 0.125).

DISCUSSION

Intra- and inter-gall analyses

The intra- and inter-gall analyses were performed in principle as preliminary tests, with the goal of evaluating the existing polymorphism and defining the number of individuals to be sampled per gall, thus, we used fewer primers. However, the low levels of heterozygosity and the plots generated by STRUCTURE (Table 2 and Figure 3), along with data on the biology of the insect, allowed us to incorporate these analyses into the final study.

Our results reveal a high similarity among individuals from the same gall, as shown in the four galls analyzed, with all individuals sharing all allelic sets in similar proportions. The analysis between galls of a given tree also revealed low heterozygosity, highly structured populations, and a lower variation within galls than between them. Such data attest the high level of similarity among individuals from the same gall, in addition to helping to define the sampling scheme for each locality. Thus, we established that each gall could be represented by a single individual and that each tree could be represented by three galls.

Intra-site analysis

The analysis carried out with samples from the same site (Jequié; Figure 1), which aimed to assess genetic variability among neighboring trees, indicated a lack of correlation between similarity and proximity (Figure 4). Such data reinforce the theory that a series of introductions of *F. benjamina* (because of its use in urban arborization and ornamentation, *F. benjamina* was probably reintroduced many times) and the subsequent effects of genetic drift are the main components underlying the variability in populations of *G. uzeli*, such that each population in each tree has its own history of foundation, even though they share some alleles. Moreover, any physical barriers (walls, curves) are actual barriers to the dispersal of *G. uzeli*, even at short distances.

Inter-site analysis

Both the UPGMA dendrogram and STRUCTURE clustered the study sites into three

groups: 1. Salvador, Jaguaquara, and Contendas do Sincorá; 2. Nazaré and Jequié, and 3. Vitória da Conquista (Figure 5). The way sites were clustered, together with the Mantel test, indicates that there is no relation between geographic and genetic proximity. However, genetic distance values (Table 3) do not necessarily inform about gene flow among populations, indicating genetic proximity that may be due to common ancestors. This would be likely considering the possibility that host plants originated from the same location.

AMOVA partitioned with hierarchical levels showed a high percentage of variation within populations (63.69%), which differs from the results found by Brito et al. (2012), who used random amplified polymorphic DNA (RAPD) and found larger variation among populations than within them. The observed value from the current study points to the inexistence or restriction of gene flow among the different sites, and a possible increase of endogamy within them. The low dispersal capacity of *G. uzeli* hinders gene flow between populations, even geographically close ones. Although *G. uzeli* shows limited active flight, its fringed wings allow for its permanence in the air and for wind-driven, long distance dispersal (Lewis, 1997). However, given the distance among sites and the paucity of host plants in between, this would only be possible by transport through saplings or branches of the host plant.

Fixation indices (F_{ST}: 0.3630 and θ_B: 0.4797) revealed strong population structure and reinforce the proposal that haplodiploidy, low dispersal capacity, and the process of foundation of new galls influence population structure. A large F_{ST} of 0.56 was also found by Brito et al. (2012). The low variability observed in all analyses was to be expected given the knowledge about the gall-forming habits of *G. uzeli*, as well as studies carried out with other haplodiploid organisms. Miranda et al. (2012), using ISSR markers, observed H_E = 0.2616 and H_B = 0.2573 for *Melipona mandacaia* (Hymenoptera: Apidade); Brito et al. (2012), using RAPD markers, found H_E = 0.30 for *G. uzeli*; and Crespi (1991), using isozymes, observed an average H_E of 0.058 for four species of thrips, a result similar to that found in a survey of 30 genera of hymenopterans (including wasps, bees, and ants) analyzed with the same technique.

According to Hedrick and Parker (1997), low genetic variation can be a consequence of small effective population size caused by haplodiploidy and of exposition of hemizygotic loci to selection, which would allow these to be removed from the population faster. In the specific case of *G. uzeli*, although it was first recorded in Brazil in 2011 (Cavalleri et al., 2011), its host plant was introduced in the 1970s (Santos and Ramalho, 1997). However, a number of introductions occurred later, which may have fostered genetic drift, thus, decreasing the gene pool of the population (Frankham et al., 2008). The galling habit of *G. uzeli* may be another critical factor in the reduction of genetic diversity, as suggested by Van Valen's (1965) hypothesis that more stable environments, such as galls, would favor a decline in heterozygosity.

Hence, geographic distance would not be the determinant factor underlying the observed population structure and genetic diversity in populations of *G. uzeli*. Such populations are probably influenced by bioecological features (galling habit, dispersal capacity, and haplodiploidy), founder effect, genetic drift, and recent common ancestry, given that a period of a few decades would not be sufficient to differentiate them significantly.

The differences between the data obtained by Brito et al. (2012) and those presented here probably result from the different sampling designs and molecular markers employed. While we sampled 30 individuals per site and individually extracted DNA from each of them, Brito et al. collected 36 individuals and extracted DNA from a pool of four individuals, i.e., nine specimens per site. Thus, variation within populations would tend to decrease and F_{ST} to increase, as was actually observed in AMOVA results. Furthermore, RAPD markers use prim-

ers that are shorter than those used for ISSR (10 bp and 16 to 20 bp, respectively), thus being less specific, which may bias the data obtained.

Studies such as the current one, which associate molecular analyses to biological data, can support the understanding of the influence of natural history on the dynamics of population structure and genetic diversity, especially in haplodiploid species.

Conflicts of interest

The authors declare no conflict of interest.

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