Applied Entomology and Zoology
https://doi.org/10.1007/s13355-020-00706-6

TECHNICAL NOTE

Characterization of microsatellite loci for three species of *Tomoplagia* (Diptera: Tephritidae) and absence of cross-species amplification

Camila Rabelo Oliveira Leal[1] · Leonardo Ré Jorge[2] · Anete Pereira de Souza[3] · Thomas Michael Lewinsohn[1] · Karina Lucas Silva-Brandão[4]

Received: 26 June 2020 / Accepted: 13 October 2020

Abstract
Tomoplagia (Diptera: Tephritidae) are endophagous herbivores on flowerheads of Asteraceae, with varying levels of host specialization. We isolated, characterized, and tested the heterologous amplification for sets of microsatellites of three species from this genus associated with native hosts in the Espinhaço mountain range, Brazil. We used two protocols to build microsatellite-enriched libraries and characterized 12 polymorphic loci for each fruit-fly species. The samples of *T. grandis*, a monophagous species, presented a lower number of alleles than the samples of the oligophagous species *T. incompleta* and *T. bicolor*. The average observed heterozygosity did not vary among species and the high values of Polymorphism Information Content suggest that the characterized microsatellite loci are quite informative. The primers developed for each *Tomoplagia* species failed to amplify the microsatellite loci for the other two species, which indicates a low conservation of the flanking nucleotide sequences in the three evaluated species. The sets of polymorphic microsatellites characterized for these species of *Tomoplagia* may be used in future ecological and evolutionary studies that aim to detect patterns of diversity on a fine scale.

Keywords Asteraceae · Endophagous insects · Fruit flies · Heterologous amplification · Rupestrian grassland

Introduction

In the last decades, the use of molecular tools to investigate patterns of variation and structuring of genetic diversity, as well as the processes responsible for such patterns at different spatial and temporal scales has become more frequent (Allendorf 2017; Creer et al. 2016; Seehausen et al. 2014). Among molecular markers, microsatellites, or simple sequence repeats (SSR), are quite effective for this aim: they are highly informative, mainly because of their codominant and multiallelic nature; have wide coverage in the genome; and present wide reproducibility, low cost, and technical simplicity for their development (Borém and Caixeta 2016). The genotyping of microsatellite loci requires a small amount of DNA, since it is a technique based on PCR (polymerase chain reaction), and this allows less invasive or destructive sampling, especially important to preserve specimens from biological collections (Allendorf 2017; Borém and Caixeta 2016). Other advantages of microsatellites are the possibility of using orthology between related groups for transferring primer pairs between close species (Augustinos et al. 2008; Barbará et al. 2007; Gupta et al. 2003).

Here, we characterize microsatellite markers for different species of *Tomoplagia* Coquilett (Tephritidae). The genus *Tomoplagia* is the most diverse group of endophagous flies on Asteraceae flowerheads in Brazil (Lewinsohn 1991; Prado et al. 2002, 2004). In most species whose biology is known, females lay eggs in the flowerheads of their host plants, in which the larvae feed on flowers and cypselae. The larvae pupate inside the flowerhead, from which they emerge as adults. Thus, the host plant represents a complete

✉ Karina Lucas Silva-Brandão
klsilva@gmail.com

[1] Instituto de Biologia, Departamento de Biologia Animal, Universidade Estadual de Campinas, Rua Monteiro Lobato, 255, Campinas, SP CEP 13083-862, Brazil

[2] Department of Ecology, Institute of Entomology, Biology Centre of the Czech Academy of Sciences, Branišovská 31, 37005 České Budějovice, Czech Republic

[3] Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, Av. Candido Rondom, 400, Campinas, SP 13083-875, Brazil

[4] Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Av. dos Estados, 5001, Santo André, SP CEP 09210-580, Brazil

Published online: 27 October 2020

habitat in which the flies take shelter and develop. The genus *Tomoplagia* is essentially Neotropical, with the majority of its 59 known species occurring in the rupestrian fields of the Espinhaço mountain range, in the state of Minas Gerais, Brazil, where this study was conducted (Prado et al. 2002, 2004, 2005).

Several studies have investigated the patterns of association of *Tomoplagia* spp. with their host plants, both on a local and regional scale (Lewinsohn 1991; Prado et al. 2002, 2004). For instance, *Tomoplagia grandis* Prado et al. 2004 is a strictly monophagous species; its only known host plant is *Lessingianthus buddleiifolius* (Mart. ex DC.) H. Rob. *Tomoplagia bicolor* Prado et al. 2004 is an oligophagous species with at least 17 recorded host plants, almost all belonging to five genera of the subtribe Lychnophorinae (Prado et al. 2004). Finally, *T. incompleta* (Williston, 1896) is also oligophagous, with more than 20 known hosts, mainly in the genera *Lessingianthus* and *Lepidaploa* of the subtribe Vernoniinae (Prado and Lewinsohn 2004). The wide range of host specialization among *Tomoplagia* species, the well-established host associations of adults reared directly from flowerheads and the geographic distribution established from previous field studies make the *Tomoplagia*–Asteraceae interaction an excellent model for ecological–evolutionary studies, such as those related to patterns of genetic diversity and processes that underlie the evolution of ecological specialization (Devictor et al. 2010; Forister et al. 2012).

