

A panel of microsatellite markers developed for solitary trap-nesting wasp *Ancistrocerus trifasciatus* (Müller, 1776) by cross-species amplification

Rita Radzevičiūtė^{1,2*},

Anna Budrienė²,

Eduardas Budrys^{1,2},

Svetlana Orlovskytė^{1,2},

Jurga Turčinavičienė¹

¹Department of Zoology,
Vilnius University,
M. K. Čiurlionio 21/27,
LT-03101 Vilnius, Lithuania

²Institute of Ecology,
Nature Research Centre,
Akademijos 2,
LT-08412 Vilnius, Lithuania

Solitary cavity-nesting Hymenoptera constitute a group of important bioindicators of terrestrial habitats. Some of them, like caterpillar-hunting potter wasp *Ancistrocerus trifasciatus* (Hymenoptera: Vespidae: Eumeninae), are quite abundant in both continuous and fragmented habitats and might be a promising model species for studying the impact of habitat fragmentation and landscape connectivity on genetic diversity of entomophagous insects. Highly polymorphic microsatellites are a powerful molecular tool for intraspecific studies but the development of this marker system *de novo* is especially laborious and time-consuming. An alternative time- and effort-effective approach to establish the microsatellite markers for the species of interest is the cross-species amplification of loci already isolated in related species. Here we present a panel of five polymorphic microsatellite loci for *A. trifasciatus* developed by cross-species amplification of twenty-nine microsatellite markers published so far for nearctic potter wasps.

Key words: *Ancistrocerus trifasciatus*, Vespidae, Eumeninae, microsatellites, cross-species amplification

INTRODUCTION

Habitat loss, degradation and fragmentation are considered to be the major factors influencing the maintenance of biodiversity (Tscharntke, Brandl, 2004). The negative impacts of habitat loss on species' persistence are well known (Debinski, Holt, 2000) whereas the effects of habitat fragmentation on biodiversity at various scales are less understood and seem to differ for taxa with different life histories and / or ecological functions (Fahrig, 2003; Steffan-Dewenter, 2003). In contrast to pollinators or ground-dwelling carabid beetles (Cane, 2001; Niemelä, 2001; Zimmermann et al., 2010) little is known about the effects of changes in habitat configuration and size on the genetic diversity of entomophagous insects capable to fly

quite long distances. The caterpillar-hunting solitary vespid wasp *Ancistrocerus trifasciatus* (Müller, 1776) might be a promising model for habitat fragmentation and landscape connectivity studies. It is one of the most abundant cavity-nesting wasp species in both continuous and fragmented forests of various types in Central Europe (Sobek et al., 2009; Budrys et al., 2009), thus allowing sufficient sampling required for most of the frequency-based statistics in population genetics (Allendorf, Luskart, 2007). The assessment of population structure of this wasp species in fragmented habitats may also be of great interest for evaluation of habitat quality. Highly polymorphic DNA markers like microsatellites could be an appropriate molecular tool for answering these questions.

Microsatellites, also known as simple tandem repeats (STRs) or simple sequence repeats (SSRs), are short sections of DNA that consist of simple

