J Appl Genetics
DOI 10.1007/s13353-015-0272-y

ANIMAL GENETICS • SHORT COMMUNICATION

# First results on the genetic diversity of the invasive signal crayfish *Pacifastacus leniusculus* (Dana, 1852) in Europe using novel microsatellite loci

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Received: 19 August 2014 / Revised: 13 January 2015 / Accepted: 14 January 2015


**Abstract** The introduction of non-native crayfish in aquatic ecosystems is very common due to human activities (e.g. aquaculture, recreational and commercial fisheries). The signal crayfish, *Pacifastacus leniusculus* (Dana, 1852), is one of the most widespread invasive species in Europe. Although several important ecological and economic impacts of this species have been reported, its European population genetic characterisation has never been undertaken using nuclear markers. Thus, the aim of this study was to develop and characterise new microsatellite markers for signal crayfish that can be useful in future studies in its invaded range, since only five are available so far. In total, 93 individuals from four geographically distinct European populations (Portugal, Great Britain, Finland and Sweden) were scored for the new markers and for those previously described, with the Bayesian analysis revealing a clear distinction among populations. These markers are suitable for future studies of the population genetic structure of this important invasive species, by increasing information about the possible pathways of introduction and dispersal, and by giving insights about the most important vectors of introduction.

**Keywords** Invasion genetics · Microsatellites · Alien crayfish · Introduction pathways · Population differentiation

Communicated by: Maciej Szydlowski

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## Introduction

The signal crayfish, *Pacifastacus leniusculus* (Dana, 1852), is native to North America and is one of the most widespread non-native crayfish in European aquatic ecosystems (Kouba et al. 2014). Starting in 1960, the species was introduced to open waters in Sweden to replace lost populations of the native noble crayfish, *Astacus astacus* (Linnaeus, 1758), which had been severely depleted by the crayfish plague (Alderman 1996). In subsequent decades, it has invaded 29 territories, mostly due to intentional introductions (Kouba et al. 2014).

*P. leniusculus* can be responsible for serious ecological impacts across several levels of organisation, from individuals to ecosystems (Nyström et al. 1996). Indeed, while this

Published online: 01 February 2015

species is widely recognised as an important consumer on eggs and juveniles of fish and amphibians, benthic invertebrates, detritus, submerged vegetation and algae, it may also represent an important prey for several mammal, fish and bird species. Furthermore, *P. leniusculus* is an important vector of the crayfish plague *Aphanomyces astaci* (Schikora, 1906) that seriously threatens native European crayfish species (Bohman et al. 2006; Filipová et al. 2013). Signal crayfish can also function as an ecosystem engineer, being responsible for important physical changes in the invaded habitat, mainly as a result of bioturbation activities (Harvey et al. 2014).

Given its important ecological and economic impacts, genetic characterisation of this invasive species is crucial in the design of future management measures, as the genetic data can increase information on the possible geographical pathways of introduction and dispersal, provide insights about the most important vectors of introduction, and about propagule pressure. However, to date, no European study has been undertaken using microsatellites, high-resolution markers, ideal for the evaluation of genetic diversity patterns both within and among populations. In fact, only five microsatellites are available for this species, and they have only been used to screen Japanese populations (Azuma et al. 2011). As for the European populations, there is only one genetic study published so far, using a barcoding approach. It revealed a substantial genetic variation of studied individuals, but without a clear geographic pattern (Filipová et al. 2013). All studied European individuals belonged to *P. leniusculus leniusculus*, whereas three subspecies are traditionally recognised in its native range (Miller 1960), and additional genetically distinct lineages have recently been revealed in populations assigned to signal crayfish (Larson et al. 2012).

Thus, the aim of this study was to develop and characterise new microsatellite markers in order to: (1) increase the number of available markers and (2) characterise in a pilot study selected European populations, since only Japanese populations have been studied so far (Azuma et al. 2011). Additionally, in order to improve genotyping throughput as well as cost-effectiveness, multiplex polymerase chain reaction (PCR) assays were designed to amplify all loci.

## Methods

A small piece of muscle tissue was isolated from a pereiopod of each crayfish and immediately preserved in 95 % ethanol. Four distinct populations were sampled, originating from Portugal ($n$=31; 41°47′54″N, 6°45′54″W, Fervença River), Great Britain ($n$=15; 54°01′42″N, 2°14′44″W, Bookill Gill Beck), Finland ($n$=22; 61°15′48″N, 28°03′33.07″E, Lake Saimaa)

**Table 1** Characteristics of ten polymorphic microsatellite loci in *Pacifastacus leniusculus*, including three markers previously described by Azuma et al. (2011)