In this study, we characterized microsatellite markers for those three species of *Tomoplagia* that are frequently found in the cerrados and rupestrian fields in the Southeast region of Brazil, i.e., *T. grandis*, *T. bicolor,* and *T. incompleta*. Since the three species belong to the same genus, we tested the heterologous amplification of the characterized microsatellites among them.

Materials and methods

Construction of libraries and design of primers

We built microsatellite-enriched libraries for each of the three *Tomoplagia* species employing two different approaches. For *T. incompleta*, microsatellite isolation was developed by Genetic Marker Services (Brighton, UK; https://geneticmarkerservices.com), and the library was built according to the protocol proposed by Edwards et al. (1996), from the genetic material of five adult individuals. The total genomic DNA was obtained from the whole *T. incompleta* individuals with a salt-extraction protocol (Aljanabi and Martinez 1997). The enzyme RsaI (New England Biolabs) was used to digest the total genomic DNA and enrichment was done using probes described in González et al. (2015).

The libraries for the species *T. grandis* and *T. bicolor* were produced with the protocol proposed by Billotte et al. (1999), from the genetic material of one and five individuals, respectively. We extracted the total genomic DNA from the whole *T. grandis* and *T. bicolor* adults using a cetyltrimethylammonium bromide protocol (CTAB; Doyle and Doyle 1987). The enzyme AfaI (Invitrogen), a RsaI isoschizomer, was used to digest the genomic DNA of the two species, and the obtained fragments were linked to the 5′-CTCTTGCTT ACGCGTGGACTA-3′ e 5′-TAGTCCACGCGTAAGCAA GAGCACA-3′ double-stranded adapters. We found transposable elements (transposons) in the digestion of *T. bicolor* DNA with the enzyme AfaI and, for this reason, we built two other libraries for this species using the enzymes BsuRI and AluI (Invitrogen).

We captured fragments that contained microsatellites in *T. bicolor* and *T. grandis* libraries with the simultaneous hybridization with two probes, $(CT)_8$ and $(GT)_8$, linked to biotin by magnetic spheres coated with streptavidin (MagneSphere Magnetic Separation Products, Promega). Microsatellite-enriched DNA fragments were separately amplified via PCR, and then cloned in pGEM-T Easy Vector (Promega) and inserted via electroporation into competent cells of *Escherichia coli* XL1-Blue (Promega). Positive clones were selected and grown on agar medium with 100 µg/mL ampicillin and 50 µg/mL X-galactosidase. After incubation for 16 h at 37 °C, we transferred the colonies obtained for each species to microplates and stored them at − 70 °C.

We sequenced 25, 48, and 48 positive clones for *T. incompleta*, *T. grandis*, and *T. bicolor*, respectively, in an ABI 3500×L automatic sequencer (Life Technologies) with Big Dye Terminator v.3.1 kit (Applied Biosystems).

We used the software SSRIT (https://archive.gramene.org/db/markers/ssrtool) to identify the presence of microsatellite repetitions in the generated sequences. To draw the primer pairs for the loci characterized for each species we applied the software Primer3Plus (Untergasser et al. 2012), using the following parameters: size of the primer between 18 and 22 bp; final size of amplification products between 100 and 280 bp; melting temperature (Mt) between 52 °C and 65 °C and maximum difference in Mt between primer pairs of 3 °C; content of GC between 40 and 60%; absence of complementarity in the same sequence or between primer pairs.

Amplification and polymorphism of loci

We tested the microsatellite loci in six individuals of the species for which each set of loci was isolated and performed the heterologous amplification tests on six individuals each of the other two species. The conditions for amplification were: 1.5 µL of genomic DNA in the standardized concentration of 5 ng/µL; 2 µL of 5× buffer; 2 µL of 5% DMSO;

1.6 µL of $MgCl_2$ at 25 nM; 0.3 µL of dNTPs at 10 nM; 0.3 µL of each direct and reverse primer at 10 nM; 0.3 µL of homemade Taq DNA polymerase; and autoclaved deionized H_2O in sufficient quantity for 20 µL of reaction. The PCR program was: initial denaturation at 94 °C (5 min); 30 cycles of denaturation at 94 °C (30 s); annealing at specific temperature (45 s) (Tables 1, 2, and 3) and extension at 72 °C (45 s); a final extension cycle at 72 °C (10 min). We evaluated the polymorphism of the microsatellite loci on a 6% polyacrylamide gel stained with silver (Creste et al. 2001).