* Corresponding author. E-mail: rita.radzeviciute@gf.stud.vu.lt

motifs (1–6 bp in length) repeated up to about 100 times (Jarne, Lagoda, 1996). They are highly abundant and distributed (almost) randomly throughout the genomes of both prokaryotes and eukaryotes (Pradeep et al., 2008). Microsatellites are co-dominant in nature and exhibit extensive levels of intraspecific polymorphism as a result of DNA polymerase slippage or gene conversion (Strand et al., 1993; Richard, Pâques, 2000). Their variability derives mainly from differences in length rather than in the primary sequence and is characterized by the presence of multiple alleles and high heterozygosity (Ellegren, 2004). These features make microsatellites a powerful molecular tool for biological studies at the intraspecific level (Chambers, MacAvoy, 2000). The advent of the multiplex PCR technique and the automated capillary electrophoresis with laser detection of different fluorescent dyes have advanced the STR genotyping and increased the throughput (Vemireddy et al., 2007). In the last two decades microsatellites became the marker of choice for linkage mapping, parentage assignment, population genetics, habitat fragmentation studies and related areas (Selkoe, Toonen, 2006). Despite the increasing application of emerging radically new genome-wide genotyping and sequencing approaches (Tautz et al., 2010) in the recent years, microsatellites still remain relevant genetic markers (Guichoux et al., 2011), especially for non-model organism studies of limited budget. A cost-effective microsatellite marker panel for the non-model species of interest can be established in a time- and effort-saving way by the cross-species amplification of already available STR primers originally designed for a species belonging to the same genus or closely related genera (Barbará et al., 2007). The flanking regions of microsatellite loci usually mutate at lower rates than the short tandem repeats themselves and in many cases serve as conserved primer annealing sites even across related taxa (Primmer et al., 1996). However, the utility of any STR system is predicted to decrease with increasing phylogenetic distance (Ellegren, 2004). Other drawbacks related to cross-species application of microsatellite markers are amplification of non-orthologous loci and size homoplasy due to indels or point mutations in the flanking regions or in the tandem repeats as well (Barbará et al., 2007). Nevertheless, it can

be solved by conducting the sequence analysis of cross-amplified PCR products prior to application of microsatellite loci to the species under study (Yue et al., 2010).

In the case of solitary cavity-nesting Hymenoptera, a group of important bioindicators of terrestrial habitats (Tscharrntke et al., 1998), there have been only four microsatellite primer notes published so far. One of them reports a STR marker system for the Red Mason bee *Osmia rufa* (Linnaeus, 1758) (Apidae: Megachilinae) (Neumann, Seidelmann, 2006) whereas the rest three deal with solitary nearctic potter wasps, *Euodynerus foraminatus* (de Saussure, 1853), *Ancistrocerus adiabatus* (de Saussure, 1852) and *Monobia quadridens* (Linnaeus, 1763) (Hymenoptera: Vespidae: Eumelinae) (Stalhut et al., 2004; Bushrow, Cowan, 2005; Foune et al., 2008). This study focuses on testing the cross-species amplification success of microsatellite loci isolated for these wasp species to one of the most abundant palearctic trap-nesting solitary predator wasp *Ancistrocerus trifasciatus*.

MATERIALS AND METHODS

Collection of specimens

Males of *A. trifasciatus* were used for cross-species PCR optimization experiments and selection of polymorphic loci because in this species (as in many haplodiploid Hymenoptera as well) males are hemizygous and should produce a single PCR product after a successful amplification. Six male wasps were collected in Kokulla (59°54'N 17°33'E), Sweden, Väike-Maarja (59°09'N 26°18'E), Estonia, Greifenhagen (51°38'N 11°26'E), Germany, Saugūniškės (54°51'N 25°02'E) and Dūkštų oak-wood (54°50'N 24°58'E), Lithuania, using trap-nests of standardised construction (Budrys et al., 2010). Specimens from distinct localities of the species' distribution area were selected to capture polymorphic loci with a lower number of samples tested. For calculation of general polymorphism statistics, 40 females of *A. trifasciatus* were sampled with the same method in three localities in Lithuania: Puvociiai (54°07'N 24°18'E, N=12), Dūkštų oak-wood (54°50'N 24°58'E, N=15) and Kiemeliai (54°52'N 25°01'E, N=13). Specimens were preserved in 96% ethanol and retained at the Institute of Ecology of Nature Research Centre, Vilnius, Lithuania.

