NOTES AND NEWS

DEVELOPMENT OF MICROSATELLITE MARKERS IN THE COPEPOD, *CALIGUS ROGERCRESSEYI* BOXSHALL & BRAVO, 2000 (COPEPODA, CALIGIDAE)

BY

SANDRA FERRADA[1,3]), CRISTIAN B. CANALES-AGUIRRE[1]), RICARDO GALLEGUILLOS[1]), ANDREA BARRERA[1]) and JOSÉ A. GALLARDO[2])

[1]) Laboratorio de Genética y Acuicultura, Departamento de Oceanografía, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Casilla 160-C, Concepción, Chile

[2]) Laboratorio de Genética Aplicada. Escuela de Ciencias del Mar, Pontificia Universidad Católica de Valparaíso, Avenida Altamirano 1480, Valparaíso, Chile

Salmon farming has been correlated with sea lice outbreaks (Krkoek et al., 2005; Penston et al., 2008) and concurrent declines of wild salmonids (Krkoek et al., 2007). The Chilean sea louse, *Caligus rogercresseyi* Boxshall & Bravo, 2000, is a native ectoparasitic copepod that, in recent years, has intensively infested farmed salmon in Chile (Bravo et al., 2008). This parasite was transmitted to the farmed fish by the native rock cod, *Eleginops maclovinus* (Cuvier, 1830) and *Odontesthes regia* (Humboldt, 1821) (cf. Carvajal et al., 1998). Several aspects relevant to their biology and interactions with the environment have been described (Marin et al., 2002; González & Carvajal, 2003; Zagmutt-Vergara et al., 2005; Bravo et al., 2009). The life cycle of *C. rogercresseyi* lasts between 30 and 45 days (González & Carvajal, 2003), and their prevalence and abundance on farmed fish, in southern Chile, is strongly dependent on salinity and temperature (Zagmutt-Vergara et al., 2005; Bravo et al., 2009). In Chile, pesticides have been intensively used to control *C. rogercresseyi*; however, there now is strong evidence that a loss of sensitivity of the parasite to some of those treatments has occurred over recent years (Bravo et al., 2008).

To date, there are no studies of the population structure and genetic diversity of *C. royercresseyi*. Furthermore, no microsatellite DNA markers have been isolated in species of the genus *Caligus* Müller, 1785. In this study, we describe the isolation and characterization of eight polymorphic microsatellite loci for *C. rogercresseyi*. Microsatellites are suitable markers for genetic studies, because

[3]) Fax: +56.412225400; e-mail: sferrada@udec.cl

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of their high level of polymorphism and co-dominant inheritance (Zhan et al., 2008), and they have also been successfully used to characterize the population structure of other sea lice (Todd et al., 2004; Nolan & Powell, 2009).

To develop the genomic library, we collected adult individuals and lecithotrophic larvae (nauplius 1) selected from samples derived from fish cultivations of the Atlantic salmon (*Salmo salar* Linnaeus, 1758) on a farm located in Caicaén, Chile (41°46′S 73°07′W). Lecithotrophic larval tissue is considered optimal for the isolation of microsatellites, because it avoids contamination by host DNA that can arise from the epidermal mucus and fish blood present in the stomach contents of the parasite (Todd et al., 2004).

The genomic library was developed by Genetic Identification Services, Chatsworth, California, using DNA from the aforementioned samples that was extracted using the salting-out protocol (Miller et al., 1988). Genomic DNA was digested with the restriction enzymes MscI, FspI, HpaI, AluI, NaeI, and DraI. Fragments between 300 and 700 bp were chosen to enrich four genomic libraries. The genomic libraries were then prepared using biotin-CA_{15}, biotin-ATG_{12}, biotin-$CATC_8$, and biotin-$TAGA_8$ as capture molecules. Recombinant plasmids were generated by cloning the digested fragments of *C. rogercresseyi* in the HindIII site of the plasmid pUC19 and introduced into *Escherichia coli* (Migula, 1895) Castellani & Chalmers, 1919 strain DH5α™ by electroporation (ElectroMaxJ™; Invitrogen). A total of 71 recombinant clones for the four libraries contained microsatellite blocks. Specifically 28 of 30 from CA enriched library, 15 of 29 from ATG enriched library, 2 of 9 from CATC enriched library, and 26 of 27 from TAGA enriched library. Recombinant clones were chosen arbitrarily for sequencing on an ABI PRISM 377, using the ABI PRISM Taq dye terminator cycle sequencing methodology. From the sequences of the 71 clones, it was possible to design 26 primer pairs, using DesignerPCR v1.03 (Research Genetics, Inc.). The product of the 26 primer pairs on over 10 adult samples of *C. rogercresseyi* was evaluated for resolution and polymorphisms. Only eight loci were polymorphic, the others loci were monomorphics or failed PCR products. The results of the PCR from various primer pairs indicated which regions would be sequenced for further characterization.