Genotyping

We selected the polymorphic loci in the initial polyacrylamide gel test by characterizing the genotypic profile of 15 individuals from *T. grandis*, 25 from *T. bicolor*, and 25 from *T. incompleta*. All individuals of each species came from one host species sampled at Serra do Cabral State Park (17° 43′ 33.96″ S; 44° 11′ 15″ W). Thus, the analyzed individuals of *T. grandis* were reared from *Lessingianthus buddleiifolius* flowerheads, and individuals of *T. bicolor* and *T. incompleta* were reared respectively from *Eremanthus elaeagnus* (Mart. ex DC.) and *Echinocoryne schwenkiifolia* (Mart. ex DC.) flowerheads. All samples were collected within a 1 km radius. The host plant of *T. grandis* is rare and restricted to some patches along its distribution, and for this reason, it was not possible to obtain more than 15 individuals from the same host/place to be evaluated in the present study. We discarded primer pairs that failed to amplify or that amplified several nonspecific fragments in the tested temperature range (53–65 °C).

The direct primers used to amplify the selected loci were synthesized with the addition of an M13 tail (TGT AAAACGACGGCCAGT) at the 5′ end, thus allowing the annealing of the FAM, VIC, PET, and NED fluorophores (Life Technologies, Carlsbad, CA, USA; Schuelke 2000). To allow the simultaneous reading of four fluorophores in an automatic sequencer we assigned a fluorescence for each locus (Tables 1, 2, and 3). All amplified samples were run on a 1.5% agarose gel with 50 mM Tris–acetate

Table 1 Characterization of 12 microsatellite loci developed for *Tomoplagia grandis*

Locus	Primer sequences (5′–3′)	Motif	At (°C)	Size (pb)	NA	H_O	H_E	*p*	PIC	GenBank Accession No.
Tgr01	F: TCAAAGGAAAAACGTCAGTCA	(GT)8	53	92–118	4	0.31	0.29	0.16	0.32	MT995914
1-FAM	R: AATGTCAGTTTACAACGCCG									
Tgr02	F: GCTGCCTTGAACTCTCTCTA	(TG)10	59	172–184	5	0.12	0.66	< 0.01	0.85	MT995915
1-VIC	R: TGCTAACACAGTTAGTTTTGC									
Tgr03	F: GCAGTGGAGAACAATTGCAT	(CA)36	53	132–238	8	0.63	0.79	< 0.02	0.86	MT995916
1-NED	R: TGTCCATTTATCTGGGGAATCA									
Tgr04	F: AGACACCTATACGAGCGTTC	(ACA)7	53	189–243	4	0.56	0.65	0.14	0.77	MT995917
1-PET	R: ATTGTCGGATCTCATCGGTC									
Tgr05	F: TTTTCCACCTGTTAACGGGA	(TG)10	59	252–286	13	0.31	0.78	< 0.01	0.85	MT995918
2-FAM	R: GCCTACATTACGCTGTGTAAC									
Tgr06	F: CCATTGAAAGCAAACGCAAA	(AC)8	61	238–270	10	0.88	0.92	0.24	0.85	MT995919
2-VIC	R: CAGTGTCTCTGGCAGTGTTA									
Tgr07	F: CACACGCTTCTTCTCAAGTG	(TG)10	59	196–220	7	0.19	0.76	< 0.01	0.86	MT995920
2-NED	R: CTACGCAACTACATGCACAC									
Tgr08	F: TCCATTCGCAGTGACACTTA	(CA)7	59	206–286	9	0.50	0.75	< 0.01	0.72	MT995921
2-PET	R: CGGTTAAAACTTTCCGCTACA									
Tgr09	F: GGGAAGAGTGGACTTAGTGG	(AC)7	61	194–220	10	0.31	0.74	< 0.01	0.79	MT995922
3-FAM	R: GCAACTTTACAAGCCAAGCT									
Tgr10	F: GCAGTGAGTTGTAATGATATGAAC	(GT)7	61	118–166	3	0.38	0.60	0.06	0.55	MT995923
3-VIC	R: CGAGACGCCCAAAAATAGAC									
Tgr11	F: ACCGCAAATAAAAAGGGCTC	(CA)8	61	216–256	5	0.31	0.78	< 0.01	0.85	MT995924
3-NED	R: TCAGCCATTGTGGTTTTGAC									
Tgr12	F: CGCCAATTAACTTTCGGTGT	(CA)8	61	246–256	5	0.69	0.54	0.34	0.49	MT995925
3-PET	R: ACAACAACAAGCAAGTGCAT									

