EUROPEAN JOURNAL OF ENTOMOLOGY
ISSN (online): 1802-8829
http://www.eje.cz
Eur. J. Entomol. **113**: 302–306, 2016
doi: 10.14411/eje.2016.037
NOTE

Characterization of 16 novel microsatellite loci for *Ephippiger diurnus* (Orthoptera: Tettigoniidae) using pyrosequencing technology and cross-species amplification

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Key words. Orthoptera, Tettigoniidae, *Ephippiger diurnus*, calling song, microsatellite polymorphism, katydid, population genetics

Abstract. A novel panel of 16 microsatellite markers, obtained by pyrosequencing of enriched genomic libraries, is reported for the flightless European bushcricket *Ephippiger diurnus* (Dufour) (Orthoptera: Tettigoniidae). Five multiplex and one simplex PCR protocols were optimized, and the polymorphism at the 16 loci was assessed in two natural populations from southern France. The mean allele number and (expected mean heterozygosity) were 8.94 (0.71) and 6.57 (0.70), respectively, in each population. Several loci were at Hardy-Weinberg disequilibrium (HWD), possibly due to the incidence of null alleles. The occurrence of null alleles has been previously reported for this species, and it is a common feature of microsatellite loci in Orthoptera. Cross-amplification tests demonstrated the transferability of some of these loci to other ephippigerine species. The microsatellite loci reported here substantially increase the number of available loci for this species and will afford an accurate picture of *E. diurnus* phylogeography, the genetic structure of its populations, and an improved understanding of the evolution of male song and other sexually-selected traits in this highly variable species.

INTRODUCTION

The European bushcricket *Ephippiger diurnus* (Orthoptera: Tettigoniidae) has attracted considerable attention among behavioral and evolutionary biologists because of its diverse calling songs (Duijm, 1990; Ritchie, 1996), large spermatophore (Barbosa et al., 2016), and a strong population genetic structure (Spooner & Ritchie, 2006). *E. diurnus* are flightless, do not migrate, and have specific habitat preferences, and previous studies showed that they are distributed in geographically isolated, genetically differentiated populations throughout their range in southern France and northeastern Spain (Party et al., 2015). These geographically separate populations generally exhibit distinctive male songs that are characterized by a specific number of syllables per call (Ritchie, 1991, 1996), and some attempts have been made to relate the song trait to phylogeography by evaluating mitochondrial DNA (*COI*) divergence (Party et al., 2015). The various populations can be crossed in the laboratory (Ritchie, 2000), but the full potential of such hybridization is unknown. To determine the phylogeography of *E. diurnus* with greater precision and to explore the evolution of song diversity, genetic markers that afford reliable, fine-level resolution of population differences are needed.

The development of a minimal number of valuable microsatellite loci for population genetics (i.e. polymorphic, easily scorable, free of null alleles and conforming to Hardy-Weinberg expectations) has proven challenging in *E. diurnus*. Indeed, a set of 16 loci had been reported for this species before our study (Hockham et al., 1999; Hamill et al., 2006). According to the authors themselves, these loci displayed strong heterozygote deficit, and the incidence of null alleles was considerable for some of them. In a preliminary trial we tested 13 of these available 16 loci on samples from highly divergent populations previously characterized for mitochondrial DNA *COI* variation (Party et al., 2015). Most loci failed to amplify and/or presented complex allelic patterns impeding their scoring. This situation significantly reduced the number of available markers to only five, which is a minimum value for population genetic analyses. We therefore applied high-throughput (pyrosequencing) technology to a partial genomic library enriched in microsatellite motifs in order to increase the number of loci and filter out those of low quality according to criteria detailed below.

