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# Eight new microsatellite loci of the Western Palearctic *Hyles euphorbiae* complex (Lepidoptera, Sphingidae)

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We describe eight new microsatellite loci for hawkmoths of the *Hyles euphorbiae* complex. They are polymorphic (except for one locus in one population) with 2–18 alleles per locus, an expected heterozygosity between 0.14 and 0.94, and an observed heterozygosity between 0.10 and 0.75. As typical for Lepidoptera, the yield of new loci was low due to the presence of microsatellite gene families and variable flanking regions. These microsatellites provide informative results in population studies of the West Palearctic *H. euphorbiae* complex since cross amplification for *H. euphorbiae* and *H. tithymali* was successful for all but one locus.

The taxa of the *Hyles euphorbiae* complex as well as more distantly related species are known to readily hybridize in nature (Harbich 1975, 1976a, 1976b, *see* overview in Hundsdoerfer *et al*. 2005b). A natural hybrid population between the two main lineages, namely the North African *H. tithymali* (Boisduval 1832) and the European *H. euphorbiae* (Linnaeus 1758), is postulated for Malta based on morphological (Harbich 1989, 2009) and mitochondrial sequence data (Hundsdoerfer *et al*. 2005a, 2005b, 2009b). Overall, the reproductive plasticity is reflected in fuzzy species delimitations caused by a high variability of the few applicable morphological characters, especially larval colour patterns (Hundsdoerfer *et al*. 2011). Hundsdoerfer *et al*. (2005a, 2005b, 2009b, and unpubl. data) and Hundsdoerfer and Wink (2006) found considerable incongruences between morphology and nuclear as well as mitochondrial sequence markers. This indicates young lineages *in statu nascendi* and/or reintegration of incompletely separated lineages which makes the species complex especially interesting for population genetic studies. To reveal the patterns of natural hybridization between the lineages a co-dominant genotype marker system is essential.

Microsatellite markers have been reported to be challenging in identification and application for lepidopteran species (Meglécz *et al*. 2004). They are rare within lepidopteran genomes and difficult to amplify (Meglécz & Solignac 1998). Lepidopteran microsatellites are associated with flanking regions characterized by repetitive sequences (Meglécz *et al*. 2004), a high incidence of single nucleotide polymorphisms and indels (Hundsdoerfer *et al*. 2009a) and mobile elements leading to multiple copies of a locus

(microsatellite families, Zhang 2004, Hundsdoerfer *et al*. 2009a). These properties can result in a deficit of heterozygotes due to the presence of null alleles (Meglécz *et al*. 2004). Nevertheless, microsatellites can be used for lepidopterans with proper attention being paid to these issues (Hundsdoerfer *et al*. 2009a).

DNA was isolated from larvae of *Hyles euphorbiae* (Iberia) and *Hyles tithymali* (Canary Islands) using the innuPREP DNA Mini Kit (Analytik Jena, Germany) to create a DNA library for each species (performed by GENterprise, Mainz, Germany): DNA was sheared by nebulization. After electrophoretic fractionation fragments of two size ranges (1.5 to 2.5 kb and > 2.5 kb) were excised and regained by electroelution, ligated into SmaI-cut, dephosphorylated pUC19 vectors and transformed into *Escherichia coli* host cells (tribe DH10B). Approximately 16 000 colonies for *H. tithymali* and *H. euphorbiae* each were screened for the microsatellite motives GA, CA, AAT, GGC, AAG and ATG by hybridization of colony filters with corresponding oligo probes.