Annealing temperature (At), number of alleles (NA), observed heterozygosity (H_O), expected heterozygosity (H_E), Fisher's exact test to HWE (*p*), and polymorphic information content (PIC). Dyes are indicated per locus. The number before each dye represents the three groups of combinations used during multiplex genotyping

Table 2 Characterization of 12 microsatellite loci developed for *Tomoplagia bicolor*

Locus	Primer sequences (5′–3′)	Motif	At (°C)	Size (pb)	NA	H_O	H_E	p	PIC	GenBank Accession No.
Tbi01	F: TTCACATCTCATCGCTTGGA	(GA)8	63	128–170	11	0.32	0.62	< 0.01	0.66	MT995902
1-FAM	R: CCGGAATGAAGAAAGCACTG									
Tbi02	F: ACGCTTTGCTTTGTTTACCA	(TG)8	63	148–204	6	0.88	0.55	< 0.01	0.53	MT995903
1-VIC	R: TAACCTGTGTCTGCAACCTT									
Tbi03	F: TCAAACGCCAACACTTGATG	(AC)8	63	92–130	6	0.20	0.52	< 0.01	0.87	MT995904
1-NED	R: TTACCTTGCGCTATCTTCGT									
Tbi04	F: GCTTTTGTTAAGTTTGGCGC	(AC)9	61	128–146	8	0.10	0.56	< 0.01	0.63	MT995905
1-PET	R: TGGACTTGCCATATGCCATA									
Tbi05	F: CATTGACGCCGTGAAATAGG	(AG)8	63	128–144	6	0.10	0.64	< 0.01	0.82	MT995906
2-FAM	R: CCCGAAATCTTAAGTGTGGC									
Tbi06	F: TGCTGTTGGGCAAATTCTTT	(AC)8	63	176–194	12	0.36	0.80	< 0.01	0.81	MT995907
2-VIC	R: GCCAAATTCCAACTGAATGC									
Tbi07	F: AGTTGAGCAAAATGGCGAGA	(GT)6	59	152–200	6	0.36	0.60	< 0.01	0.70	MT995908
2-NED	R: CACAAAAGTGCCCTCACACG									
Tbi08	F: CCTCAAAAGGGTACATCATATATGT	(TG)9	63	146–186	10	0.36	0.68	< 0.01	0.76	MT995909
2-PET	R: GCATCGTTGAAAGCCCTAAG									
Tbi09	F: AGGACAAAGGTGTGTGGAAA	(AC)10	61	162–202	13	0.40	0.76	< 0.01	0.79	MT995910
3-FAM	R: GCTTTTTCACAACTCCCTCC									
Tbi10	F: ACATGCATGCGAAACCTTAC	(AC)9	63	146–188	13	0.24	0.76	< 0.01	0.84	MT995911
3-VIC	R: AAGTGGTAAGTGGTATGCGT									
Tbi11	F: CGTCTGCTGAACATTCAAT	(TG)6	63	158–182	10	0.44	0.64	0.01	0.74	MT995912
3-NED	R: AGATGTGCAGTTTGGCAAAG									
Tbi12	F: AATATTGCCGATTGAGGGGA	(TACA)7	61	212–264	13	0.16	0.72	< 0.01	0.64	MT995913
3-PET	R: ATTAGTCACCAGCCGATTGT									

Annealing temperature (At), number of alleles (NA), observed heterozygosity (H_O), expected heterozygosity (H_E), Fisher's exact test to HWE (p), and polymorphic information content (PIC). Dyes are indicated per locus. The number before each dye represents the three groups of combinations used during multiplex genotyping

(TAE) buffer (pH 7.5–7.8) to test the amplification. We carried out the PCRs with the addition of 0.1 µL of each of the fluorophores, plus a second step in the amplification program for the annealing of the fluorophore to the tail. This step consisted of eight cycles of denaturation at 94 °C (30 s), annealing at 53 °C (45 s), and extension at 72 °C (45 s). We diluted the PCR products (10–20%) and then combined them in a multiplex with four loci marked by different fluorophores for genotyping in the ABI 3500xL sequencer. We used Liz GeneScan 600 marker (Life Technologies) as a molecular weight standard.

We read the electropherograms with the plugin for microsatellite analysis in Geneious Prime v.2019.0.4 (Kearse et al. 2012). Alleles were determined according to their size (peak calling) and bins were overlapped on the fluorescence peaks to identify and standardize alleles.