MATERIAL AND METHODS

Sample collection and DNA extraction

Fifty-one specimens of *E. diurnus* were collected from nine localities in southern France between 2011 and 2014 (Fig. 1). Hind femora were dissected and preserved in 95% ethanol for DNA

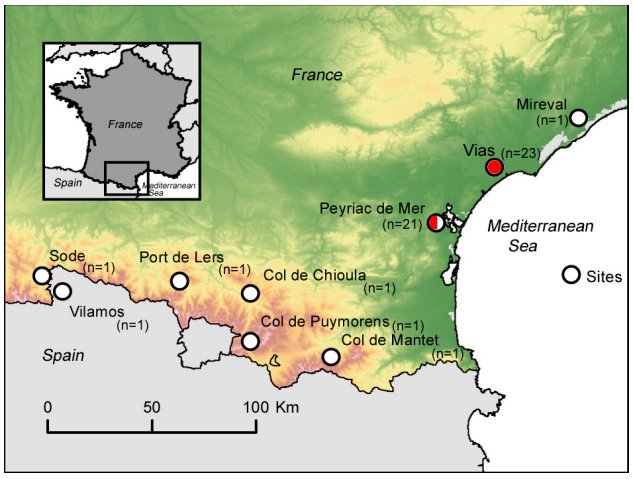

Fig. 1. Localities and number of sampled specimens (n) in south France. Circles represent sampled localities for microsatellite enriched DNA libraries construction, PCR amplification test (white) and/or polymorphism analysis (red).

extraction. Whole genomic DNA was extracted using the DNA Easy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA quality and molecular weight were assessed in a 1% agarose gel and with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Villebon sur Yvette, France).

Microsatellite isolation

Five µg of DNA were obtained by pooling individual DNA extracts from eight insects sampled in eight of nine localities (Fig. 1). The DNA pool was sent to Genoscreen, Lille, France (www.genoscreen.fr) for microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries following the approach described by Malausa et al. (2011). Briefly, enriched libraries were constructed using eight microsatellite probes (TG, TC, AAC, AAG, AGG, ACG, ACAT, ACTC), and the resulting library was sequenced on a GsFLX PTP. The resulting 72,447 reads were analyzed using the program QDD (Meglecz et al., 2010) and sorted according to the following criteria: number of microsatellite repeats ≥ 5, microsatellite motif not interrupted by any other bases or sequences, fragment size ≥ 80 bp. A fasta file with 5,027 reads containing a microsatellite repeat and a list of optimized primer pairs for 503 reads (size range: 90–319 bp) was provided by Genoscreen. Within these reads, 323 primer pairs with expected fragment sizes ≥ 120 bp were chosen. Special attention was paid to homologous sequences shared among distinct reads: short, repeated sequences in the vicinity of microsatellites are frequently shared among distinct loci and impede consistent single locus PCR amplifications if primers overlap them (Meglécz et al., 2007). To avoid this problem, sequences homologous among different reads identified after an "all-against-all" BLASTn analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were masked before primer design. In the same line, all sequences were checked and masked for the presence of known annotated repeated elements in the flanking regions by the RepeatMasker software (http://www.repeatmasker.org/) and the related domestic silkworm database. Finally, 100 loci were chosen to proceed with the first PCR screening using unlabeled primers (Eurofins Genomics, Ebersberg, Germany). For some of these loci new primers were designed when the expected amplification size was not adequate for the posterior multiplexing PCR procedure and or when primers provided by Genoscreen were located in zones of high homology according to our BLASTn analysis (Table S1).

Because of the high level of divergence among mtDNA *COI* clades (see Fig. 4 in Party et al., 2015) we tested PCR amplification of the 100 loci in four specimens of *E. diurnus* collected in Mireval, Sode, Port de Lers, and Col de Mantet (Fig. 1), and belonging to the two main *COI* clades (Fig. 1 in Party et al., 2015). Sequences and primers for the 100 loci are given in Table S1. Thus, we could retain only those loci amplifying unambiguously in all clades (Table S1). All amplifications were achieved with an ABI GeneAmp PCR System 2700 thermal cycler. PCR reactions were carried out in a 10-µl solution containing the following: 2 mM $MgCl_2$, 2 mM DNTPs, 1 × PCR Buffer, 0.5 unit of Go*Taq* G2 polymerase (Promega, Charbonnieres, France), 2 µM of each forward and reverse primer and ~10 ng of template DNA. PCR cycling conditions were as follows: 95°C 3 min, followed by 30 cycles at 94°C 30 s, 60 s at 60°C, 72°C for 45 s, and a final extension step of 10 min at 72°C. PCR products were resolved in a 2% agarose gel. Twenty-one primers not amplifying in all four specimens (i.e. partial PCR amplification) were not considered. Forty-two primer pairs that showed clear, reproducible and unique fragments in the four specimens were retained for further analysis. Among them, eight loci showed an incidence of smear or amplified nonspecific bands, thereby justifying a +3°C increase of the annealing temperature (Table S1). Lastly, 37 loci did not amplify at 60°C and were tested at 52°C using the same PCR conditions as above but were not tested in the following steps described below (Table S1).