DNA extraction and cross-species PCR optimization

DNA was extracted from wasps' thorax musculature using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) following manufacturer's recommendations. 29 microsatellite loci (18 of *M. quadridens* (Foune et al., 2008), 6 of *A. adiabatus* (Bushrow, Cowan, 2005) and 5 of *E. foraminatus* (Stalhut et al., 2004)), polymorphic in their respective species, were tested in cross-species amplification experiments. Microsatellites isolated in *A. adiabatus* (*Aa1*, *Aa2*, *Aa3*, *Aa4*, *Aa5*, *Aa9*) and *E. foraminatus* (*Efo01*, *Efo02*, *Efo03*, *Efo04*, *Efo07*) had dinucleotide tandem repeat units whereas loci for *M. quadridens* (*Mq9*, *Mq24*, *Mq32*, *Mq46*, *Mq88*, *Mq96*, *Mq101*, *Mq132*, *Mq133*, *Mq143*, *Mq151*, *Mq160*, *Mq176*, *Mq242*, *Mq253*, *Mq256*, *Mq259*, *Mq261*) contained trinucleotide repeat motifs. Initially singleplex PCR reactions were carried out applying primers and PCR thermal conditions published in aforesaid articles, in a total reaction volume of 20 µl. PCR mixture contained 2 µl DNA (~20 ng), 2 µl of each primer (10 µM), 2 µl 10 × Platinum buffer (Invitrogen), 2 µl 2mM dNTP mixture (Thermo Fisher Scientific), 1 µl 50 mM MgCl₂ (Invitrogen), 0.5 U Platinum *Taq* DNA polymerase (Invitrogen) and 9 µl double-distilled water. All PCRs were performed with the MasterCycler personal (Eppendorf) thermocycler. If a specific PCR product was not found on 4% GenAgarose™ Tiny II agarose (Inno-Train) gel in

0.5 × TBE buffer (Eppendorf), PCR was repeated by changing the primer annealing temperature (±5 °C), duration of PCR steps, number of cycles or concentrations of PCR reagents. Size and concentration of PCR products were measured with GenLadder™ 100 DNR ladder (Inno-Train). Detailed conditions of optimized PCR for each polymorphic successfully cross-amplified locus are given in Table 1. They were applied to prepare samples for DNA sequencing.

DNA sequencing

Precipitated PCR products of three male specimens (from distinct localities) for each polymorphic locus were shipped to MacroGen Inc., South Korea, where they were purified and sequenced in both directions with the 3730xl genetic analyser (Applied Biosystems), using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and PCR primers. Sequences were obtained to confirm that (1) orthologous loci were amplified; (2) observed length polymorphism arises from differences in the number of tandem repeat units and not from indels or point mutations in flanking regions; (3) no interruptions are present in perfect tandem repeats. Forward and reverse sequences and subsequently sequences of different samples were manually aligned with BioEdit 7.0.9.0 (Hall, 1999). Sequence data were deposited in GenBank database (<http://www.ncbi.nlm.nih.gov/genbank>) under the accession numbers JQ995782-99 and JX014253-54.

Table 1. Optimized PCR conditions for cross-species amplified loci found to be polymorphic in *A. trifasciatus*. In all cases initial denaturation took 10 min at 94 °C, and final extension 10 min at 72 °C. * indicates primer annealing temperature that was not changed during PCR optimization. [Mg²⁺] refers to final concentration of Mg²⁺ in PCR reaction mixture

Locus	Source species	Reference	Number of cycles	Denaturation		Annealing		Elongation		[Mg ²⁺], mM
				Duration, s	T, °C	Duration, s	T, °C	Duration, s	T, °C	
<i>Aa1</i>	<i>Ancistrocerus adiabatus</i>	Bushrow, Cowan, 2005	30	30	94	30	55.0	45	72	1.5
<i>Aa3</i>										
<i>Aa9</i>										
<i>Efo02</i>	<i>Euodynerus foraminatus</i>	Stalhut et al., 2004	35	30	94	30	56.0	45	72	2.5
<i>Efo07</i>										
<i>Mq9</i>	<i>Monobia quadridens</i>	Foune et al., 2007	30	45	94	45	56.3	60	72	1.5
<i>Mq24</i>							48.3*			
<i>Mq96</i>							60.0			
<i>Mq261</i>							61.5			

Microsatellite genotyping

Confirmed STR loci were subsequently applied to genotype female samples. Forward primers for each locus were labelled with fluorescent dye (either 6-FAM, VIC or TET (Applied Biosystems)), depending on amplification intensity and ranges of allele length in their source species. PCRs were carried out in a final volume of 15 µl containing 9 µl of True Allele PCR Premix (Applied Biosystems), 1 µl of primer mix (5 µM each), 4 µl double-distilled water and 1 µl DNA (~10 ng). *Aa1*, *Aa3*, *Aa9* loci were amplified using a bit modified cycling conditions recommended by the manufacturer for PCRs with True Allele PCR Premix: initial denaturation 12 min at 96 °C, followed by 30 cycles of 15 s at 95 °C, 15 s at 55 °C, 30 s at 72 °C and final extension 60 min at 72 °C. This PCR thermal regime did not perform well for other loci, so optimized PCR conditions used before (Table 1) were applied, only the final extension was changed to 60 min at 72 °C in order to shift all PCR products consistently towards “plus A” peaks in the results of fragment length analysis.