One hundred and ninety two positive clones (96 per species) were sequenced (GENterprise) of which 146 (85 for *H. euphorbiae* and 61 for *H. tithymali*) contained microsatellite repeats. The flanking regions of 59 clones in total were appropriate to design primer pairs (GENterprise). Only eight loci (14%) for *H. euphorbiae* (and seven for *H. tithymali* respectively) were reliably PCR amplified and showed to be polymorphic without indication of microsatellite families (multiple alleles per individual). These eight loci (Table 1) of which five were originally found in *H. tithymali* and three in *H. euphorbiae* were used to genotype 20 non-sibling individuals each from two populations of *H. euphorbiae* (southeast Bulgaria, BUL; northeast Spain, ESP) and two populations of *H. tithymali* (Gran Canaria, GC; La Palma, LP). PCR was performed in a volume of 20 $\mu$l following the protocol of Schuelke (2000) containing 1 U *Taq* polymerase (Bioron, Ludwigshafen, Germany), PCR reaction buffer with a final concentration of 2.5 mM $MgCl_2$ (Bioron), 0.2 mM of each dNTP (Fermentas, St. Leon-Rot, Germany), 0.4 $\mu$M FAM labeled M13-Primer, 0.4 $\mu$M reverse Primer, 0.1 $\mu$M forward Primer with M13 tail at the 5´ end and approximately 10–30 ng DNA template. The PCR comprises 38 cycles of denaturation at 94 °C for 30 s (5 min for the first cycle), primer annealing at 56 °C for the first 30 cycles and 53 °C for the last 8 cycles for 45 s, and extension at 72 °C for 45 s (10 min for final elongation). Primers for loci Hti63 and Hti67 performed unsufficiently with dye-labeling after the protocol of Schuelke (2000), but worked well as directly labeled forward primers (0.4 $\mu$M) with a modified protocol (94 °C for 3 min, 39 cycles of 30 s at 94 °C, 30 s at 59 °C for Hti63 or 57 °C for Hti67 respectively and 1 min at 72 °C and final 20 min at 72 °C). Fragment size was determined on an ABI3130 automatic sequencer using an internal size standard (Liz 600, ABI) and the software GeneMapper (ver. 4, ABI).

The eight loci were polymorphic in all four tested populations of both species (2–18 alleles, Table 1), except for locus Heu76 which was not reliably PCR amplified in *H. tithymali* and locus Hti63 in which one allele got fixed in the Bulgarian population of *H. euphorbiae* (Table 1). Although this respective allele is missing the microsatellite we argue that locus Hti63 still has some population genetical value because of a cline with higher frequencies of alleles containing a repeat motif in more southwestern populations. Significant differences between expected heterozygosity (ranging from 0.14 and 0.94) and observed heterozygosity (ranging from 0.10 and 0.75) were found at five loci (Table 1, calculated and tested using Arlequin ver. 3.1.1, Excoffier *et al*. 2005). The heterozygote deficit at these loci can be explained by the presence of null alleles (Table 1; inferred by the software MicroChecker ver. 2.2.3, van Oosterhout *et al*. 2004). A significant heterozygote excess was found at one locus in one population (inferred by Genepop on the web ver. 4.0: http://genepop.curtin.edu.au/, Raymond & Rousset 1995, Rousset 2008; Table 1). The definition of populations (i.e. pooling of specimens from different localities) might also be the reason for Hardy-Weinberg disequilibrium in some cases. Furthermore, these problems could result from the peculiar nature of *H. euphorbiae* for which the hybrid influx from other species of the genus has been hypothesized (Harbich 1976a). Linkage disequilibrium was

**Table 1.** Characteristics of eight microsatellite loci studied in *Hyles euphorbiae* from Europe (Bulgaria & Spain) and *H. tithymali* from the Canary Islands (Gran Canaria & La Palma). Abbreviations: Pop = population, BUL = population from Southeast Bulgaria, ESP = population from Northeast Spain, GC = population from Gran Canaria, LP = population from La Palma, *n* number of specimens analyzed, $H_E$ expected heterozygosity, $H_O$ observed heterozygosity, $p_{HWE}$ probability that population is in Hardy Weinberg equilibrium.