Fragment analysis of 42 loci followed the cost-effective M13 fluorescent protocol described by Schuelke (2000) with modifications described below. Each forward primer was tagged at its 5' end with one 18–19 bp tail described in Culley et al. (2013) and one fluorescent label depending on the expected amplification size to allow posterior PCR multiplexing (Table S1). The combination of tails and fluorescent labels were as follows: M13 modA-NED, M13 modB-PET, T7 term-VIC and M13 (-21)-FAM (Applied Biosystems, Warrington, UK, see Table 1). Simplex PCR tests were performed on four to eight specimens to confirm PCR amplification with tailed primers. The 6.25 µl PCR reaction contained: 3.25 µl Multiplex PCR Master mix (Qiagen), 1 µl of a primer mix per locus containing: 2 µM of each reverse and labeled tail primer and 0.5 µM of the forward tailed primer (ratio: 1 : 1 : 1/4, see Culley et al., 2013), 1 µL H_2O and 1 µl of DNA (~10 ng/µl). PCR cycling conditions followed a denaturing step of 15 min at 95°C, then 30 cycles at 94°C for 30 s, 60°C or 63°C (Table S1) for 45 s and 72°C for 45 s; and then 8 cycles of 94°C for 30 s, 53°C for 45 s and 72°C for 45 s and a final elongation step of 10 min at 72°C. PCR products were visualized on a 2% agarose gel. Fragment analysis was conducted on a 3730 xl DNA Analyzer (Applied Biosystems) using the GeneScan 500 LIZ as internal size standard (Applied Biosystems) and 1 to 2 µl of PCR product (1 : 20 dilution). After visual screening of electropherogram profiles in GeneMapper version 5.0 (Applied Biosystems), 25 primers were selected for their scoring in additional specimens. Finally, 16 primer pairs showing polymorphism and unambiguous profiles were retained. For loci showing noisy electropherograms, the annealing temperature was increased by 3°C (Table S1).

Multiplex PCR amplification

We used Multiplex Manager version 1.0 (Holleley & Geerts, 2009) to determine the best combination of loci in a multiplexed PCR amplification protocol. Five multiplex PCR amplification reactions and one simplex PCR were defined for the final set of 16 loci, and these were amplified in two populations of *E. diurnus* (Vias and Peyriac de Mer; Table 2) belonging to each of the two main mtDNA *COI* clades described in Party et al. (2015, see

Table 1. Characteristics of 16 microsatellite loci from *Ephippiger diurnus*. Abbreviations: F – the forward primer sequences; R – the reverse primer sequences; Ta – annealing temperature.

