Gaël Aleix-Mata [1,2,*], Míriam Expósito [1], Francisco J. Ruiz-Ruano [3,4], Ana María López-Beceiro [5], Luis E. Fidalgo [5], Carlos Martínez-Carrasco [6], María Rocío Ruiz de Ybáñez [6], Mathieu Boos [7,8], Jesús M. Pérez [2,9], Antonio Sánchez [1,*]

Development and characterization of 15 novel polymorphic microsatellite loci for two important bot flies (Diptera, Oestridae) by next-generation sequencing

[1] Department of Experimental Biology, Jaén University, E-23071, Jaén, Spain.

[2] Department of Animal and Plant Biology, and Ecology, Jaén University, E-23071, Jaén, Spain.

[3] Department of Genetics, Faculty of Sciences, Granada University, E-18071, Granada, Spain.

[4] Department of Ecology and Genetics, Evolutionary Biology Centre, Uppsala University, SE-752 36, Uppsala, Sweden.

[5] Department of Anatomy, Animal Production and Veterinary Clinical Sciences, University of Santiago de Compostela, E-27002, Lugo, Spain.

[6] Department of Animal Health, Murcia University, E-30100, Murcia, Spain.

[7] Research Agency in Applied Ecology, Naturaconst@, 67270 Wilshausen, France.

[8] Pôle Scientifique, Fédération Nationale des Chasseurs, 13 rue du Général Leclerc 92136 Issy-Les-Moulineaux, Cedex, France.

[9] Wildlife Ecology & Health group (WE&H)

* Corresponding author: abaca@ujaen.es or galeix@ujaen.es

Gaël Aleix-Mata: galeix@ujaen.es; ORCID: 0000-0002-7429-4051

Míriam Expósito: mem00010@red.ujaen.es

Francisco J. Ruiz-Ruano: fjruizruano@ugr.es; ORCID: 0000-0002-5391-301X

Ana María López Beceiro: Anam.lopez.beceiro@usc.es

Luis E. Fidalgo: luis.fidalgo@usc.es

Carlos Martínez-Carrasco: cmcpleit@um.es; ORCID: 0000-0002-8742-0109

María Rocío Ruiz de Ybáñez: rocio@um.es

Mathieu Boos: mboos.naturaconst@free.fr

Jesús M. Pérez: jperez@ujaen.es; ORCID: 0000-0001-9159-0365

Antonio Sánchez: abaca@ujaen.es; ORCID: 0000-0002-6715-8158

Abstract

Cephenemyia stimulator and *Oestrus ovis,* are two important parasitic bot flies (Oestridae) species causing myiasis, with a potential negative impact on the welfare of the host. Using next-generation sequencing approach and bioinformatics tools a large panel of possible microsatellites loci was obtained in both species. Primer pairs were designed for 15 selected microsatellite loci in *C. stimulator* and other 15 loci in *O. ovis* for PCR amplification. Loci amplification and analysis were performed in four populations of each species. The results demonstrated that all selected loci were polymorphic, with the number of alleles ranging from 2 to 6 per locus in *C. stimulator* and 3 to 13 per locus in *O. ovis*. This is the first time to describe these microsatellite loci for *C. stimulator* and *O. ovis*. These two sets of microsatellite markers could be further used for biogeographic and population genetics studies.

Keywords: *Oestrus ovis*, *Cephenemyia stimulator*, microsatellites, next-generation sequencing

Introduction

The family Oestridae embraces about 170 dipteran species belonging to 29 genera grouped into four subfamilies: Cuterebrinae, Gasterophilinae, Hypodermatinae and Oestrinae (Scholl et al. 2019). In their larval phases, oestrids are obligate parasites of a wide variety of mammals (including man), developing in the nostrils, throat, trachea, bronchioles, lungs, oesophagus, stomach, subcutaneous tissues, genitalia and central nervous system of their hosts (Zumpt 1965; Hall and Wall 1995; Colwell 2001; Colwell et al. 2006). These types of myiasis have a negative impact on the welfare of parasitized animals and can cause restlessness, sinusitis, sneezing, coughing, nasal discharge, mucopurulent exudates, dyspnea and difficulty swallowing, among other symptoms (Shcherban 1973; Ilchmann et al. 1976; Dorchies et al. 1998, 1993; Scholl et al. 2019). Larviposition by gravid females may lead to stress and a drop in food intake by hosts (Colwell 2001). Moreover, oestrid infestations can increase the likelihood of occurrence of opportunistic infections and in severe cases can provoke cranial lesions and even the death of the host (Allen and Bunch 1982; Mozaffari et al. 2013). Parasitization by oestrid flies is usually characterized by complex ecological interactions between these dipterans and their hosts, further complicated by intrinsic and extrinsic factors (e.g., environmental conditions and host behaviour and density) that influence the distribution and prevalence of these parasites (Papavero 1977; Colwell 2001; Colwell et al. 2006).

