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Genetic population structure and hybridization in two sibling species, *Tomoplagia reticulata* and *Tomoplagia pallens* (Diptera: Tephritidae)

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ABSTRACT. *Tomoplagia reticulata* and *T. pallens* are sibling species that are specialists on *Eremanthus glomerulatus*. Besides adult terminalia, they show slight morphological differences and distinct geographic distributions. Once, however, they were found sympatrically. Using data from allozyme and mtDNA, we examined patterns of intra- and interspecific genetic structure, and investigated the possible occurrence of gene flow between them. Both species showed low diversity and high genetic structure, which can be linked to their high degree of specialization. Larval development occurs within flower heads, tissues that are available only during a short period of the year. Afterward, as they do not hibernate, they probably suffer a great reduction in population size, which leads to low genetic diversity. As monophagous insects, their population structure may correspond to the fragmented distribution of *E. glomerulatus*, which could isolate fly populations and increase inbreeding within them. One population exhibited a mixed genetic composition, compatible

with one hybridization season when species were sympatric. This hybridization seems to be a rare event, due to *T. pallens* unusual range expansion.

Key words: Allozymes; Asteraceae; Campo rupestre; Cerrado; *Eremanthus glomerulatus*; mtDNA

INTRODUCTION

Close species deriving from a common ancestor may show genetic divergence without conspicuous morphological changes and are classified as sibling species. They are known in all groups of animals, yet they seem to be much more common in some groups, such as parasites and phytophagous insects. Tephritidae (Diptera) is a family of phytophagous comprising more than 4250 species of worldwide distribution (Norrbom et al., 1999). The larvae of most species breed in living plant tissues, such as fruits and flower heads. The subfamilies Trypetinae and Dacinae have been the focus of many studies, because several species are important fruit pests. Much less is known about species of Tephritinae, whose larvae feed in flower heads or vegetative parts of Asteraceae (Headrick and Goeden, 1998). *Tomoplagia* is a New World and predominantly Neotropical genus of Tephritinae with 45 species described, of which 43 are restricted to Central and South America (Norrbom et al., 1999). In Brazil, *Tomoplagia* is the most diverse and abundant genus reared from Asteraceae flower heads (Prado et al., 2002).

Abreu et al. (2005) described two sibling species in this genus, *Tomoplagia reticulata* and *T. pallens*. They breed on the same host plant, *Eremanthus glomerulatus* Less (1829), laying their eggs in the flower heads, where the larvae develop. Besides adult terminalia, they show slight morphological differences and distinct geographic distributions. *Tomoplagia pallens* has a pale yellowish body color, wings with faint markings, and occurs mainly in Goiás State. *Tomoplagia reticulata* has a dark yellow to orange body color, pigmented wings, and is found only in Minas Gerais State (Abreu et al., 2005).

The host plant, *E. glomerulatus* (Asteraceae), occurs throughout the central part of Brazil, in Minas Gerais, Goiás, and adjacent regions in Bahia and São Paulo (MacLeish, 1987). It is commonly found in large colonies, in *cerrado*, that are surrounded by distinct vegetation physiognomies according to their geographic location. In Minas Gerais, the surrounding vegetation is composed of scattered small trees, with a dense grassy ground layer in between. In Goiás, *cerrado* has a great influence of grassland, corresponding to a rich ground layer (herbs, subshrubs and small shrubs) without trees or with rare small trees, which are much more scattered than in the typical *cerrado* (Rizzini, 1997).

Over several years of sampling, we never found these two species in sympatry, except for year 2000, when they both were collected in Minas Gerais. This finding led us to investigate whether they display incomplete reproductive isolation when co-occurring. As they can be distinguished by four loci, *PGI*-1, *ME*, *MDH*, and *G6PD* (Abreu et al., 2005), if they are not fully isolated we would expect some heterozygotes after this contact. Using data from allozyme and mitochondrial DNA (mtDNA), we examined patterns of intra- and interspecific genetic structure of *T. reticulata* and *T. pallens* with the aim of comparing levels of differentiation between parasites of the same host, and investigating a possible occurrence of gene flow between this pair of closely related species, when in sympatry.

MATERIAL AND METHODS

Collection of material

Flower heads of *Eremanthus glomerulatus* were collected during 4 consecutive seasons of flowering, from 1999 to 2002, and placed in plastic jars covered with a cotton cloth. After emergence, adults were fed for four days with honey and water and then frozen in liquid nitrogen until genetic analysis.