Locus	Repeat	Primer sequence (5'–3')	Expected size (bp)	Dye	Tail [a]	Ta (°C)	PCR reaction	Primer mix (μM)
Ediur_3	$(GA)_9$	F: TTG CAA TGA AAC GTT CTT CCT R: TGA CCA GCA TTC TGT CTT GG	216	FAM	M13 (-21)	60	Multiplex 1	F tailed + tail = 0.7 R = 2
Ediur_4	$(ATAC)_{12}$	**F**: TCA GAG GGG TTT CCT TTC CT R: AGC GAT ACC AAG ACG ACG AT	235	VIC	T7 term	63	Simplex	F tailed + tail = 0.7 R = 2
Ediur_6	$(TGT)_6$	F: GAC ACT CTT CCG GAG CTT CA R: TGA TCC TCG ATT AGT CGC AA	205	VIC	T7 term	60	Multiplex 1	F tailed + tail = 0.7 R = 2
Ediur_8	$(TGTA)_7$	**F**: ACT GAG TTC GCA CAC ACG AC **R**: CGA ATC GAG CCG ATC TTA TT	175	VIC	T7 term	60	Multiplex 2	F tailed + tail = 0.7 R = 2
Ediur_20	$(ACA)_5$	**F**: CAC AAA CAT CTG GTT GTC GAA **R**: ACT GCC CGA TAA CGT ACA CTT	236	FAM	M13 (-21)	60	Multiplex 2	F tailed + tail = 0.7 R = 2
Ediur_21	$(TTG)_9$	F: AGG AAT GAG AAA ACT GCC GA R: CAG AGG CAG CTG CAA ACA TA	227	PET	M13 modB	60	Multiplex 4	F tailed + tail = 1 R = 2.3
Ediur_22	$(AC)_{12}$	F: CCC CTC AAA TAT CCC AAC AC **R**: CGC AAT GTC GAA CAC ATT TT	189	FAM	M13 (-21)	63	Multiplex 5	F tailed + tail = 0.7 R = 2
Ediur_25	$(AAG)_7$	**F**: AAA GCT ATT GGG TTT GTG GAA **R**: CCA CCA ACT GAA CAG TGT CCT	214	PET	M13 modB	60	Multiplex 1	F tailed + tail = 1 R = 2.3
Ediur_47	$(TGA)_{10}$	F: TGG GAA ACA TGG AAA GGT GT R: CCT GTC ATT TGC TGC TTC CT	149	VIC	T7 term	63	Multiplex 5	F tailed + tail = 0.7 R = 2
Ediur_48	$(AC)_6$	F: AAC CCA CCG GCC TAT TAA CT R: GGC AAA CTC CAG TCA TCC AT	227	VIC	T7 term	60	Multiplex 4	F tailed + tail = 0.7 R = 2
Ediur_55	$(ATAG)_{13}$	F: GCA CCG CAG CCA TAG ATA AG R: ATG TGA GTT ACG AGG CAA GC	135	PET	M13 modB	60	Multiplex 2	F tailed + tail = 1 R = 2.3
Ediur_57	$(CA)_7$	F: TGA ACA AGA AAT AGA GGC GAG A R: ATT TCT GTG GCG TGA TGT GA	123	VIC	T7 term	60	Multiplex 3	F tailed + tail = 0.7 R = 2
Ediur_59	$(TGAT)_6$	**F**: TCT GGC CAT ATT AAG GGA AAT G **R**: TGA CAT GGA GTG ATG GAT GG	237	PET	M13 modB	60	Multiplex 3	F tailed + tail = 1 R = 2.3
Ediur_75	$(AC)_{16}$	F: TTT GGT GCT AGC GTT GAG TG R: CGT GCT TGA CAG ACC TCA GA	160	NED	M13 modA	60	Multiplex 4	F tailed + tail = 1 R = 2.3
Ediur_85	$(ATG)_8$	F: TGT CGG TCT AAG GCC CAT AC **R**: CAA TTG ACC TAA TGC AGG TGA C	240	FAM	M13 (-21)	60	Multiplex 4	F tailed + tail = 0.7 R = 2
Ediur_86	$(TTG)_9$	F: AAT GCA TCC AAC CGA CTA CC R: TGC TAA CTT ATT CCG GTG GC	297	VIC	T7 term	60	Multiplex 2	F tailed + tail = 0.7 R = 2

[a] Tail primer sequences from Culley et al. (2013): M13 (-21) TGT AAA ACG ACG GCC AGT, M13 modA TAG GAG TGC AGC AAG CAT, M13 modB CAC TGC TTA GAG CGA TGC, T7 term CTA GTT ATT GCT CAG CGG T. Modified F and R primers are highlighted (see Table S1).

Table 2. Characteristics of 16 microsatellite loci from two populations of *Ephippiger diurnus*. Abbreviations: N – number of genotyped specimens; Na – number of alleles; *Ho* – observed heterozygosity; *He* – expected heterozygosity.