PCR products were diluted with double-distilled water. 15 µl of Hi-Di formamide (Applied Biosystems) for each sample were mixed with 0.25 µl GeneScan-500 LIZ internal size standard (Applied Biosystems) and 1 µl of diluted PCR product. Two poolplexes of PCR products were designed, no positive controls were used. Genotyping was performed on the ABI 3730 genetic analyzer (Applied Biosystems) with the 50 cm length four-capillary array (Applied Biosystems) and POP7 polymer (Applied Biosystems). Allele sizes were determined by both automated allele calling and subsequent visual inspection using the GeneMapper 4.0 software (Applied Biosystems).

Statistical methods

MICRO-CHECKER (Van Oosterhout et al., 2004) was used to detect possible null alleles and large allele drop-out. General polymorphism statistics (observed and expected heterozygosity, allelic richness), Hardy-Weinberg equilibrium and linkage disequilibrium tests were calculated using Arlequin 3.5.1.3 (Excoffier, Lischer, 2010). Significance across multiple tests was determined after the standard Bonferroni correction (Rice, 1989).

RESULTS AND DISCUSSION

Seven of twenty-nine tested STR loci (*Efo01*, *Mq88*, *Mq133*, *Mq143*, *Mq253*, *Mq256* and *Mq259*) failed consistently to cross-amplify with all *A. trifasciatus* samples. *Efo03*, *Mq32*, *Mq46*, *Mq101*, *Mq151*, *Mq176*, *Mq242* yielded non-specific products even after application of various PCR optimization strategies. Finally, the remaining fifteen loci amplified successfully but only nine of them proved to be polymorphic (*Aa1*, *Aa3*, *Aa9*, *Efo02*, *Efo07*, *Mq9*, *Mq24*, *Mq96* and *Mq261*). As it was expected, the STR loci isolated in the congeneric *A. adiabatus* demonstrated the highest cross-species amplification success (six loci of six tested amplified, three of them variable in *A. trifasciatus* males from distinct populations).

Reliable sequence data were obtained for all polymorphic microsatellites but *Mq96* and *Mq261*. As the microsatellite origin of these loci could not be confirmed, they were discarded from further analysis. However, *Mq96* and *Mq261* might be included in the STR marker panel for *A. trifasciatus* in the near future because applying the new “sequencing by synthesis” approach overcomes the problem of DNA polymerase slippage during the sequencing of repetitive DNA and sequences of these loci might be obtained (Morozova, Marra, 2008). Primers for the remaining loci (*Aa1*, *Aa3*, *Aa9*, *Efo02*, *Efo07*, *Mq9*, *Mq24*) amplified PCR products of orthologous loci. With the exception of *Efo07*, where two base pairs were missing just before the start of tandem repeats in the allele of a specimen from Sweden, sequences of other loci showed no length polymorphism in flanking regions due to indels. Perfect tandem repeats were not interrupted by point mutations either.

As expected, almost no or at least noticeably less stutter peaks were characteristic of microsatellite loci containing trinucleotide than dinucleotide repeats (Ellegren, 2004) but the latter demonstrated higher levels of polymorphism (Table 2). The *Efo02* locus yielded a particularly ambiguous pattern with many stutter peaks of the same intensity in the electrophoretograms of automated fragment length analysis. Thus it was impossible to identify the true alleles of this locus. Further improvement of PCR and/or capillary electrophoresis conditions is required to obtain obvious results of this polymorphic microsatellite marker in *A. trifasciatus*.