For PCR, genotyping, and fragment analysis, the following conditions were used: in a 10-μL reaction volume, 20 ng template DNA was combined with 2 mM $MgCl_2$, 0.2 mM dNTP's, 0.4 μM of reverse and dye-labelled forward primer (6-FAM, VIC, NED, PET), and 0.025 U/μL Taq DNA polymerase (Invitrogen®). The amplifications were carried out in a PCT-200 thermocycler (MJ-Research®) with an initial denaturation at 94°C for 3 min. followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 55-57°C for 40 s (see table I), extension at 72°C for 30 s, and a final extension at 72°C for 4 min. The fragments were visualized on an

TABLE I

Details of eight microsatellite loci in *Caligus royercresseyi* Boxshall & Bravo, 2000, including primer sequence, locus name, repeat motif, size range (bp), annealing temperature (T_a), number of alleles (N_a), expected (He) and observed (Ho) heterozygosities, significance of deviation from probability of Hardy-Weinberg equilibrium (P_{HW}), null alleles, and GenBank accession number

Locus	Repeat motif	Primer sequences (5′-3′)	Size range	T_a (°C)	N_a	Ho	He	P_{HW}	Null alleles	Accession no.
CalA121	$GT_{(23)}$	F: PET-TGAGAGTGAGTGTCTGAGATGT R: GCAAACACAGTGAAACAGTG	278-314	56	12	0.441	0.882	0.000	0.141	GQ379196
CalA127	$GT_{(66)}$	F: VIC-TTCCTCCTTTCACAATAACAG R: GCGATCAAAACTGTATGAATC	233-299	56	16	0.265	0.786	0.000	0.219	GQ379197
CalA115	$CA_{(16)}$	F: NED-GCGATAGGGCTACTGTTAATG R: AAGAACACAGGGAACGTACAC	196-288	56	21	0.937	0.946	0.999	0.000	GQ379194
CalB107	$ATG_{(5)}$	F: FAM-TATTCATCGTGTTGGGAGTGT R: GCAGCCTCTTGTATCTTTTAGG	112-130	56	5	0.824	0.622	0.000	0.163	GQ379198
CalB116	$CCT_{(5)}$	F: PET-TCAGCCGAATACTTCAAACG R: CGAGAACCTCAGCGATACTG	179-185	56	3	0.382	0.699	0.000	0.020	GQ379199
CalC1	$GGAT_{(5)}$	F: NED-ACGAACCTATTCCCATGAAC R: CCAAATGAGGGGTTGTCTAC	123-158	56	8	0.765	0.803	0.000	0.155	GQ379200
CalA101	$TA_{(7)}TG_{(11)}TA_{(5)}$	F: FAM-CGACCTCGTAATGTTGACC R: TAGGCAAATCGTAGATGTGATG	108-122	57	8	0.862	0.872	0.409	0.052	GQ379193
CalA119	$CA_{(9)}$	F: VIC-CCTGGCTTAGAGATACAATG R: CATGGCACTTACATAGTCATT	123-143	55	7	0.912	0.835	0.000	0.115	GQ379195

ABI PRISM 3730xl capillary sequencer using LIZ 500 as internal size standard. Assignment of allele size was carried out using Peak Scanner v1.0 (Applied Biosystems).

The polymorphisms of eight loci were evaluated using 34 individual samples from farmed *S. salar*, from Caicaén (41°46′S 73°07′W) in April 2008, and Lin Lin (42°23′S 73°27′W) in August 2008, Chile. The individual samples were collected from different fishes in different cages and at different locations, in order to minimize the possibility of capturing consanguineous individuals. The number of alleles was estimated using Genalex v6 (Peakall & Smouse, 2006), the observed and expected heterozygosity were estimated in Arlequin v3.01 (Excoffier et al., 2005). To detect significant differences from the Hardy-Weinberg equilibrium, we followed the procedure of Guo & Thompson (1992) implemented in Arlequin v3.01 (Excoffier et al., 2005). Tests for linkage disequilibrium for all pairs of loci in the Hardy-Weinberg equilibrium were performed using Genepop v3.4 (Raymond & Rousset, 1995). Finally, the presence of null alleles was estimated using the Brookfield algorithm (Brookfield, 1996) and large allele drop out and scoring errors due to stutter peaks were implemented in Micro-Checker v2.2.3 (Van Oosterhout et al., 2004).