The sheep bot fly, *Oestrus ovis,* is a cosmopolitan parasite that affects sheep and goats (Scala et al. 2001), as well as a variety of wild hosts including European mouflon (*Ovis aries musimom*), bighorn sheep (*Ovis canadensis*), aoudad (*Ammotragus lervia*), Alpine ibex (*Capra ibex*), Asiatic ibex (*Capra sibirica*), Iberian ibex (*Capra pyrenaica*), white-tailed deer (*Odocoileus virginianus*) and llama (*Lama glama*) (Zumpt 1965; Allen and Bunch 1982; Moreno et al. 1999; Colwell et al. 2006; Gómez-Puerta et al. 2013;

74 Barroso et al. 2017; Sánchez et al. 2017; our unpublished data for the Iberian Ibex).
75 Ophthalmomyiasis and rhinomyiasis in humans due to *O. ovis* infestation are both
76 relatively frequent (Fries et al. 2018; Brini et al. 2019). By contrast, *Cephenemyia*
77 *stimulator* is more host-specific and in the Palaearctic usually only parasitizes roe deer
78 *Capreolus capreolus* (Colwell et al. 2006; Király and Egri 2007; Calero-Bernal and
79 Habela 2013).

80 In some cases, taxonomical doubts can arise when attempting to identify third-
81 instar oestrid larvae using only morphobiometric criteria (Otranto et al. 2005). These
82 limitations may become even more patent with first- and second-instar larvae (Wetzel and
83 Bauristhene 1970). The description and use of new molecular markers (e.g., COI, 28S,
84 ITSs and mitogenomes) has improved the accuracy of the characterizations and
85 identifications of oestrid species, and of phylogenetic reconstructions (Otranto and
86 Stevens 2002; Weigl et al. 2010; Marinho et al. 2012; Moreno et al. 2015; Zhang et al.
87 2016; Yan et al. 2019).

88 Within members of the order Diptera, microsatellites have been described and
89 used in ecological, demographic, biogeographic and genetic studies in a number of
90 Calliphoridae taxa (Florin and Gyllenstrand 2002; Torres et al. 2004; Torres and Azeredo-
91 Espin 2005, 2008; Diakova et al. 2018; Rodrigues et al. 2009). Microsatellites have
92 proven to be useful in the family Oestridae for addressing relationships between
93 specimens from different host populations and for assessing genetic diversity and
94 structure at population level (Milton et al. 2011; Bitarello et al. 2009; Cheng et al. 2014;
95 Liu et al. 2018).

96 In this study, we used next-generation sequencing and bioinformatics to select
97 novel polymorphic microsatellite loci, 15 for *Cephenemyia stimulator* and 15 for *Oestrus*
98 *ovis*, which we tested with PCR amplification for specimens from different populations

of both species. In addition, to test cross-amplification in related dipteran species we analysed the amplification of the 15 microsatellite loci of *C. stimulator* in a specimen of *C. auribarbis*. The main objective of this work is to describe and analyse for the first time microsatellite loci in *C. stimulator* and *O. ovis*, that in the future could be useful for biogeographic and populations genetics studies.

Material and methods

Genome sequencing and microsatellite primers selection

For library construction and genome sequencing DNA was extracted from one larva of *C. stimulator* from roe deer (*Caprelous capreolus*) (Lugo, Spain: 43°00'N, 7°33'W) and from one larva of *O. ovis* from domestic goat (*Capra aegagrus*) (Almería, Spain: 37º03'N, 2º34'W) using the Quick-DNA Tissue/Insect kit (Zymo Research). Genomic DNAs (3 µg) was used to construct two libraries, one for *C. stimulator* and other for *O. ovis*, with 350 bp fragments. About 20 Gbp of sequences were obtained from each species (coverage about ~20x) using Illumina® Hiseq™ 2000 platform in paired-end reads with length 2x150 nt.