Table 2. Allele characteristics and polymorphism data for six cross-species amplified polymorphic microsatellite loci based on genotypes of 40 *A. trifasciatus* females. N – number of individuals, A – allelic richness, H_o – observed heterozygosity, H_e – expected heterozygosity. • significant deviation from Hardy-Weinberg equilibrium ($P < 0.0006$, adjusted value after Bonferroni correction for multiple tests)

Locus	Repeat motif	N	Allele size range (bp)	A	H_o	H_e	P-value
<i>Aa1</i>	(TC) _n	40	196–240	21.164	0.917	0.946	0.209
<i>Aa3</i>	(CT) _n	40	294–328	7.865	0.500	0.634	0.703
<i>Aa9</i>	(CT) _n	40	203–235	18.081	0.917	0.942	0.701
<i>Efo07</i>	(TA) _n	40	174–180	5.790	0.083	0.511	• 0.0001
<i>Mq9</i>	(TAC) _n	40	125–161	14.554	0.500	0.761	0.016
<i>Mq24</i>	(ATG) _n	40	156–185	9.782	0.750	0.815	0.237

Only one locus, *Efo07*, showed significant deviation ($P < 0.0006$ after Bonferroni correction) from Hardy-Weinberg equilibrium (Table 2) which resulted in homozygote excess. Results of analysis with MICRO-CHECKER suggested this was due to null alleles at *Efo07*. More than 50% of the alleles at this locus belonged to the same allele size class, so binomial analysis could not be performed. Genotyping errors due to stuttering, large allele drop-out or null alleles were not detected for *Aa1*, *Aa3*, *Aa9*, *Mq9* or *Mq24*. No evidence for linkage disequilibrium was detected either in separate or pooled populations. Overall polymorphism was moderate for the entire data set, with half of loci having allelic richness higher than ten (Table 2).

CONCLUSIONS

A microsatellite panel of five proved polymorphic scorable loci (*Aa1*, *Aa3*, *Aa9*, *Mq9*, *Mq24*) was established for solitary vespid wasp *A. trifasciatus* after the cross-species amplification experiments. These markers can be applied in future studies of population structure of *A. trifasciatus* in continuous and fragmented habitats. Additional three loci (*Mq96*, *Mq261*, *Efo02*) might also be included into the marker panel after the further improvement of PCR and/or capillary electrophoresis.

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Rita Radzevičiūtė, Anna Budrienė, Eduardas Budrys,
Svetlana Orlovskytė, Jurga Turčinavičienė

**MIKROSATELITŲ ŽYMENŲ SISTEMA LIZDA-
VIETĖSE-GAUDYKLĖSE APSIGYVENANČIAI
KLOSČIAVAPSVĖI ANCISTROCERUS TRIFASCIA-
TUS (MÜLLER, 1776), SUDARYTA PRITAIKIUS
NEARKTINĖMS KLOSČIAVAPSVĖMS SPECIFI-
NIUS MIKROSATELITŲ PRADMENIS**

Santrauka

Nebendruomeniniai įvairiose ertmėse lizdus įsirengiantys plėviasparniai vabzdžiai yra svarbūs sausumos ekosistemų bioindikatoriai. Šiai grupei priskiriama drugių vikšrus medžiojanti klosčiavapsvė *Ancistrocerus trifasciatus* (Hymenoptera: Vespidae: Eumeninae), kuri yra gana gausiai paplitusi tiek vientisoje, tiek suskaidytose miškų buveinėse. Ši vapsvų rūšis galėtų būti modeline rūšimi buveinės fragmentacijos įtakos vabzdžių-entomofagų genetinei įvairovei tyrimams. Dideliu kintamumu pasižymintys mikrosatelitai paprastai taikomi vidurūšiniams tyrimams, tačiau šių genetinių žymenų sistemos kūrimui *de novo* reikia daug laiko, laboratorinių procedūrų ir įrangos. Greitesnis ir paprastesnis mikrosatelitų žymenų sistemos sudarymo alternatyvus būdas yra artimoms rūšims nustatytų mikrosatelitų lokusų pritaikymas tikslinei rūšiai. Straipsnyje pateikiama *A. trifasciatus* populiaciniams tyrimams pritaikyta penkių mikrosatelitų žymenų sistema, kuri sudaryta patikrinus dvidešimt mikrosatelitų pradmenų, taikytų kitoms devynioms nearktinių klosčiavapsvių rūšims.

Raktažodžiai: *Ancistrocerus trifasciatus*, Vespidae, Eumeninae, mikrosatelitai