Locus	Vias (N = 23)					Peyriac de Mer (N = 21)				
	Size range (bp)	Na	*Ho*	*He*	Null alleles frequency	Size range (bp)	Na	*Ho*	*He*	Null alleles frequency
Ediur_3	226–232	4	0.04	0.59**	0.34	228–238	4	0.50	0.61	
Ediur_4	229–284	14	0.50	0.91**	0.21	225–280	11	0.58	0.83**	0.14
Ediur_6	220–247	6	0.22	0.61**	0.24	226–238	4	0.76	0.69	
Ediur_8	143–261	20	0.61	0.95**	0.17	161–241	13	0.62	0.86**	0.12
Ediur_20	247–271	7	0.35	0.82**	0.26	234–259	8	0.25	0.82**	0.31
Ediur_21	205–247	8	0.65	0.82	0.09	219–244	5	0.45	0.45	
Ediur_22	186–207	10	0.61	0.77	0.09	186–195	5	0.70	0.64**	
Ediur_25	207–237	6	0.68	0.71		210–240	6	0.81	0.79	
Ediur_47	160–190	11	0.54	0.85**	0.16	160–200	7	0.38	0.56	0.12
Ediur_48	236–245	7	0.58	0.73		235–243	5	0.67	0.68	
Ediur_55	120–260	18	0.65	0.85	0.11	121–204	6	0.37	0.72**	0.20
Ediur_57	137–142	4	0.30	0.33		137–145	3	0.43	0.41	
Ediur_59	244–305	9	0.12	0.87**	0.40	244–305	7	0.41	0.79**	0.21
Ediur_75	157–182	9	0.27	0.78**	0.28	150–174	9	0.76	0.82	
Ediur_85	245–291	9	0.91	0.82		242–272	7	0.86	0.80	
Ediur_86	301	1	0	0		301–339	5	0.30	0.76**	0.26
Mean		8.94	0.44	0.71			6.57	0.55	0.70	
SE		1.25	0.06	0.06			0.67	0.05	0.03	

** Significant deviations from Hardy-Weinberg equilibrium after Bonferroni correction ($P = 0.003$).

Table 3. Cross-amplification of 16 microsatellite loci isolated for *E. diurnus*. The INRA collection ID code for each voucher specimen is given within parentheses. Abbreviations: Lat – Latitude; Long – Longitude.

Taxon (INRA collection ID code)	Sampling locality Lat / Long	Locus							
		Ediur_3	Ediur_4	Ediur_6	Ediur_8	Ediur_20	Ediur_21	Ediur_22	Ediur_25
E. provencialis (JSTR02143_0101)	43.6389 / 5.9356	+	–	–	–	–	+	–	+
E. provencialis (JSTR02144_0101)	43.6389 / 5.9356	+	–	–	–	–	+	–	+
E. terrestris (JSTR02148_0101)	43.7241 / 6.6297	+	–	–	+	–	–	–	+
E. terrestris (JSTR02149_0101)	43.7241 / 6.6297	+	–	–	+	–	–	–	+
U. rugosicollis (JSTR02145_0101)	44.0419 / 4.8971	–	–	+	–	+	+	–	+
U. rugosicollis (JSTR02146_0101)	44.0419 / 4.8971	–	–	+	–	+	+	–	+
		Ediur_47	Ediur_48	Ediur_55	Ediur_57	Ediur_59	Ediur_75	Ediur_85	Ediur_86
E. provencialis (JSTR02143_0101)		–	+	–	+	–	+	+	–
E. provencialis (JSTR02144_0101)		–	+	–	+	–	+	+	–
E. terrestris (JSTR02148_0101)		–	+	–	+	–	+	+	–
E. terrestris (JSTR02149_0101)		–	+	–	+	–	+	+	–
U. rugosicollis (JSTR02145_0101)		–	+	–	+	–	+	+	+
U. rugosicollis (JSTR02146_0101)		–	+	–	+	–	+	+	+

Figs 1 and 4). The multiplexed PCR reactions were carried out in a final volume of 6.25 µl using 3.25 µl Multiplex PCR Master mix (Qiagen), 1 µl of multiplex primer mix (see Table 1), 1 µl of DNA (~10 ng/µl) and 1 µl H_2O. PCR cycling conditions for the five multiplex reactions were: 15 min at 95°C, then 30 cycles at 94°C for 45 s, 60°C or 63°C for 90 s and 72°C for 60 s; and then 8 cycles of 94°C for 30 s, 53°C for 45 s and 72°C for 45 s and a final elongation step of 30 min at 60°C. PCR cycling conditions for the simplex PCR followed the same simplex PCR protocol described above.