The localities sampled are shown in Figure 1. Sample identifications, sizes and years are summarized in Table 1. As herbivore demes can be adapted to individual host plants (reviewed in Mopper and Strauss, 1998), we pre-defined a population as all flies emerging from flower heads of a single tree. *T. pallens* was sampled in Brasília (Distrito Federal), Goiás and, only in the year 2000, Santana do Riacho (Minas Gerais), whereas *T. reticulata* was sampled only in Santana do Riacho (Minas Gerais). The samples SR-1, SR-3, GO-1, GO-2, and BSB-2 had already been investigated for allozyme polymorphism by Abreu et al. (2005).

Figure 1. Sampling localities. See Table 1 for sample names. BA = Bahia; GO = Goiás; MG = Minas Gerais; SP = São Paulo; ES = Espírito Santo; MS = Mato Grosso do Sul; RJ = Rio de Janeiro; MT = Mato Grosso; DF = Distrito Federal.

Table 1. Sampling localities, sampling year and sample size of *Tomoplagia reticulata* and *T. pallens* used in allozyme and restriction fragment length polymorphism (RFLP) analyses.

Species	Locality (State)	Sample code	Sampling year	Coordinates	Allozyme (N)	RFLP (N)
T. pallens	Brasília (DF)	BSB-1	1999	15º 36' 24''S 47º 34' 32''W	55	31
		BSB-2	2001	15º 36' 50''S 47º 41' 30''W	25	30
		BSB-3	2002	15º 40' 50''S 47º 52' 03''W	-	30
		BSB-4	2002	15º 42' 17''S 47º 52' 38''W	-	24
	Pirenópolis (GO)	GO-1	2001	15º 53' 30''S 48º 53' 19''W	32	29
	Anápolis (GO)	GO-2	2001	16º 03' 49''S 48º 51' 15''W	34	30
		GO-3	2001	16º 12' 05''S 48º 54' 39''W	34	29
		GO-4	2001	16º 12' 05''S 48º 54' 39''W	-	30
		GO-5	2001	16º 13' 14''S 48º 55' 20''W	31	30
		GO-6	2001	16º 24' 27''S 48º 59' 08''W	41	30
		GO-7	2002	16º 13' 16''S 48º 55' 22''W	-	25
		GO-8	2002	16º 12' 06''S 48º 54' 40''W	-	30
		GO-9	2002	16º 02' 38''S 48º 51' 07''W	-	20
	Pirenópolis (GO)	GO-10	2002	15º 53' 36''S 48º 52' 52''W	-	30
	Santana do Riacho (MG)	SRp-1	2000	19º 17' 58''S 43º 36' 08''W	24	-
	Santana do Riacho (MG)	SRp-2	2000	19º 17' 29''S 43º 36' 06''W	3	-
T. reticulata	Santana do Riacho (MG)	SRr-1	2000	19º 17' 58''S 43º 36' 08''W	24	-
		SRr-2	2000	19º 17' 29''S 43º 36' 06''W	47	24
		SR-3	2001	19º 17' 58''S 43º 36' 08''W	32	30
		SR-4	2001	19º 17' 58''S 43º 36' 08''W	-	20
		SR-5	2001	19º 17' 51''S 43º 36' 09''W	-	20
		SR-6	2001	19º 17' 29''S 43º 36' 06''W	-	30
		SR-7	2002	19º 17' 56''S 43º 36' 09''W	-	21
		SR-8	2002	19º 17' 28''S 43º 36' 06''W	-	17
		SR-9	2002	19º 17' 28''S 43º 36' 06''W	27	28

Allozyme analysis

The tissues were homogenized in 30 µL system III gel solution (see below). The extract of each fly was absorbed onto filter paper wicks (Whatman #1) and then applied to an 8.5% starch gel (Sigma). Three buffer systems were used: I) electrode: 0.3 M boric acid, 60 mM NaOH, pH 8.0; gel: 10 mM Tris, pH 8.5; II) electrode: 10 mM LiOH, 3 mM EDTA and 90 mM boric acid, pH 8.0; gel: electrode solution diluted 1:10, and III) electrode: 0.34 M Tris, 78 mM citric acid, pH 8.6; gel: 38 mM Tris, 2.5 mM citric acid, pH 8.6. System I was used for esterase (EST - EC 3.1.1.1), aconitase (ACO - EC 4.2.1.3), 3-hydroxybutyrate dehydrogenase (HBDH - EC 1.1.1.30), glutamate dehydrogenase (GDH - EC 1.4.1.3), and leucylalanine peptidase (PEP - EC 3.4.11); system II was used for phosphoglucose isomerase (PGI - EC 5.3.1.9), 6-phosphogluconate dehydrogenase (6PGD - EC 1.1.1.44), glucose 6-phosphate dehydrogenase (G6PD - EC 1.1.1.49), and fumarase (FUM - EC 4.2.1.2), and system III was used for isocitrate dehydrogenase (IDH - EC 1.1.1.42), phosphoglucomutase (PGM - EC 5.4.2.2.), malate dehydrogenase (MDH - EC 1.1.1.37), malic enzyme (ME - EC 1.1.1.40), and aldehyde oxidase (AO - EC 1.2.3.1). When there was more than one locus, they were numbered in ascending order from the locus with lowest mobility. The alleles were scored according to their mobility relative to the most common allele of sample BSB-1. The allele frequencies at enzyme loci were calculated by direct counting of alleles.