Descriptive analyses of microsatellite loci

We characterized the polymorphic loci of each set of microsatellites by the total number of alleles per locus and the observed (H_O) and expected (H_E) heterozygosities, using the *hierfstat* package (Goudet 2005) in R (R Core Team 2014). We calculated the Polymorphism Information Content (PIC) with the *PopGenKit* package (Paquette 2012) and applied Fisher's exact test to detect deviations from the Hardy–Weinberg equilibrium (HWE) for each locus using the *pegas* package (Paradis 2010).

Table 3 Characterization of 12 microsatellite loci developed for *Tomoplagia incompleta*

Locus	Primer sequences (5′–3′)	Motif	At (°C)	Size (pb)	NA	H_O	H_E	*p*	PIC	GenBank Accession No.
Tin01	F: TTTTCCAATGTAGAATGGGTTTT	(TG)8	59	208–228	9	0.39	0.80	< 0.01	0.70	MT995890
1-FAM	R: TCCATTCGTATGTGCTTCTTG									
Tin02	F: AATGTTCGTGGCGGAGTAT	(TG)8	59	158–200	7	0.19	0.69	< 0.01	0.88	MT995891
1-VIC	R: TGCAAAGAAAGGTCAGTGAA									
Tin03	F: GGCAATCATCGAAGCAAAGT	(TTG)8	57	162–201	8	0.19	0.60	< 0.01	0.89	MT995892
1-NED	R: TGACAATGAGTGCAGATATGAAAA									
Tin04	F: TGAGCTACCAAACGCTGCTA	(TG)9	63.3	140–172	6	0.12	0.69	< 0.01	0.87	MT995893
1-PET	R: GAACGATTGAAAGGCGAAAA									
Tin05	F: CCGTCAACAATCGAGGAAAA	(TG)9	59	96–160	12	0.42	0.81	< 0.01	0.88	MT995894
2-FAM	R: CGCTTTAATACTTCGCACACA									
Tin06	F: TGAGTTTGCTCTTGCACTTGA	(AC)9	59	120–144	10	0.05	0.78	< 0.01	0.83	MT995895
2-VIC	R: CCAGCTTGACACCGACTCTC									
Tin07	F: GCAGCAAAAGAAGGAAGCAC	(GT)13	57	148–202	14	0.38	0.76	< 0.01	0.88	MT995896
2-NED	R: AACACACTCCACGCACAAAC									
Tin08	F: GTATTTGTGGGAATGCCTTG	(AC)11	63	88–150	8	0.27	0.53	< 0.01	0.33	MT995897
2-PET	R: GTACACATGTGCGTGATTATGG									
Tin09	F: CCGGCTTTTGTTTTATGCAA	(TG)9	59	80–120	5	0.27	0.73	< 0.01	0.82	MT995898
3-FAM	R: TTCTTTGCCACACTTTTGGTT									
Tin10	F: TGGCGCTCCTAAAATCACTT	(AC)9	59	86–120	6	0.12	0.65	< 0.01	0.61	MT995899
3-VIC	R: GTGCCACAGACCAGTTTGAA									
Tin11	F: TCGCAGCAACGATGATTTAG	(TG)8	57	116–174	10	0.19	0.77	< 0.01	0.88	MT995900
3-NED	R: TTCCGAGTCTAGATATCCAACCA									
Tin12	F: CTTGACGTTGATTACGACACAA	(AC)9	63.3	114–156	19	0.39	0.73	< 0.01	0.89	MT995901
3-PET	R: CGCTTTTTGTCATTGCATGT									

Annealing temperature (At), number of alleles (NA), observed heterozygosity (H_O), expected heterozygosity (H_E), Fisher's exact test to HWE (*p*), and polymorphic information content (PIC). Dyes are indicated per locus. The number before each dye represents the three groups of combinations used during multiplex genotyping

Results

We found microsatellites in 42% of the sequences obtained for *T. grandis*, in 76% of *T. bicolor* sequences, and in 36% of *T. incompleta* sequences. With these sequences, we designed primer pairs to amplify 20 loci for *T. grandis*, 19 loci for *T. bicolor*, and 17 loci for *T. incompleta*. Of the 20 pairs of primers developed for *T. grandis*, we successfully amplified 14, of which 12 were polymorphic and two monomorphic (Table 1). The number of alleles per locus ranged from 3 to 13 (mean = 6.9). We observed significant deviations from the HWE in seven loci, always with observed values lower than expected. H_O ranged from 0.12 to 0.88 (mean = 0.43) and H_E ranged from 0.29 to 0.92 (mean = 0.68). PIC values ranged between 0.32 (locus Tgr01) and 0.86 (locus Tgr07), and ten loci presented a high information content (PIC > 0.5; Table 1).