To identify the microsatellite primers we used bioinformatics protocol and the Illumina libraries for each species separately. Firstly, we joined read pairs with ends overlapping by at least six nucleotides using the fastq-join program of the FASTX Toolkit (Gordon and Hannon 2010). Then, we searched for dinucleotide microsatellites and designed primers to amplify them whenever possible with Msatcommander (Faircloth 2008) using the default options. Next, we used the script written by Schoebel et al. (2013) to select unique primer pairs to avoid microsatellites in repetitive elements, and obtained 1,932 and 4,367 possible microsatellite primer pairs for *C. stimulator* and *O. ovis*,

123 respectively. Then, we mapped the Illumina paired-end reads for each library against the
124 joined reads containing the selected microsatellites using SSAHA2 (Ning et al. 2001).
125 We visualized the mappings with IGV (Thorvaldsdóttir et al. 2013) to perform a manual
126 selection of microsatellites that present two alleles in the sequenced specimen of each
127 species. Finally, we selected 30 putative microsatellites primers, 15 for each species
128 (Tables 1 and 2).

129 Analysis of microsatellite loci

130 For microsatellite amplification, we used the DNA of the larvae extracted with the
131 Quick-DNA Tissue/Insect kit (Zymo Research) eluting in 80 µl of H_2O. We performed
132 one 'control PCR' to evaluate the DNA yield by direct amplification of a ~250 bp
133 fragment of the microsatellite locus C.sti-12 and O.ovis-12 for *C. stimulator* and *O. ovis*,
134 respectively. The control PCR (13 µl of reaction) contained 20-60 ng of template DNA,
135 6.5 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific) (final
136 concentration: 0.05 U/µl DreamTaq DNA polymerase, dATP, dCTP, dGTP and dTTP,
137 0.2 mM each, and 2 mM $MgCl_2$), and 0.2 µM of each primer. The cycling conditions were
138 conducted as indicated by the supplier's instructions (see below) (40 amplification
139 cycles); PCR amplicons were resolved in 2% agarose gels. We tested 37 larval DNAs
140 from each species. In all, 32 *C. stimulator* samples (from four populations) and 24 *O. ovis*
141 samples (also from four populations) yielded amplifications on control PCRs and were
142 then used for the 15 microsatellite loci amplifications (Table 3).

143 For microsatellite analysis, we used the 15 selected primer pairs. All forward
144 primers were modified on 5' by the incorporation of the universal M13 primer sequence;
145 PCR amplifications were performed as described by Schuelke (2000). The forward M13
146 primer was labelled with four fluorescents dyes (FAM, HEX, ATTO550, ATTO565;
147 Isogen Life Science). The PCRs were performed on 13 µl of reaction mix containing 1.53-

4.61 ng/µl of template DNA, 6.5 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific), 0.2 µM of the unlabelled M13-tailed forward primer, 0.2 µM of the fluorescently labelled M13 primer, and 0.5 µM of the corresponding reverse primer. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min; 30 cycles for 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C; and a final extension of 15 min at 72 °C.

To read the results, microsatellite PCR amplifications for each larva in both species were combined in four mixes (three mixes with four loci and one with three loci) to avoid fluorescent dye overlapping (Tables 1 and 2). Mixes contained 3 µl of the different loci PCRs and 3 µl of H_2O to give a final dilution of 1:5 and 1:4 in the mixtures of the four and three loci, respectively. The mixes were run on a Genetic Analyzer 3500 (Applied Biosystem; available in Jaén University). Alleles were sized using the GeneMapper Software version 4.1 (Applied Biosystems). We used GIMLET software (Valiére 2002) to calculate the values of expected heterozygosity (H_E), observed heterozygosity (H_O) and allelic frequency. The frequency of null alleles, and the probabilities of identity (PID) (unbiased and between siblings; Waits et al., 2001) was also calculated using CERVUS v 3.0.7 software (Marshall et al. 1998). In addition, the Hardy-Weinberg exact probability tests were performed with Genepop (Raymond and Rousset 1995).

Results and discussion

With the approach of next-generation sequencing and bioinformatics tools, 1932 and 4367 putative dinucleotide microsatellite loci were found from the library of *C. stimulator* and *O. ovis,* respectively. Selected fifteen loci from each library were tested with each species. All of those microsatellite loci were successfully amplified and were polymorphic (Tables 1 and 2).