Mitochondrial DNA analysis

Total DNA was extracted from frozen specimens. A modified phenol-chloroform extraction (Azeredo-Espin et al., 1991) was used. A 2325-bp fragment of the mitochondrial genes cytochrome oxidase I and II (COI/COII) was amplified using the primers TY-J-1460 and TK-N-3785 described by Simon et al. (1994). Three microliters of total DNA solution was used as template in 75-µL reactions containing 3.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM each primer and 3 U *Taq* DNA polymerase. Polymerase chain reaction (PCR) conditions were as follows: 4 min at 94°C; 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 60 s and extension at 71°C for 2 min, and a final extension cycle at 71°C for 4 min.

Eight microliters PCR product was digested for 2 h, at 37°C with 1 U enzyme in the appropriate buffer. DNA fragments were digested with six endonucleases (*Dde*I, *Eco*RV, *Hae*III, *Pvu*II, *Rsa*I, and *Xba*I). Electrophoresis was performed on 2.5% agarose gels, which were stained with ethidium bromide, and visualized under ultraviolet light.

Statistical methods

Allozymes

Measures of genetic diversity (percentage of polymorphic loci, mean number of alleles per locus, and the observed and expected heterozygosity) were estimated using the GENETIX 4.05.2 program (Belkhir et al., 1996-2004). F-statistics (Wright, 1951) were calculated by the method of Weir and Cockerham (1984) (estimators θ and *f*), and their significance (5% level) was tested by bootstrapping using GENETIX 4.05.2 (Belkhir et al., 2004). Tests for deviations from Hardy-Weinberg equilibrium (by exact tests) and for linkage disequilibrium were calculated using the GENEPOP 3.3 software (Raymond and Rousset, 1995). The sequential Bonferroni was used to correct for multiple comparisons. Genetic divergence among samples was estimated from allele frequencies with the method of Nei (1978) using GENETIX 4.05.2 (Belkhir et al., 2004), and these distances were subjected to neighbor-joining cluster analyses using the MEGA 3.1 software (Kumar et al., 2004).

Polymerase chain reaction-restriction fragment length polymorphism

Data from restriction site patterns were analyzed using the ARLEQUIN software (Excoffier et al., 2005). Intra- and intersample genetic diversity were measured by haplotype and nucleotide diversity (*h* and π; Nei, 1987), Nei's raw (D) and net (D_A) intersample nucleotide divergence (Nei and Li, 1979). The matrix of net intersample nucleotide divergence was used to build a neighbor-joining phenogram of the samples using MEGA 3.1 (Kumar et al., 2004). To investigate the genetic structure of samples, we performed an analysis of molecular variance (AMOVA).

The assignment of individuals to populations and the individual admixture proportion were implemented in the STRUCTURE program (Falush et al., 2007). The model probabilistically assigns individuals to source clusters (or jointly to two or more clusters in cases of admixture) on the basis of their genotypes. Twenty independent runs were carried out for each value of *K* (*K* from 1 to 6) of clusters. For each run, 500,000 iterations were carried out after a burn-in period of 50,000 iterations. The true value of *K* was chosen on the basis of the second order rate of change of the log likelihood function with respect to *K* (Δ*K*; Evanno et al., 2005). Analyses were initially

performed without prior assumptions concerning the population (phenotype or geographic location). Simulations were then carried out for $K = 2$ (2 clusters), using yellow and orange phenotypes (yellow - *T. pallens* and orange - *T. reticulata*) as prior information for clustering, and the probability of admixture in the last four generations was inferred for each specimen.

RESULTS

Allozyme variation

Only samples SR-1 and SR-2 showed both species. Ten loci (of 17 screened) were polymorphic in both species and four of them (*MDH*, *PGI*-1, *G6PD*, and *ME*) distinguished the two species (Table 2). *T. pallens* exhibited lower variation than *T. reticulata* (Table 2). The fixation index (θ) was 0.069 (± 0.047) in *T. pallens*, and 0.117 (± 0.061) in *T. reticulata*, which indicates genetic differentiation among the samples of each species.

