International Journal of Odonatology, 24.2021, 227–232 Wachholtz

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Isolation and characterization of 10 polymorphic microsatellite loci in the rarest European damselfly, *Coenagrion hylas* (Odonata: Coenagrionidae)

https://doi.org/10.23797/2159-6719_24_17

Received: 22 December 2020 – Accepted: 21 April 2021 – Published: 03 Dezember 2021

Abstract: Within Europe, the damselfly *Coenagrion hylas* has a very limited distribution and is regarded as a vulnerable species. For studying migration and population connectivity in the Central European populations, 10 microsatellite markers were developed for this species. The loci were screened on 24 individuals collected at Lech valley, Tyrol, Austria. The values for expected and observed heterozygosity ranged from 0.192 to 0.802 and from 0.208 to 0.917, respectively. All developed markers were polymorphic.

Keywords: dragonfly, conservation, NGS, population genetics

Introduction

The Siberian damselfly *Coenagrion hylas* (Trybom, 1899) is a postglacial relict in Europe and represents a cold-stenothermal fauna that probably colonized the continent during the late Pleistocene and early Holocene (Bernard & Daraż, 2010; Dumont, 1971). Apart from a few sites in the Ural and Pinega region of Russia, vital populations in Europe currently are exclusively known from an extremely limited area of occurrence of only about 42 km² in the Northern Calcareous Alps of Tyrol, Austria (Landmann, 2013; Wildermuth & Martens, 2019; Landmann et al. 2021). Accordingly, *C. hylas* may be regarded as the rarest European Zygoptera species and is assessed as vulnerable in the IUCN Red List (Kalkman et al., 2010) and listed in Annex II of the EU-FFH directive. It thus deserves special attention and protection on an international scale.

The known sites of occurrence of this rare species in the Alps are partly separated from each other by high mountain chains and other barriers. The genetic diversity and connectivity of the mostly small and isolated subpopulations are unknown. In addition, the taxonomic rank of the central European relict population, which has even been described as a separate species or subspecies, *C.* (*hylas*) *freyi* by Bilek (1954, 1957), has been the object of controversial debates (eg. Kiauta & Kiauta, 1991), and the genetic differentiation of the Central European, Russian, and east Asian populations of the taxon is still unknown.

For developing conservation and management plans for the endangered Central European relict populations of *C. hylas*, we opted not to rely on microsatellites published for congeners (e. g. Johansson et al., 2012) and developed the first microsatellite markers for this species. Here, we present 10 new species-specific microsatellite markers from *C. hylas*, verified by basic population-genetic analyses.

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Material & Methods

DNA sampling

For the construction of a DNA-library, DNA was extracted from a male individual's thorax (id = 100361), which was caught with gloves to minimize possible cross-contamination. For the validation of the new loci, we used tissue from mid-leg tibiae. For 24 male individuals of *C. hylas*, captured at one site in the Lech valley (Tyrol, Austria), one tibia each was cut off with cuticle scissors and put in 96% ethanol immediately after sampling. We randomly chose individuals based on their fit to the primers, and all these individuals happened to be males. Samples were stored at -20 °C until DNA extraction. DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) using the manufacturer's instructions. To enhance the yield, DNA was eluted twice with 25 µl H_2O. The incubation step after the first elution was prolonged to five minutes.

Primer design

The genomic DNA of the thorax was divided into two samples, checked for human contamination with ALU-J-primers, and sent to the sequencing facility on dry ice. Sequencing was performed in a Illumina MiSeq run using 300 bp paired-ends.

The sequencing results were used to search for repetitive single sequence repeats and to identify potential primers. For this purpose, we merged two respective sets of paired-end reads using *bbmerge.sh* from the bbtools suite v38.37 (BBMap) before concatenation, to create one final set of interleaved sequence reads.

The unmerged paired-end reads were not used for the downstream analyses to avoid computational overhead and to focus on just the longest reads. A read adapter and quality trimming were performed, using *bbduk* and *clumpify* from bbtools. For *bbduk*, we used a kmer length of 23 for contaminant search, minimum kmer length of 11, a maximum Hamming distance for reference kmers of 1, and a minimum quality of 6 for read trimming; both ends were trimmed when the base quality was below said minimum quality.