For the 19 pairs of primers developed for *T. bicolor*, we successfully amplified 15, 12 of which were polymorphic and three monomorphic (Table 2). The number of alleles per locus ranged from 6 to 13 (mean = 9.5). We observed significant deviations from the HWE in 12 loci, with observed values lower than expected, except for locus Tbi02. H_O ranged from 0.10 to 0.88 (mean = 0.33) and H_E ranged from 0.52 to 0.80 (mean = 0.65). All loci showed high PIC, with values ranging from 0.53 to 0.87 (Table 2).

For *T. incompleta*, we were able to amplify 14 of the 17 primer pairs developed, of which 12 were polymorphic and two monomorphic (Table 3). The number of alleles per locus ranged from 5 to 19 (mean = 9.5). We observed significant deviations from the HWE in 12 loci, with observed values lower than expected in all loci. H_O ranged from 0.05 to 0.42 (mean = 0.25) and H_E ranged from 0.53 to 0.81 (mean = 0.71). Eleven loci showed high PIC, except locus Tin08 (PIC = 0.33), and the PIC value ranged from 0.33 to 0.89 (Table 3). Summarized results for each *Tomoplagia* species are presented in Table 4.

The three sets of microsatellite markers characterized in this study were not transferable between the species of *Tomoplagia* studied. We observed both the absence of

Table 4 Summary of variation found in the number of alelles (NA) and observed (H_O) and expected (H_E) heterozygosity for three species of *Tomoplagia* (Tephritidae)

Species	Host plant	NA	Min–max H_O (average)	Min–max H_E (average)
T. grandis	*Lessingianthus buddleiifolius*	3–13 (6.9)	0.12–0.88 (0.43)	0.29–0.92 (0.68)
T. bicolor	*Eremanthus elaeagnus*	6–13 (9.5)	0.10–0.88 (0.33)	0.52–0.80 (0.65)
T. incompleta	*Echinocoryne schwenkiifolia*	5–19 (9.5)	0.05–0.42 (0.25)	0.53–0.81 (0.71)

amplification products and the amplification of nonspecific regions.

Discussion

We predominantly found AC/GT repetition motifs in the sets of microsatellite loci that we developed for *T. grandis*, *T. bicolor*, and *T. incompleta*, irrespective of probes used for library construction. This combination is also the most frequent in the genome of other Tephritidae species (Augustinos et al. 2008; Bonizzoni et al. 2000; Shearman et al. 2006) and arthropods in general (Tóth et al. 2000).

We observed a greater number of alleles at the loci characterized for *T. incompleta*, but mean allele numbers were similar for *T. incompleta* and *T. bicolor* (mean = 9.5); the lower allele number was observed for *T. grandis* (mean = 6.9). The mean H_O values vary among the 12 loci evaluated for each of the three species, with higher values found for *T. grandis* and lower values for *T. incompleta* (Table 4) and revealed low levels of observed heterozygosity compared to the expected values for the three species (mean H_E between 0.65 and 0.71). We found the lowest H_O value at locus Tin06 (0.05—*T. incompleta*) and the highest value at loci Tbi02 for *T. bicolor* and Tgr06 for *T. grandis* ($H_E = 0.88$). The number of alleles found for *Tomoplagia* species is consistent with those found in some other species of Tephritidae, such as *Anastrepha fraterculus* (7–18 alleles per locus, mean = 12.9; Manni et al. 2015) and *Ceratitis capitata* (8–22 alleles per locus, mean = 13.6; Bonizzoni et al. 2000), but exceed those found for *Anastrepha suspensa* (2–12 alleles per locus, mean = 6.1; Fritz and Schable 2004) and *Bactrocera tau* (2–9 alleles per locus, mean = 4.5; Yan et al. 2015). Yan et al. (2015) also found a locus with low observed heterozygosity for *Bactrocera tau* ($H_O = 0.069$), as we found in *T. incompleta* (locus Tin06). The observed heterozygosity values that we found for the three *Tomoplagia* species were lower than those of the abovementioned species.

The heterozygote deficit which we observed compared to expectation under the HWE may be due, among other factors, to the presence of null alleles or even to the Wahlund effect (Meeûs 2018). Null alleles occur due to changes in the bases in the flanking region of the microsatellite locus, which compromise primer annealing and the success of the sequence amplification in the PCR. The Wahlund effect reflects the combination of individuals belonging to different populations in a single population or aggregated sample. In this study, the frequency of null alleles was lower than 0.15 for all loci evaluated in the three *Tomoplagia* species. The knowledge about the reproductive biology of *Tomoplagia* is scarce, as well as information about its dispersion capability. Probably, the heterozygote deficit which we found is due to some degree of kinship between the individuals evaluated, and it is possible that some of these individuals belong to the same offspring. We must consider the potential inbreeding in the populations of the three species of *Tomoplagia* as a plausible factor affecting the heterozygote deficit observed.