Polymorphism analysis

The allele number, heterozygosity and Hardy-Weinberg equilibrium (HWE) for each loci were computed in GENEPOP version 4.3 (Rousset, 2008). *P* values were adjusted for multiple tests of significance using the sequential Bonferroni correction at the 5% nominal level (Rice, 1989). Incidence of null alleles was assessed on Micro-checker version 2.2.3 (Van Oosterhout et al., 2004), and when significant their frequency was obtained using the Brookfield 1 method. GenBank numbers (KU512644-KU512669) were attributed only to primary sequences (Table S1).

Cross-amplification

The final set of 16 loci was cross-amplified in two other species of *Ephippiger* and in one species of *Uromenus* using the simplex PCR amplification protocol described above (Table 3).

RESULTS AND DISCUSION

Microsatellite loci isolated from *E. diurnus* showed moderate to high levels of allelic diversity and were polymorphic in both populations analyzed, except for locus Ediur86 in Vias. The number of alleles ranged from four to 20 in Vias (N = 23), with a mean of 8.94 alleles per locus, and from three to 13 in Peyriac de Mer (N = 21), with a mean of 6.57 alleles per locus. The expected heterozygosity ranged from 0 to 0.95 in Vias, with a mean of 0.71, and from 0.41 to 0.86 in Peyriac de Mer, with a mean of 0.70. Heterozygote deficiency and significant departure from HWE were detected for several loci in both populations after Bonferroni correction (Table 2). The analysis of the distribution of homozygote size classes on Micro-checker suggested the incidence of null alleles that might contribute to the observed heterozygote deficiency and HWD in both populations (Table 2). No scoring errors due to stuttering or large allele drop-out were detected. The frequency of null alleles ranged from 0.09 to 0.40 in Vias and from 0.12 to 0.31 in Peyriac de Mer. A high proportion and prevalence of null alleles at microsatellite loci are common in Orthoptera (Zhang et al., 2003; Chapuis et al., 2005; Chapuis & Estoup, 2007), and *Ephippiger diurnus* seems not to be an exception. Previous reports on microsatellite characterization for this species also showed a considerable prevalence of null alleles (Hockham et al., 1999; Hamill et al., 2006). The distribution of *E. diurnus* in small, genetically differentiated populations, an outcome of low dispersal and specific habitat preferences, probably contributes to the observed heterozygote deficiency.

The microsatellite markers we report for *E. diurnus* will be valuable for fine level phylogeographic analysis and studies at larger geographical scale, and for studying the diversity of the male calling song and female preferences. Cross-amplification tests showed the transferability of this set of microsatellite markers to other *Ephippiger* species as well as to another ephippigerine, *Uromenus rugosicollis* (Table 3).

ACKNOWLEDGEMENTS. We thank J.-C. Streito and A. Foucart for collecting specimens for the cross-amplification tests. F. Cerqueira, E. Desmarais and T. Cantinelli (Centre Méditerranéen Environnement Biodiversité; Labex CEMEB) provided technical support with genotyping. The DGIMI (Diversité, génome & interactions microorganisms-insectes, UMR INRA 1333 – Université de Montpellier) research unit made its laboratory facilities available for our analyses. This study was funded by grant ANR-11-BSV7-025-01 (EVOLCHOR) from the Agence Nationale de la Recherche de France.

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Received January 22, 2016; revised and accepted March 4, 2016
Published online April 5, 2016

Supplementary file:
Table S1 (http://www.eje.cz/2016/037/S01.xls).