For *clumpify*, we used the trimmed reads with the "dedupe" option, to remove duplicate reads when both pairs matched. We further searched for single-sequence repeats with custom Bash and Python scripts, using PERF (Avvaru et al. 2018) and Primer3 version 2.4.0 (Rozen & Skaletsky, 2000). For the repeat search with PERF, we used a minimum motif size of two and a minimum of eight repeat units. For primer design with Primer3, we further used an optimal size of 20, minimum and maximum primer size of 18 and 20, respectively, and minimum and maximum GC content of 43 and 57, respectively, for left and right primer search. The output of Primer3 was converted into a list of primers with a custom Python script, in which we kept the first primer pair candidate found. All relevant scripts and settings for the full workflow can be found at https://git.uibk.ac.at/c7701188/meg_projects/tree/master/msats. Of the resulting primers, we assessed the variability of 10 consistently amplifying loci on the collected mid-leg tibiae.

Genotyping and analyses

PCR was done in 5-µl reaction volumes with 0.5 µl template DNA, 1.0 µl 5× Q5 reaction buffer (New England BioLabs, Germany), 0.1 µl 10 mM dNTP mix, 0.25 µl 10 mM M13 labelled forward primer and reverse primer, 0.03 µl 10 µM M13-tailed forward primer, 0.05 µl Q5 High-Fidelity Polymerade (New England BioLabs) and ddH_2O.

Reactions were run on a UnoCycler (VWR, USA) using the following conditions: initial denaturation at 94 °C for 2 min followed by 39 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 45 s (for primers Cohy_1 to Cohy_7, Cohy_10) or 68 °C for 45 s (for primers Cohy_8 & Cohy_9) respectively, and extension at 72 °C for 45 s. Final extension was performed at 72 °C for 10 min (for detailed information about each locus, see Table 2). Fragment analysis was performed by a commercial pro-

vider (CRC Sequencing Facility, Chicago, USA) using an ABI 3130 instrument (Applied Biosystems, Foster City, USA) and Eurofins (Germany) also using an ABI 3130 instrument. All samples were cleaned using sephadex-columns prior to analysing, and LIZ500 was used as internal size standard.

Table 1: Characteristics for 10 microsatellite loci developed for *Coenagrion hylas*: Primer name, primer sequence, repeat motif, annealing temperature in degree Celsius, fluorescent dye used, and the GenBank accession number are given for each locus.

Locus	Primer sequences (5‘-3‘)	Repeat motif	Annealing tempera-ture (°C)	Size range (bp)	Fluro-rescent dye	GenBank accession number
Cohy_4	F: CACGACGTTGTAAAACGACATCGTGCAGTCAGGAAGGTG R: CAACCGTCTCTCAAGCCCTT	$(GA)_{12}$	64	154–162	FAM	MW289833
Cohy_16	F: CACGACGTTGTAAAACGACTTGGCTGAGATCGGAGTGTG R: TCGCATAGGGGAGTGATCCT	$(GA)_{9}$	64	123–135	NED	MW289834
Cohy_19	F: CACGACGTTGTAAAACGACTGACGTCACGAAGTTCCCTG R: CGCTCGCTTTAAGCACCATC	$(GA)_{9}$	64	197–207	PET	MW289835
Cohy_24	F: CACGACGTTGTAAAACGACGCGTGTGTGTTTGTGGGTAC R: GTGGGTTTGCGTAAGGGGTA	$(TG)_{9}$	64	150–162	PET	MW289836
Cohy_33	F: CACGACGTTGTAAAACGACCAAATATCACTGGTGGCCGG R: GCCCAACCCATTTGTCCCTA	$(AT)_{24}$	64	85–91	HEX	MW289837
Cohy_42	F: CACGACGTTGTAAAACGACAGTACCTACATACGCGCGTG R: ATGGAGATGGACGACGATGG	$(GA)_{10}$	64	235–241	FAM	MW289838
Cohy_3	F: CACGACGTTGTAAAACGACAGGAAGGAATGAAGGGCTGC R: ATGAGCTCTCTTTGGTGGCC	$(ATA)_{13}$	64	282–316	HEX	MW289839
Cohy_43	F: CACGACGTTGTAAAACGACCCACAGGAGGAACGGACAAA R: CGAGCTTTCGTGAGTGTCCT	$(AT)_{18}$	68	254–282	FAM	MW289840
Cohy_45	F: CACGACGTTGTAAAACGACCGAGCTTTCGTGAGTGTCCT R: CCTTCCGATTACGCGTCAGA	$(TA)_{16}$	68	275–285	NED	MW289841
Cohy_54	F: CACGACGTTGTAAAACGACTCCTTCCGGAAAAGTGGCTC R: ACAGACAGCCAAGCCAAGAA	$(TC)_{13}$	64	178–190	PET	MW289842

Alleles were scored with GeneMarker 3.0.1 (Softgenetics, USA). The number of alleles (Na), allelic richness (A_R), and Hardy-Weinberg-tests were calculated using PopGenReport v3.0.4 (Adamack & Gruber, 2014) in R v3.6.1 (R Core Team, 2019). Bonferroni-Holm-corrected p-values were calculated for the Hardy-Weinberg-results. Expected (H_e) and observed (H_o) heterozygosity as well as F_{IS}-values were calculated using hierfstat v0.5-7 (Goudet, 2003; Goudet & Jombart, 2020) in R v3.6.1.