Table 2. Distribution of allelic frequencies of diagnostic allozyme loci.

Locus	Allele	*T. reticulata*				*T. pallens*								
		SRr-1	SRr-2	SR-3	SR-9	SRp-1	SRp-2	BSB-1	BSB-2	GO-1	GO-2	GO-3	GO-5	GO-6
ME	0.78	0.50	0.33	0.50	0.52	0.07	-	-	-	-	0.03	-	-	-
	0.90	0.50	0.58	0.50	0.48	0.29	-	0.14	-	-	-	-	0.03	-
	1.00	-	0.09	-	-	0.43	1.00	0.81	0.96	1.00	0.94	0.91	0.97	1.00
	1.05	-	-	-	-	0.21	-	0.05	0.04	-	0.03	0.09	-	-
PGI-1	0.33	-	-	-	-	-	-	-	0.04	0.06	-	-	-	0.02
	1.00	-	0.06	0.02	0.04	1.00	0.83	0.95	0.96	0.94	1.00	1.00	1.00	0.98
	3.90	1.00	0.94	0.98	0.96	-	0.17	0.05	-	-	-	-	-	-
MDH	0.75	0.57	0.76	0.67	1.00	-	-	0.07	0.02	0.02	-	-	-	-
	0.82	0.43	0.24	0.33	-	-	-	-	-	-	-	0.02	0.03	-
	1.00	-	-	-	-	1.00	1.00	0.93	0.98	0.91	1.00	0.98	0.97	0.97
	1.15	-	-	-	-	-	-	-	-	0.07	-	-	-	0.03
G6PD	0.84	1.00	0.88	1.00	0.94	-	-	-	-	-	-	-	-	-
	1.00	-	0.12	-	0.06	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
P		46.7	41.2	23.5	23.5	33.3	16.7	33.3	17.6	29.4	23.5	29.4	29.4	11.8
H_e		0.195	0.188	0.131	0.080	0.146	0.06	0.149	0.103	0.090	0.072	0.087	0.080	0.061
(SD)		(0.38)	(0.40)	(0.23)	(0.16)	(0.41)	(0.66)	(0.40)	(0.18)	(0.18)	(0.17)	(0.17)	(0.16)	(0.14)
A		1.6	1.8	1.5	1.5	1.6	1.2	2.1	1.8	1.7	1.7	1.8	1.8	1.8
f		0.81	0.82	0.67	0.83	0.90	0	0.72	0.63	0.53	0.58	0.62	0.45	0.41

Genetic variability (P = proportion of polymorphic loci, H_e = mean expected heterozygosity + standard deviation (SD); A = mean number of alleles per locus) and inbreeding coefficient (*f*) at all allozyme loci for *Tomoplagia reticulata* and *T. pallens*.

Significant departures from the Hardy-Weinberg equilibrium (at 5% significance level), in at least one sample, were detected in both species: in *T. reticulata*, in the loci *ME*, *MDH*, *PGM*, *6PGD*, and *G6PD*, and in *T. pallens* in *ACO*, *AO*, *ME*, *MDH*, *PGM*, and *6PGD*. All significant deviations had positive *f* values, indicating deficiencies of heterozygote. Mean *f* value was 0.549 (± 0.214) in *T. pallens* and 0.798 (± 0.113) in *T. reticulata*. There was linkage disequilibrium only in *T. reticulata*, between loci *ME-MDH* in sample SRr-2 and *IDH-MDH* in SR-3.

Genetic relationships between samples are presented in Figure 2. Mean genetic identity was 0.764 between species. The intraspecific identities ranged from 0.928 to 1 among *T. pallens* samples and from 0.966 to 0.982 in *T. reticulata.* SRp-1 and SRp-2 were the samples with higher differences to other conspecific samples (BSB and GO). In diagnostic loci *PGI*-1 and *G6PD* (according to Abreu et al., 2005), each species had its typical allele fixed or very nearly fixed. Exceptions were populations SRr-2 and SR-9, which had *pallens* alleles in both loci, mostly in heterozygosis, and SRp-2, which had one heterozygous individual in *PGI*-1 (Figure 2).