| Primer name and sequence | | Repeat sequence[a] | GenBank acc. no. | Species | Pop | *n* | No. of alleles | Allele size[b] | $H_E$ | $H_O$ | $p_{HWE}$ | Null allele bias |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hti62F | 5´-GAGAACGTCGGCAAATAATCGT-3´ | $(AC)_{10}$ | JF412019 | *H. euphorbiae* [X] | BUL | 20 | 8 | 369–427 | 0.86 | 0.10 | < 0.001 | Yes |
| Hti62R | 5´-GTGCAGATGGCAGTCTTATCG-3´ | | | *H. euphorbiae* [X] | ESP | 18 | 18 | 359–432 | 0.93 | 0.50 | < 0.001 | Yes |
| | | | | *H. tithymali* | GC | 20 | 5 | 359–378 | 0.58 | 0.40 | 0.224 | No |
| | | | | *H. tithymali* | LP | 18 | 4 | 363–378 | 0.64 | 0.72 | 0.035 | No |
| Hti63F | 5´-TATATAGAATGGAGGGTGACATTG-3´ | $(TC)_5$ | JF412020 | *H. euphorbiae* [X] | BUL | 20 | 1 | 269 | – | – | – | – |
| Hti63R | 5´-AAGCAACCTAATATTCAACGCCA-3´ | | | *H. euphorbiae* [X] | ESP | 20 | 3 | 268–283 | 0.14 | 0.10 | 0.071 | No |
| | | | | *H. tithymali* | GC | 20 | 2 | 269–283 | 0.47 | 0.70* | 0.041 | No |
| | | | | *H. tithymali* | LP | 19 | 3 | 269–283 | 0.53 | 0.42 | 0.349 | No |
| Hti65F | 5´-CTGGCTAAAGTGTTCATGAGCT-3´ | $(AC)_5(CA)_2$ | JF412021 | *H. euphorbiae* [X] | BUL | 20 | 5 | 313–322 | 0.64 | 0.75 | 0.214 | No |
| Hti65R | 5´-CTCAATATCTGTTCGTACTCGAG-3´ | | | *H. euphorbiae* [X] | ESP | 20 | 8 | 282–324 | 0.63 | 0.45 | 0.136 | No |
| | | | | *H. tithymali* | GC | 20 | 4 | 239–322 | 0.64 | 0.60 | 0.055 | No |
| | | | | *H. tithymali* | LP | 20 | 3 | 239–322 | 0.66 | 0.65 | 0.378 | No |
| Hti66F | 5´-AAGATCTTGCCCTGGTTGAAC-3´ | $(CA)_8$ | JF412022 | *H. euphorbiae* [X] | BUL | 20 | 2 | 250–260 | 0.45 | 0.15 | 0.005 | Yes |
| Hti66R | 5´-ACCTCTACAATCGTGGAGCC-3´ | | | *H. euphorbiae* [X] | ESP | 20 | 3 | 250–261 | 0.44 | 0.50 | 1.000 | No |
| | | | | *H. tithymali* | GC | 20 | 2 | 250–260 | 0.51 | 0.40 | 0.391 | No |
| | | | | *H. tithymali* | LP | 19 | 2 | 250–260 | 0.46 | 0.37 | 0.612 | No |
| Hti67F | 5´-TTAGGGTGGTGCAGAGACCT-3´ | $(GT)_7(GA)_8$ | JF412023 | *H. euphorbiae*[X] | BUL | 20 | 10 | 360–403 | 0.77 | 0.25 | < 0.001 | Yes |
| Hti67R | 5´-CATCACGTTCGAGGAGTTGCT-3´ | | | *H. euphorbiae*[X] | ESP | 20 | 15 | 360–403 | 0.94 | 0.30 | < 0.001 | Yes |
| | | | | *H. tithymali* | GC | 20 | 6 | 360–382 | 0.78 | 0.30 | < 0.005 | Yes |
| | | | | *H. tithymali* | LP | 18 | 5 | 358–382 | 0.65 | 0.47 | 0.083 | No |
| Heu68F | 5´-ACAACTTCAACGTGCCGC-3´ | $(CA)_{15}(CG)_4 + (CA)_9 + (TC)_6$ | JF412024 | *H. euphorbiae* | BUL | 20 | 9 | 344–441 | 0.84 | 0.25 | < 0.001 | Yes |
| Heu68R | 5´-TAGCCTTAAATTTCCATCACC-3´ | | | *H. euphorbiae* | ESP | 20 | 16 | 347–431 | 0.94 | 0.25 | < 0.001 | Yes |
| | | | | *H. tithymali* [X] | GC | 20 | 8 | 352–386 | 0.82 | 0.25 | < 0.001 | Yes |
| | | | | *H. tithymali* [X] | LP | 20 | 5 | 354–382 | 0.76 | 0.30 | < 0.001 | Yes |
| Heu72F | 5´-GAAGGCTACAAACAGCACG-3´ | $(TA)_3 + (TA)_4 + (TA)_6$ | JF412025 | *H. euphorbiae* | BUL | 17 | 4 | 451–508 | 0.61 | 0.65 | 0.272 | No |
| Heu72R | 5´-ACTTTCTAGCTATCACGGC-3´ | | | *H. euphorbiae* | ESP | 20 | 9 | 426–512 | 0.67 | 0.40 | < 0.001 | Yes |
| | | | | *H. tithymali* [X] | GC | 20 | 4 | 450–508 | 0.35 | 0.35 | 0.253 | No |
| | | | | *H. tithymali* [X] | LP | 19 | 2 | 451–508 | 0.15 | 0.16 | 1.000 | No |
| Heu76F | 5´-TGTGACGCAAGTCTGTCG-3´ | $(GT)_{11}$ | JF412026 | *H. euphorbiae* | BUL | 19 | 5 | 238–244 | 0.48 | 0.47 | 0.506 | No |
| Heu76R | 5´-CAGAGCAGACACTTGGAG-3´ | | | *H. euphorbiae* | ESP | 19 | 7 | 236–248 | 0.69 | 0.42 | < 0.001 | Yes |
| | | | | *H. tithymali* [X] | GC | – | – | – | – | – | – | – |
| | | | | *H. tithymali* [X] | LP | – | – | – | – | – | – | – |