The species-specific microsatellites that we isolated and characterized in this study are not transferable between the three species of *Tomoplagia* that we evaluated. The success of the transferability of microsatellite loci is highly variable between taxa and may reflect different levels of conservation of the flanking nucleotide sequence of the microsatellite regions between less related species (Barbará et al. 2007). Augustinos et al. (2008) managed to amplify 26 of 29 microsatellite loci developed for *Bactrocera oleae* in 11 other species of Tephritidae, including four congeneric species and other in the genera *Anastrepha* (4), *Ceratitis* (2), and *Rhagoletis* (1). According to the only phylogenetic hypothesis available for 19 species of the genus *Tomoplagia* (Yotoko et al. 2005), *T. grandis*, *T. bicolor*, and *T. incompleta* are phylogenetically distant. Moreover, although they co-occur widely in the study region (the rupestrian fields of the Espinhaço mountains in Minas Gerais), the three species are ecologically disjunct, since they are trophically associated with distinct subsets of Asteraceae (respectively, the compartments or modules 3, 4, and 6 of the insect-plant network analyzed in Prado and Lewinsohn (2004)). Thus, the phylogenetic distance between species and their ecological differences may engender the low conservation of microsatellite flanking nucleotide sequences between the three species and act as barriers to hybridization.

The microsatellite markers characterized here are the first for the genus *Tomoplagia* and will contribute to the knowledge of the biology and ecology of this tropical group, whose advantages as a study system have been mentioned before. In addition, these markers will allow future ecological and

evolutionary studies to elucidate mechanisms that generate or constrain diversification as well as enabling comparisons of patterns of diversification with other organisms.

Acknowledgements We would like to thank graduate students Danilo Sforza, Fernanda Oliveira, and Thamiris Deo for their help during the construction of the microsatellite libraries at LAGM/UNICAMP. Sampling permits were provided by Instituto Estadual de Florestas (IEF-MG; authorization number 029/2016). We also thank IEF for logistical support during the fieldwork. The present study is registered in the SISGEN (AE426B9).

Author contributions All authors have contributed to the study conception and design. Sampling was performed by CROL and TML. Material preparation, data collection, and analyses were performed by CROL and LRJ. The first draft of the manuscript was written by CROL and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding The field and laboratory work were financed by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP Grant 2016/24821-1 to TML). This study is part of the PhD thesis of CROL in the Graduate Program in Ecology at UNICAMP, with funding from FAPESP (Doctoral Grant 2015/10236-7) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brazil (CAPES)–Finance Code 001. The construction of the microsatellite library *of T. incompleta* was funded by FAPESP (LRJ, Doctoral Grant 2009/54806-0). KLSB thanks FAPESP (Grant 2012/16266-7). TML received a Productivity Research Grant from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (311800/2015-7).

References

Aljanabi SM, Martinez I (1997) Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Res 25:4692–4693

Allendorf FW (2017) Genetics and the conservation of natural populations: allozymes to genomes. Mol Ecol 26:420–430. https://doi.org/10.1111/mec.13948

Augustinos AA, Stratikopoulos EE, Drosopoulou E et al (2008) Isolation and characterization of microsatellite markers from the olive fly, *Bactrocera oleae*, and their cross-species amplification in the Tephritidae family. BMC Genom 9:618. https://doi.org/10.1186/1471-2164-9-618

Barbará T, Palma-Silva C, Paggi GM et al (2007) Cross-species transfer of nuclear microsatellite markers: potential and limitations. Mol Ecol 16:3759–3767. https://doi.org/10.1111/j.1365-294X.2007.03439.x

Billotte N, Lagoda PJL, Risterucci AM, Baurens FC (1999) Microsatellite-enriched libraries: applied methodology for the development of SSR markers in tropical crops. Fruits 54:277–288

Bonizzoni M, Malacrida AR, Guglielmino CR et al (2000) Microsatellite polymorphism in the Mediterranean fruit fly, *Ceratitis capitata*. Insect Mol Biol 9:251–261. https://doi.org/10.1046/j.1365-2583.2000.00184.x

Borém A, Caixeta ET (2016) Marcadores moleculares. Editora UFV, Viçosa

Creer S, Deiner K, Frey S et al (2016) The ecologist's field guide to sequence-based identification of biodiversity. Methods Ecol Evol 7:1008–1018. https://doi.org/10.1111/2041-210X.12574

Creste S, Neto AT, Figueira A (2001) Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. Plant Mol Biol Rep 19:299–306. https://doi.org/10.1007/BF02772828

Devictor V, Clavel J, Julliard R et al (2010) Defining and measuring ecological specialization. J Appl Ecol 47:15–25. https://doi.org/10.1111/j.1365-2664.2009.01744.x

