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Polymorphic microsatellite loci in the endangered butterfly *Lycaena helle* (Lepidoptera: Lycaenidae)

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Abstract. Six polymorphic microsatellite loci were isolated in the endangered butterfly *Lycaena helle*. Five of them provided interpretable results. We detected four to 34 alleles per locus in a total of 235 samples (males and females) collected from meadows in the Ardennes-Eifel (Germany, Luxemburg and Belgium) and the Westerwald (Germany). We collected one leg for DNA-extraction as a non-lethal method. The expected heterozygosities ranged from 48.6% to 83.1%, depending on the locus analysed. These markers are currently being used in our studies of the species' phylogeography over its western Palearctic distribution area and for the analysis of the conservation status of the fragmented populations in Central Europe.

Lycaena helle (Denis & Schiffermüller, 1975) is a species with a boreo-mountainous distribution (Ebert & Rennwald, 1991; Tolman & Lewington, 1997; Kudrna, 2002). During the early postglacial, this lycaenid butterfly may have been homogenously distributed over major parts of the western Palearctic. Climatic changes over the past thousands of years have led to a disjunct distribution scattered over parts of Scandinavia and the European mountain systems (e.g. Pyrenees, Alps, and Carpathians). Habitats of *Lycaena helle* are characterised by a moisty character and cool climate. Anthropogenic land use changes (e.g. agricultural intensification, afforestation of non-wooded habitats) combined with climate changes have resulted in the extinction of this species in many parts of Central Europe (Fischer et al., 1999). Sites still colonized by *Lycaena helle* are mostly scattered over regions of higher altitude (e.g. in Central Europe the Massif Central, Vosges, Ardennes, Eifel, Westerwald). These sites are often isolated and most probably represent small remnants of ancient population networks. All these

factors enforce the collapse of interconnected habitat networks (IUCN, 1996; Van Swaay & Warren, 1999).

With the aid of microsatellite markers we analysed the phylogeography and the genetic status of this disjunctly distributed species with its extant metapopulations in the Alps and isolated and small relict populations in Central Europe (cf. Luikart et al., 1998).

Six recombinant clones were randomly selected from a microsatellite enriched library (Gruenstein & Hogness, 1975; Hamilton et al., 1999) for sequencing analysis, and PCR primers were designed for six loci. One microsatellite locus (Lhe13) was abandoned because of problems during the optimization of the PCR amplification process.

Polymorphism of five PCR-primers generating the expected PCR products was tested with 235 *Lycaena helle* samples collected from different sites in the Ardennes, the Eifel and the Westerwald. DNA was extracted from individuals using a Qiagen DNeasy™ Tissue Extraction Kit (Hilden, Germany).

TABLE 1. Characteristics of six polymorphic microsatellite loci in *Lycaena helle*. Abbreviations: F – forward primer; R – reverse primer; T_a – annealing temperature; A – mean number of alleles; H_o – observed heterozygosity; H_e – expected heterozygosity; 235 individuals were analysed for each locus.

Locus	GenBank Accession no.	Primer sequence (5'–3')	Repeat motif	Size of sequenced allele (bp)	T _a (°C)	A	H _o	H _e
<i>Lhe03</i>	EU117181	F: GCGCAAACTATTTCAGTTTAC R: ACTTAAATGTTTCTCGGCTC	(GACA) ₅	271	53	4	54.2	48.6
<i>Lhe13</i>	EU117182	F: ACTTTTGGTGATCTCTTAAG R: AACATAATGGTTTGCCGCTG	(CA) ₁₅	299	55	–	–	–
<i>Lhe14</i>	EU117183	F: GTTGTTTTTCGAGCCAAGGAG R: AGTGACGATTCCGATGACTC	(AT) ₁₈	281	56	26	55.3	83.1
<i>LheB06</i>	EU117178	F: CCTTTCCACATATAGTTGGC R: CATCGTCAGGTCACCTTCATC	(TA) ₈	486	54	28	52.5	78.0
<i>LheE12</i>	EU117179	F: AAATCAAGTCACGCATAC R: CCTTAATCGATGGCAATTTTC	(TG) ₂₆	448	53	13	78.8	73.8
<i>LheF12</i>	EU117180	F: AGTAGCCATTTTACCGTCAG R: CTACGCAACGGATTTTGATG	(GT) ₂₄	401	54	34	68.5	72.1

Microsatellite loci were amplified in a reaction mixture of ThermoZym Mastermix (Molzym, Bremen, Germany) and about 20–100 ng diluted DNA, depending on the locus analysed. To reduce costs and laboratory work, two microsatellite markers were multiplexed. The multiplex contained two pairs of primers, Lhe03 and LheE12 loci were amplified. The loci LheF12, LheB06, and LheE14 were amplified separately. The PCR amplifications were performed in a total of 27.6 µl for the multiplex which contained 25 µl ThermoZym Mastermix, 3 pmol for each primer of the Lhe03 locus and 10 pmol for each primer of the LheE12 locus. For LheF12, LheB06, and LheE14 a total of 16 µl contained 10 µl Mastermix and 5 pmol for each primer. Amplification was initiated with 1 min of denaturation at 94°C followed by 45 cycles involving 30 s of denaturation at 94°C, 30 s of primer annealing at a temperature ranging from 53 to 56°C (T_a) and 1 min of extension at 72°C, and a final extension step at 72°C for 2 min following a cool down to room temperature (see Table 1). 10 µl of the PCR products were loaded and PCR fragments resolved by electrophoresis on 2.4% agarose gels stained with ethidium bromide as a control before scoring the microsatellites using an automated sequencer running Megabace software (GE Healthcare, USA).

Significant deviations from Hardy-Weinberg equilibrium ($p < 0.05$) were detected for two of these loci. With the program Micro-checker (van Oosterhout et al., 2004) we found strong evidence for null alleles for the loci Lhe B06 and Lhe E14. No significant linkage disequilibrium was detected among any two loci ($p > 0.05$). The total number of alleles ranged from four to 34 per locus with a total value of 105 alleles detected over all populations. The expected heterozygosity averages over all populations analysed for the five loci ranged from 48.6% to 83.1% (Table 1). The expected and observed heterozygosity for these loci was calculated using GenALEX (Peakall & Smouse, 2006).

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