In *C. stimulator* we amplified and analyzed the 15 microsatellites loci for each larva (N=32) and calculated the allele size range, the allelic frequency, H_E, H_O and the percentage of amplification (Table 1). The percentage of amplification for each locus varied between 84.38% (for C.sti-14 locus) to 100% for (C.sti-8 locus). We detected 54 alleles, and the number of alleles per locus varied from 2 to 6. For each locus, we also calculated H_E, which varied between 0.17 and 0.64; the H_O values varied between 0.19 and 0.63 for C.sti-8 and C.sti-13, respectively (Table 1). Mean values of H_E and H_O were 0.48 and 0.44, respectively, and the statistical analysis of the data indicated that the values of H_E and H_O fitted the Hardy-Weinberg (HW) equilibrium.

In addition, the specimen of *C. auribabis* was tested for the amplification with *C. stimulator* microsatellite primer set. Ten of 15 loci were amplified; six loci are homozygous and other four loci are heterozygous, with alleles of the same size range as presented in *C. stimulator*. However, we found new nine alleles in this sample (Table 1).

We amplified 15 microsatellites loci for each *O. ovis* specimen analysed (N=24) and performed the same calculations as for *C. stimulator* (Table 2). The percentage of amplification varied between 50% (loci O.ovis-8 and O.ovis-14) and 100% (locus O.ovis-10). In *O. ovis*, 114 alleles were detected and the number of allele for locus varied from 3 to 13. For each locus, the frequency of each allele in each population was very low, due to the high variability in the number of alleles. Total of the 114 identified alleles, only 14 alleles presented in all populations, while the rest (100 alleles) did not. We also calculated H_E, which varied between 0.16 and 0.88, and H_O, which varied between 0.17 and 0.71 from microsatellites O.ovis-13 and O.ovis -5, respectively (Table 2). H_E and H_O were more varied than in *C. stimulator*, although the statistical analysis of all data indicated that the values of H_E and H_O generally fitted the Hardy-Weinberg (HW) equilibrium, except in the loci O.ovis-3 and O.ovis-4, which deviated significantly ($p < 0.05$).

197 Loci C.sti-6, O.ovis-2, O.ovis-6, and O.ovis-7 had two alleles with a large
198 difference in size (more than 50 nt) (Tables 1 and 2). The smaller alleles could be
199 amplified and showed the signal more efficiently than the larger ones, leading to allelic
200 dropout. Therefore, use of these four loci with precaution may be required, for example,
201 using a multi-tube approach (Taberlet et al., 1996).

202 Finally, in *C. stimulator* with this panel of 15 loci, we achieved a probability of
203 identity, unbiased, $P_{(ID)}$ of $6x10^{-8}$ and a probability of identity for sibling $P_{(ID)\ sib}$ of
204 $3.61x10^{-4}$. In *O. ovis* with this panel of 15 loci, we obtained a probability of identity $P_{(ID)}$
205 of $2.43x10^{-16}$ and a probability of identity for sibling $P_{(ID)\ sib}$ of $1.68x10^{-6}$. These
206 probabilities indicated that the described microsatellite loci in both species are suitable to
207 be used in biogeographic and population genetics studies, as the obtained values are
208 according to the previously estimated and recommended ($P_{(ID)} < 10^{-4}$)(Waits et al. 2001,
209 Latorre-Cardenas et al. 2020).

210 Microsatellite loci were originally developed by construction and screening of
211 repetitive sequences enriched genomic libraries, and the sequences of positive clones
212 were used to design PCR primers (Vieira et al. 2016). According to the results, the next-
213 generation sequencing and the bioinformatics analysis performed to select the
214 microsatellite loci have proven to be an efficient methodology (Light et al. 2018). This is
215 the first time to describe two sets of 15 microsatellite loci for *C. stimulator* and *O. ovis*,
216 respectively. These novel and polymorphic loci may be used for further biogeographical
217 and population genetics studies, for examples, defining management units (Bergamo et
218 al. 2018), in inbreeding (Abe and Pannebakker 2017), epidemiology, host-specificity,
219 gene flow and spatial genetic structure studies (Rasero et al. 2010; Harimalala et al. 2017;
220 Cao and Wu 2019), and monitoring abundance and effective population size (Marí-Mena
221 et al. 2019), in these two important bot flies.

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