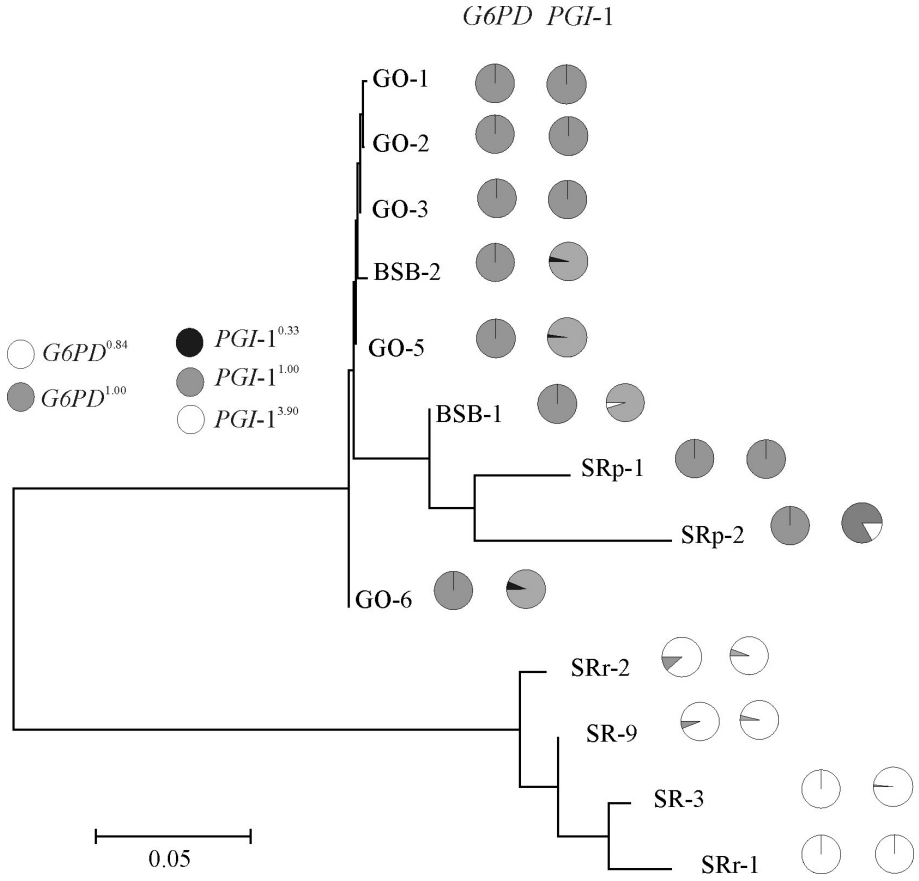

Figure 2. Neighbor-joining cluster analysis based on Nei's (1978) genetic distance estimated among the samples of *Tomoplagia pallens* and *T. reticulata*, using allozyme data. Pie diagrams indicate allele frequency of *G6PD* and *PGI*-1 loci.

Mitochondrial DNA variation analysis

The enzyme *Dde*I had 6 restriction patterns; *Hae*III, 3; *Pvu*II, 2; *Eco*RV, 4; *Xba*I,

2, and *Rsa*I, 2, totalizing 15 restriction sites and 24 composite haplotypes (A-X). In *T. pallens*, there were 15 haplotypes; haplotype A was the most frequent in all samples. In *T. reticulata*, there were 11 haplotypes and the most frequent was J, except for sample SR-5. Only haplotypes A and B were found in both species, A at very high frequencies in *T. pallens*, and B at low frequencies in both species. Some specimens of samples SRr-2, SR-4 and SR-5, morphologically assigned to *T. reticulata*, had haplotype A (Figure 3).

Measures of genetic diversity are shown in Table 3. Both the nucleotide and haplotype diversity varied among samples of the two species. As in allozymes, *T. reticulata* showed higher variability, especially sample SR-2, which had the greatest values for both diversity measures. *T. reticulata* showed greater sample differentiation (ϕ_{ST} = 0.237) than *T. pallens* (ϕ_{ST} = 0.111), and the larger part of the observed variation was within samples in both species (76.30 and 88.97%, respectively), according to AMOVA.

Table 3. Haplotype (*h*) and nucleotide diversity (π) of *Tomoplagia pallens* and *T. reticulata* samples.