We doublechecked and rescored all samples to look for potential genotyping errors that could explain negative Fis-values, but without any changes in the outcome. Therefore, negative Fis-values are likely to have biological reasons and/or to be artefact of the small sampling size.

Results & Discussion

The raw data for the two samples of individual 100361 consisted of 1.48 million and 1.29 million reads. After merging and trimming, this amounted to a total of 2.23 million reads and a total of 2.21 million non-duplicate reads. In those 2.21 million reads, we found 9350 possible repeat regions, and 232 possible primer pair candidates. According to Watts (2009), the number of repeat motifs in the majority of microsatellites for odonates are quite short (10 or less); our results are thus in line with this observation (see Tab. 1).

Genotyping 24 individuals of *C. hylas* showed that five loci deviated from the Hardy-Weinberg equilibrium (Cohy_3, Cohy_24, Cohy_33 Cohy 45, Cohy54), but all loci were polymorphic with five showing heterozygote deficits (Cohy_3, Cohy_16, Cohy_24, Cohy_45, Cohy_54) and five showing higher observed than expected heterozygosity (Cohy_4, Cohy_19, Cohy_33, Cohy_42, Cohy_43; see Table 2). All inbreeding coefficients were statistically insignificant. Three to eight alleles were found per polymorphic locus. Given the number of individuals genotyped, the numbers of alleles retrieved is relatively low, which is in line with what has been found for microsatellites in other odonates (Watts, 2009). When studying more samples from different subpopulations within the range of the relict population of *C. hylas*, these numbers might well be higher. Observed heterozygosity ranged from 0.208 to 0.917. Deviations from Hardy-Weinberg equilibrium could be explained by strong population structure or inbreeding in the samples, since no null alleles were present, which often are accountable for these deviations (Waples and Allendorf, 2015). Inbreeding coefficients ranged between -0.067 and 0.590. On the one hand, the elevated values for some loci could be due to the small sample size (e.g., Nonaka et al. 2019). On the other hand, these elevated values could also indeed indicate inbreeding, considering the fact that our samples are not (part of) a panmictic population and therefore are not ideal for calculating Hardy-Weinberg deviations.

Table 2: Details for 10 microsatellite loci developed for *Coenagrion hylas*: number of alleles (Na), observed and expected heterozygosity (Ho, He), inbreeding coefficient (FIS), significance for Hardy-Weinberg equilibrium (HWE, Bonferroni-Holm-adjusted p-values), number of genotyped individuals (n), and number of alleles per locus (a). Asterisks represent significant values for a confidence level of 95%.

Locus	N_a	H_o	H_e	F_{IS}	HWE	a
Cohy_4	3	0.208	0.192	-0.065	1.000	3
Cohy_16	5	0.292	0.297	0.039	0.087	5
Cohy_19	5	0.792	0.727	-0.067	0.778	5
Cohy_24	7	0.500	0.822	0.410*	0.000*	7
Cohy_33	2	0.917	0.500	-0.827	0.000*	2
Cohy_42	5	0.583	0.556	-0.029	0.121	5
Cohy_3	8	0.458	0.762	0.417*	0.007*	8
Cohy_43	6	0.708	0.533	-0.310	0.694	6
Cohy_45	6	0.333	0.780	0.590*	0.000*	6
Cohy_54	7	0.375	0.582	0.375*	0.004*	7

The 10 species-specific polymorphic microsatellite markers for *C. hylas* developed using next-generation sequencing will be useful in studying the Central European *C. hylas* population (see already Landmann et al. 2021). Additionally, they might be helpful in clarifying the relationships of this isolated relict population with the Ural and Pinega populations in Russia.

Acknowledgements

Permission for DNA sampling was obtained from the Tyrolean government, Dept. of Nature Conservation. We thank Philipp Andesner (assistance in the lab) and Florian Reischer (GenBank accession) for their help. The study was implemented in and partly funded by an EU-LIFE Project (LIFE Lech – Dynamic River System Lech: LIFE15 NAT/AT/000167).

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