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Edwards KJ, Barker JHA, Daly A et al (1996) Microsatellite libraries enriched for several microsatellite sequences in plants. Biotechniques 20:758–760. https://doi.org/10.2144/96205bm04

Forister ML, Dyer LA, Singer MS et al (2012) Revisiting the evolution of ecological specialization, with emphasis on insect–plant interactions. Ecology 93:981–991. https://doi.org/10.1890/11-0650.1

Fritz AH, Schable N (2004) Microsatellite loci from the Caribbean Fruit Fly, *Anastrepha suspensa* (Diptera: Tephritidae). Mol Ecol Notes 4:443–445. https://doi.org/10.1111/j.1471-8286.2004.00699.x

González C, Harvey N, Ornelas JF (2015) Development and characterization of microsatellite loci in the mistletoe *Psittacanthus schiedeanus* (Loranthaceae). Appl Plant Sci 3:1400099. https://doi.org/10.3732/apps.1400099

Goudet J (2005) hierfstat, a package for r to compute and test hierarchical *F*-statistics. Mol Ecol Notes 5:184–186. https://doi.org/10.1111/j.1471-8286.2004.00828.x

Gupta PK, Rustgi S, Sharma S et al (2003) Transferable EST-SSR markers for the study of polymorphism and genetic diversity in bread wheat. Mol Genet Genom 270:315–323. https://doi.org/10.1007/s00438-003-0921-4

Kearse M, Moir R, Wilson A et al (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Lewinsohn TM (1991) Insects in flower heads of Asteraceae in Southeast Brazil: a tropical case study on species richness. In: Price PW (ed) Plant-animal interactions: evolutionary ecology in tropical and temperate regions. Wiley, New York, pp 525–559

Manni M, Lima KM, Guglielmino CR et al (2015) Relevant genetic differentiation among Brazilian populations of *Anastrepha fraterculus* (Diptera, Tephritidae). ZooKeys 540:157–173. https://doi.org/10.3897/zookeys.540.6713

Meeûs T (2018) Revisiting F_{IS}, F_{ST}, wahlund effects, and null alleles. J Hered 109:446–456. https://doi.org/10.1093/jhered/esx106

Paquette SR (2012) PopGenKit: useful functions for (batch) file conversion and data resampling in microsatellite datasets. R package version 1

Paradis E (2010) pegas: a R package for population genetics with an integrated-modular approach. Bioinformatics 26:419–420. https://doi.org/10.1093/bioinformatics/btp696

Prado PI, Lewinsohn TM (2004) Compartments in insect–plant associations and their consequences for community structure. J Anim Ecol 73:1168–1178

Prado PI, Lewinsohn TM, Almeida AM et al (2002) The fauna of Tephritidae (Diptera) from capitula of Asteraceae in Brazil. Proc Entomol Soc Wash 104:1007–1028

Prado PI, Norrbom AL, Lewinsohn TM (2004) New species of *Tomoplagia* Coquillett (Diptera: Tephritidae) from capitula of Asteraceae in Brazil. Neotrop Entomol 33:189–211

Prado P, Abreu A, Solferini V, Norrbom A (2005) Genetic and morphological diagnosis and description of two cryptic species of flower head-infesting Tephritidae (Diptera). Insect Syst Evol 36:361–370. https://doi.org/10.1163/187631205788838528

R Core Team (2014) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18:233–234. https://doi.org/10.1038/72708

Seehausen O, Butlin RK, Keller I et al (2014) Genomics and the origin of species. Nat Rev Genet 15:176–192. https://doi.org/10.1038/nrg3644

Shearman DCA, Gilchrist AS, Crisafulli D et al (2006) Microsatellite markers for the pest fruit fly, *Bactrocera papayae* (Diptera: Tephritidae) and other *Bactrocera* species. Mol Ecol Notes 6:4–7. https://doi.org/10.1111/j.1471-8286.2006.01024.x

Tóth G, Gáspári Z, Jurka J (2000) Microsatellites in different eukaryotic genomes: survey and analysis. Genome Res 10:967–981. https://doi.org/10.1101/gr.10.7.967

Untergasser A, Cutcutache I, Koressaar T et al (2012) Primer3—new capabilities and interfaces. Nucleic Acid Res 40:115–115. https://doi.org/10.1093/nar/gks596

Yan W, Liu L, Huang S et al (2015) Development and characterization of microsatellite markers for the fruit fly, *Bactrocera tau* (Diptera: Tephritidae). Appl Entomol Zool 50:545–548. https://doi.org/10.1007/s13355-015-0359-2

Yotoko KSC, Prado PI, Russo CAM, Solferini VN (2005) Testing the trend towards specialization in herbivore-host plant associations using a molecular phylogeny of *Tomoplagia* (Diptera: Tephritidae). Mol Phylogenet Evol 35:701–711