Sample	Haplotype diversity (SD)	Nucleotide diversity (SD)
BSB-1	0.615 (0.096)	0.096 (0.065)
BSB-2	0.453 (0.104)	0.058 (0.046)
BSB-3	0.193 (0.095)	0.012 (0.018)
BSB-4	0.235 (0.109)	0.020 (0.024)
GO-1	0.254 (0.100)	0.033 (0.031)
GO-2	0.067 (0.061)	0.004 (0.009)
GO-3	0.191 (0.093)	0.012 (0.017)
GO-4	0.067 (0.061)	0.004 (0.010)
GO-5	0.067 (0.061)	0.004 (0.010)
GO-6	0.067 (0.061)	0.008 (0.014)
GO-7	0.347 (0.108)	0.022 (0.025)
GO-8	0.067 (0.061)	0.008 (0.014)
GO-9	0.521 (0.042)	0.032 (0.032)
GO-10	0.067 (0.061)	0.004 (0.010)
SRr-2	0.670 (0.043)	0.137 (0.087)
SR-3	0.637 (0.081)	0.102 (0.068)
SR-4	0.279 (0.123)	0.035 (0.033)
SR-5	0.419 (0.127)	0.076 (0.055)
SR-6	0.480 (0.053)	0.076 (0.057)
SR-7	0.467 (0.112)	0.080 (0.057)
SR-8	0.118 (0.101)	0.020 (0.024)
SR-9	0.542 (0.070)	0.093 (0.063)

In parentheses, the standard deviation (SD).

Nucleotide divergence was higher among samples of *T. reticulata* than of *T. pallens*. SRr-2 was the *T. reticulata* sample that showed the highest similarity with *T. pallens* samples, being in some cases closer to samples of the other species (SRr-2-BSB-1 = 0.840) than to its own (SRr-2-SR-5 = 1.146). In the phenogram (Figure 3), samples SR-5 and GO-9 are apart from the others, with longer branches. SR-5 is the only *T. reticulata* sample in which the most frequent haplotype is not J but Q. GO-9 has haplotype M in almost the same frequency as A. In other *T. pallens* samples, haplotype A is the most frequent and the other ones are in a very low frequency.

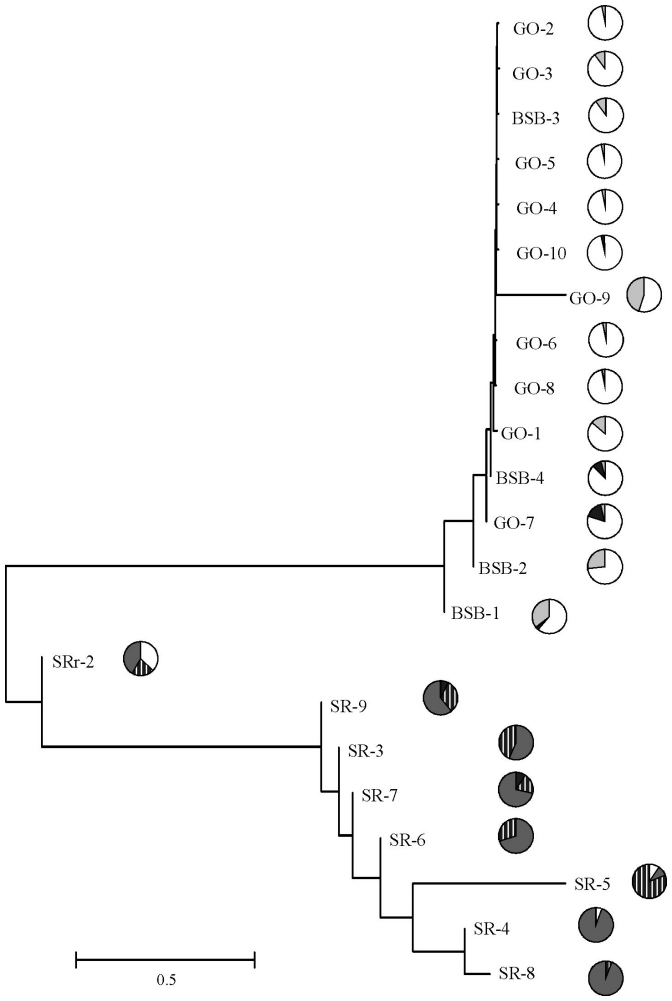

Figure 3. Neighbor-joining cluster analysis based on net intersample nucleotide divergence among *Tomoplagia reticulata* and *T. pallens* samples, using mtDNA data. Pie diagrams indicate haplotype frequency of the following categories: shared haplotypes between species, A (most frequent haplotype in *T. pallens* - white) and B (black); most frequent haplotype in *T. reticulata* (dark gray), pooled rare haplotypes in *T. reticulata* (hatched); pooled rare haplotypes in *T. pallens* (light gray).