The number of alleles per polymorphic locus ranged from 3 to 21 and the values of observed heterozygosity and expected heterozygosity ranged from 0.265 to 0.937 and from 0.622 to 0.946, respectively (table I).

There was no evidence of linkage disequilibrium between any pair of loci in the sample ($p < 0.05$). Significant deviations from the Hardy-Weinberg equilibrium were found in the following loci: CalA121, CalA127, CalB107, CalB116, CalA119, and CalC1.

There are several possible explanations for the deficits of heterozygosity in most of these developed loci. Rico et al. (1997) reviewed the possible causes of excess homozygosity, evaluating various hypotheses concerning genotyping errors, the presence of null alleles, the Wahlund effect (Wahlund, 1928), inbreeding, assortative mating, and/or selection. We assessed some of these hypotheses in an attempt to explain the heterozygote deficits observed in the loci CalA121, CalA127, CalB116 and CalC1.

In assessing for the hypotheses for genotyping errors and null alleles, none of the loci showed large allele drop out and/or slip-strand mispairing during the polymerase chain reaction that can cause stutter bands (cf. Van Oosterhout et al., 2004). However, of the four loci with heterozygote deficits, three had a high frequency of null alleles (cf. Pemberton et al., 1995), ranging from 11% to 21% (table I), suggesting that null alleles could be a cause contributing to this outcome. Null alleles are found in most taxa (Dakin & Avise, 2004) but seem to be particularly common in populations with high effective populations sizes (Chapuis

& Estoup, 2007). As null alleles create false homozygotes, they are problematic for measures of genetic differentiation and parentage analysis. However, if the allele frequencies are corrected for this situation, they can then be used to assess levels of population differentiation (cf. Van Oosterhout et al., 2004). Carlsson (2008) using an analysis based on simulations, demonstrated that microsatellite loci affected by null alleles would probably not alter the overall outcome of assignment testing, and therefore could be included in these types of studies. However, null alleles may not be the only factor causing disequilibrium.

Alternatively, the heterozygosity deficiency may be due to the Wahlund effect, either in time (overlapping of genetically differentiated generations), or in space (an admixture of individuals sampled over a large geographical area that may include more than one panmictic unit), that might occur with individuals of *C. rogercresseyi* collected in two seasons and locations separated by more than 100 kilometers.

On the other hand, a high rate of inbreeding in ectoparasites confined to the culture system, the assortative mating or selection against heterozygotes, could cause a part of these deficits. In fact, a strong selection pressure due to the use of chemotherapeutic drugs has been suggested for this species (Bravo et al., 2008). We therefore cannot reject a priori the possibility that inbreeding, assortative mating, and/or differential survival of genotypes plays a part in these heterozygote deficiencies, although we have no direct evidence for this hypothesis.

Because we found two loci in disequilibrium with an excess of heterozygotes, CalB107 and CalA119, the assumptions previously given cannot explain all the disequilibria. In this context, heterozygote excess in populations is not as common as heterozygote deficiency, and therefore has not been fully explored theoretically. Overdominant selection favouring heterozygotes, associative overdominance, and negative assortative mating are generally used to explain heterozygote excess in natural populations (cf. Stevens et al., 2007).

Therefore, we cannot conclude which of the possible causes explains the deviations from the Hardy-Weinberg expectations observed, and no single factor alone provides a satisfactory explanation.

This is the first description of the polymorphic microsatellite loci for the Chilean sea louse *Caligus rogercresseyi*. This work provides a significant contribution to studies of the population genetics of the parasite on farmed fish. Furthermore, it will assist in studies of the ecology and conservation of the natural hosts *Eleginops maclovinus* and *Odontesthes regia* (cf. Frost & Nilsen, 2003; Todd et al., 2004; Tribble et al., 2007). Adequate knowledge of the genetic structure of the population of this parasite in southern Chile may permit an improvement of ectoparasite control.

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