[a] Repeat motif of the original clone, [b] Alleles not following repeats of the motif are allowed due to indels (1–73 bp) in the flanking region (verified by sequencing homozygotes), [X] Cross amplification (species from/for which the locus was not designed originally), * significant heterozygote excess ($F_{IS}$ = –0.526, $p$ = 0.0277; inferred by Genepop on the web ver. 4.0).

solely found for the Bulgarian population of *H. euphorbiae* among three pairs of loci: Hti62/Heu68, Hti65/Heu68, Hti62/Heu76 (Genepop on the web ver. 4.0). Divergence of the two populations of each species was inferred by a locus by locus AMOVA (*H. euphorbiae*: $F_{ST}$ = 0.036, $p$ = 0.010, *H. tithymali*: $F_{ST}$ = 0.044, $p$ = 0.005; Arlequin ver. 3.1.1). In case of three loci (Hti62, Hti67, Heu68) allele size ranges deviated relatively wide between the two species (Table 1).

The low amplification success, the presence of null alleles, and the high frequency of alleles which cannot be explained by the modulus of the microsatellite motif is indicative for peculiar genomic features of microsatellite flanking regions in Lepidoptera. Local BLAST searches based on the 192 clones provided by GENterprise and 146 clones from the study of Hundsdoerfer *et al*. (2009a) revealed the presence of microsatellite families (high sequence similarity of the flanking region) as already found by Hundsdoerfer *et al*. (2009a). Nevertheless, the local BLAST search indicated that the primer pairs presented in Table 1 are specific to a single locus. The existence of microsatellite alleles of a length inconsistent with the repeat modulus was verified by sequencing 109 homozygote specimens using standard reaction conditions (25 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C). This analysis shows a high incidence of single nucleotide polymorphisms and indels (1–73 bp) within the flanking regions suggesting that mutations might also affect primer binding sites preventing amplification (null alleles).

By describing eight new simple sequence repeat regions for the *H. euphorbiae* complex, this study corroborates earlier reports about difficult genomic features of microsatellite loci in lepidopterans. Nevertheless, these microsatellites can be used to analyze population structure in the species complex if particular attention is paid to verify allele length (due to instability of flanking regions) to detect homoplasies and if the presence of null alleles is considered. Together with 11 loci developed for *H. tithymali* (Hundsdoerfer *et al*. 2009a), which have to be tested for cross amplification in *H. euphorbiae* (but are promising to perform well inferred from the results of this study), these loci provide a solid base for population studies of the whole species complex.

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