Cluster analysis with the STRUCTURE software revealed that the most likely genetic structure for the whole data set consisted of two clusters ($\Delta K = 2$). One grouped all eight populations displaying *T. reticulata* morphology, and the other, the fourteen populations with *T. pallens* phenotype. When we used phenotypic information as prior clustering information ($K = 2$), 99.5% (397/399) of the *T. pallens* specimens and 91.62% (175/191) of the *T. reticulata* specimens were assigned to the appropriate cluster with a probability ≥0.90. The remaining 2 *pallens* individuals (2 BSB-1) and 16 *reticulata* individuals (9 SRr-2, 1 SR-4, 2 SR-5, 2 SR-7, and 2 SR-9) had an assignment probability below 0.90, indicative of mixed ancestry (Figure 4).

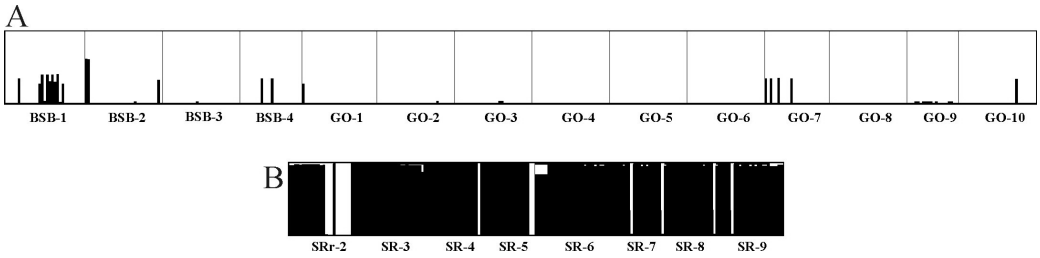

Figure 4. Best clustering result and detection of hybrids ($K = 2$) by the STRUCTURE software, using mtDNA data. Each individual is represented as a vertical line partitioned into colored segments, the length of which is proportional to the individual's estimated K cluster membership coefficients. **A.** *Tomoplagia pallens* samples. **B.** *T. reticulata* samples.

DISCUSSION

The Tephritid life cycle is closely associated with the host plants. They spend most of their lifetime on them or in parts thereof, and the most intimate part of the life cycle is larval development, which occurs within living plant tissues. Some of these tissues, such as flowers and fruits, are available only during a short period of the year. Polyphagous species can use other hosts when the main resource is scarce but specialists do not have any choice (Fitt, 1986; Aluja and Birke, 1993). Their peak oviposition should be synchronized with the peak flowering/fruiting of the host plant (Zwölfer, 1982), and afterward, either the species hibernate until the next favorable period or they will suffer a great reduction in population size. *E. glomerulatus* has a flowering period from March to October, having few flowers during the rest of the year. There are no studies describing *Tomoplagia*'s life cycle, but our own observations indicate that adults live for around 30 days and females start mating and laying eggs only one week after emergence.

Data on other tephritids suggest that tropical species are multivoltine and do not have diapause (Bateman, 1972). In this way, *T. reticulata* and *T. pallens* may have a peak population during peak flowering and then experience a great reduction in population size between flowering periods. These annual and recurrent size fluctuations are expected to lead to low genetic diversity in both *T. pallens* and *T. reticulata*. Indeed, the allozyme genetic diversity observed in *T. pallens* ($H_e = 0.094$) and *T. reticulata* (0.148) is lower than in other Tephritinae that have diapause during winter. *Urophora cardui* induces galls on *Circium arvense*; the larvae overwinter in galls and adults emerge in summer (Peschken and Harris, 1975). *U. cardui* has an H_e between 0.29 and 0.32 (Johannesen and Seitz, 2003). *Tephritis bardanae* is an achene parasite of *Arctium* that hibernates, right after emergence, for up to 200 days (Straw, 1989) and has an H_e of 0.127-0.232 (Eber et al., 1991).

When we compared genetic diversity between them, *T. pallens* had a lower genetic diversity, using both allozymes and mtDNA. As *T. pallens* has a broad geographic distribution and lower inbreeding levels (f), this was not expected. This low nuclear and mtDNA diversity could be a sign of a recent demographic expansion. *T. pallens* also showed a haplotype network with limited divergence among the existing haplotype variants (star-like). According to Avise (2000), this network shape is usually interpreted as

the signature of a recent population expansion, reinforcing the hypothesis of recent demographic expansion in this species.

Both species showed moderate to high genetic structure. As monophagous insects, their population structure may correspond to the fragmented distribution of *E. glomerulatus*. Host plant structure influences movement patterns of herbivores within and among patches, and therefore, it also has an effect on their population structure (McCauley, 1987). The host's fragmented distribution could isolate fly populations and increase inbreeding within them and genetic differentiation between them, as seen in other studies on invertebrates (e.g., Britten and Rust, 1996; Keyghobadi et al., 1999; Britten et al., 2003).

When we compared the genetic structure between parasites, *T. pallens* showed less structure than *T. reticulata*. Samples of *T. pallens* were taken from trees that were up to 663 km apart and *T. reticulata* was sampled along 0.9 km. Minas Gerais has a high topographic relief compared to the plateau area of Goiás, and the vegetation around *E. glomerulatus* patches is denser in Minas Gerais than in Goiás. The higher genetic structure in *T. reticulata* could be due to efficient physical barriers between patches, which would restrict the movement of flies, promoting genetic differentiation between populations. Roland et al. (2000) found that forests were twice as resistant to butterfly movement as meadows. Besides physical barriers, it seems that *T. pallens* can migrate longer distances than *T. reticulata*, as it reached *T. reticulata* distribution but the opposite was not seen. This higher migration capacity could maintain higher levels of gene flow among populations, diminishing differentiation levels.

Hybridization

Within each species, all samples had a very similar genetic composition: one most common allele/haplotype and some rare alleles/haplotypes. The most frequent haplotype of *T. pallens* was A while in *T. reticulata*, it was J. However, SRr-2, a *T. reticutala* sample, had haplotypes A and J at similar frequencies. This sample and SR-9 showed *pallens* alleles at two diagnostic allozyme loci, *PGI*-1 and *G6PD*, mostly in heterozygosis (Figure 2). This could be a sign of secondary hybridization but also of retention of ancestral polymorphism.

If species diverged recently, the shared haplotypes could be retention of ancestral polymorphism. The derived morphological characters would have arisen rapidly and recently, and this differentiation cannot be seen yet in mtDNA. Along time, random lineage extinctions would eliminate ancestral mtDNAs, while derived mtDNAs would be created by mutation. Thus, some individuals could be closer to members of other species than to their conspecifics, only due to particular patterns of survival and extinction of maternal lineage following the process of speciation (Tajima, 1983; Neigel and Avise, 1986). However, as signs of hybridization were also observed with the other marker, allozymes, the hypothesis of retention of ancestral polymorphism becomes a less probable alternative to explaining the genetic patterns observed.

Considering that we collected *T. pallens* and *T. reticulata* in sympatry in 2000, some successful interspecific matings could have occurred in Minas Gerais. If so, we would expect that SRr-2 should show stronger signs of hybridization than SR-9. This was indeed observed. The pattern is compatible with one hybridization season, when both spe-

cies had contact, followed by backcrosses with *T. reticulata*, which caused a decrease in the frequency of the *T. pallens* genotype in this area over the years. Pie diagrams (Figures 2 and 3) show evidence of this decline along with the decrease in mean nucleotide diversity from 0.137 in 2000 to 0.074 in 2002. Also, there was linkage disequilibrium in sample SR-2, indicating recent input of genotypes in this gene pool.

Most potential hybrids had a *reticulata* morphotype and haplotype A, which is the most frequent one in *T. pallens*. The rather low density of the invading *T. pallens* compared with the resident *T. reticulata* could have favored hybridization matings due to restricted mate choice (Hubbs' principle - Hubbs, 1955). If females were to remain unpaired, they would choose heterospecific mates. In a review of animal hybridization, Wirtz (1999) found support for this hypothesis and proposed that "hybrid matings are usually between the females of a rare species and the males of a common species, but not vice versa". Therefore, the rarer of the two parental species is the "mother species", whose mtDNA would pass to hybrids, as we observed.

Feder et al. (1999) described an event of hybridization between the cryptic species *Rhagoletis zephyria* and *R. pomonella*, also using allozymes and mtDNA restriction fragment length polymorphism. In these species, like in many other tephritid cryptic species, the reproductive isolating mechanism is the mating site. Usually, adult flies tend to mate and oviposit in the same host species in which they had fed as larvae (Feder and Filchak, 1999), which works as a reproductive barrier when close species have different hosts. Nevertheless, as *T. reticulata* and *T. pallens* parasitize the same host species, the mating site does not act as a barrier to them. Future studies may characterize courtship and ovipositing behavior of each species and clarify if there is also a behavioral barrier to mating. Anyway, this study indicates that sporadic hybridization events may not break down the phenotypic and genetic integrity of a species.

In conclusion, *T. pallens* and *T. reticulata* have the same host species and when their ranges overlap, a hybrid offspring can be produced. This hybridization seems to be a rare event, due to *T. pallens* unusual range expansion. As we probably documented a short period of sympatry, further investigation is needed to quantify the extent of gene flow between the two species. We cannot determine what caused this migration, but it could be related to an unusual rise in temperature, for example, as the species adapted to warmer environments migrated to a cooler one.

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