| Locus | Primer sequences (5′–3′) | Fluorescent dye | Primer concentration | GenBank accession no. | Repeat | Na | Allele size range (bp) | HWE |
|---|---|---|---|---|---|---|---|---|
| MixA | | | | | | | | |
| LPL6 | F: TGTCGGATCATAGTCGTCGT<br>R: GGCTCTCAGTAACCTACCAGG | VIC | 0.45 | KP161863 | $(ttg)_{12}$ | 14 | 121–172 | 0.567 |
| LPL22 | F: CTTCTGTCTTAATTACTGAGGATGATT<br>R: TGTATGCGATGTATGTGATGTATGT | NED | 3 | KP161860 | $(cata)_{15}$ | 11 | 82–126 | 0 |
| LPL26 | F: AAATAAGACCCGACAAAGCG<br>R: ATGAGGAGCCGCAAGTGTAA | FAM | 0.5 | KP161861 | $(tg)_{11}$ | 5 | 323–337 | 0.510 |
| LPL40 | F: CAGTCGATATTTCTTTATATCCCTTCA<br>R: CTGGTTCCAGATAGCAGCGT | PET | 0.6 | KP161862 | $(caa)_9$ | 4 | 128–137 | 0.176 |
| MixB | | | | | | | | |
| LPL15 | F: TGTCGGATCATAGTCGTCGT<br>R: AGGCTCTCAGTAACCTACCAGG | PET | 0.7 | KP161864 | $(ttg)_{12}$ | 15 | 122–173 | 0.791 |
| LPL32 | F: AAAGCGGACAACATGGAAGT<br>R: GCCGCAAGTGTAAGCTGAA | FAM | 2.5 | KP161865 | $(gt)_7$ | 5 | 304–318 | 0.003 |
| LPL45 | F: TCTAGAAACAGACTGGTCTCATGG<br>R: CACCTACGGGCTATTCATGC | FAM | 1 | KP161866 | $(gat)_6$ | 3 | 102–114 | 0.879 |
| MixC | | | | | | | | |
| Scop1 | F: GCCCTCTGCTTACTTTCTCAC<br>R: CTCATGGAGTACGAGTCCAGA | NED | 0.4 | AB610897 | $(ca)_{25}$ | 15 | 139–183 | 0 |
| Scop9 | F: GCTGAAATGGAGGGATGA<br>R: TGTGCCTTTTCTAAGCTGT | FAM | 0.4 | AB610898 | $(ga)_{17}$ | 4 | 164–170 | 0 |
| Scop31 | F: GATCTGGACGTCGACGTCTT<br>R: CCCTGTACCATTCATGATTG | VIC | 0.4 | AB610901 | $(ca)_{19}$ | 22 | 198–256 | 0 |

*F* forward primer sequence; *R* reverse primer sequence; *Na* number of observed alleles per locus; *HWE* Hardy–Weinberg equilibrium exact test *p*-value

and Sweden ($n$=25; 59°24′13″N, 18°12′03″E, Lake Träsksjön). Total genomic DNA was extracted using a standard high-salt protocol (Sambrook et al. 1989) and sent to Genoscreen (Lille, France) for microsatellite-enriched library preparation and sequencing by 454 Genome Sequencer FLX Titanium (454, Roche Applied Science) using the method described by Malausa et al. (2011).

Multiplex PCR reactions were designed following Froufe et al. (2013). Ten primers pairs were chosen, taking into account several criteria, such as the size of the resulting amplicons (small and large loci to maximise their capillary separation), different motif classes (we chose three perfect motifs, with simple repeats only) and the compatibility of primers from different loci for PCR multiplexing. We added a GTTT-sequence 'pig-tail' tag to the 5′ end of all reverse primers (reduces stutter bands; see Brownstein et al. 1996) and a combination of the NED, VIC, PET or 6FAM (Applied Biosystems, Foster City, CA, USA) dyes to the forward primers according to our multiplex assay (Table 1). Allelic variation was initially tested on 20 individuals (five per population) with PCR reactions in simplex to validate selected loci and ascertain optimal annealing temperatures. In total, we repeated the same procedure with three sets of 30 different primers each. Additionally, we also combined the five described microsatellites (Azuma et al. 2011) in a third multiplex PCR reaction, in order to test their usefulness in European populations, since they were previously described and scored in Japanese populations only.

All PCR amplifications were performed in 10-μL reactions containing 1 μL of 25 ng of template DNA, 5 μL of Qiagen Multiplex PCR Master Mix, 1 μL of Primer Mix (variable concentrations of primer pairs and fluorescent dyes according to prior optimisation of multiplex reactions; see Table 1) and 3 μL of distilled $H_2O$. PCR reactions were performed on a DNA Engine Dyad® Peltier Thermal Cycler (Bio-Rad Laboratories), consisting of a denaturing step at 95 °C for 15 min, followed by 13 cycles of denaturation at 95 °C for 30 s, 45 s of annealing at 56 °C, where the annealing temperature is lowered by 0.5 °C with each consecutive cycle, and 30 s of elongation at 72 °C; 27 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s; and a final extension at 60 °C for 30 min. All labelled PCR amplicons were resuspended in 10 μL of Hi-Di™ Formamide and their sizes determined in an Applied Biosystems 3100 DNA analyser, with 500 LIZ as an internal size standard. Alleles were scored using GeneMapper® v.4.0 (Applied Biosystems).

Allelic diversity, observed ($H_O$) and expected ($H_E$) heterozygosity, and inbreeding coefficients ($F_{IS}$) were estimated in GENETIX v.4.05 (Belkhir et al. 1996–2004). Frequencies of null alleles were estimated by using the expectation maximisation algorithm implemented in FreeNA (Chapuis and Estoup 2007). Deviations from the Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were tested

**Table 2** Genetic variation of *Pacifastacus leniusculus* from four European populations for ten microsatellite loci

| | $N_a$ | | | | $H_E$ | | | | $H_O$ | | | | $F_{IS}$ | | | | | Null allele frequency | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus | GB | PT | FI | SW | GB | PT | FI | SW | GB | PT | FI | SW | GB | PT | FI | SW | $F_{ST}$ | GB | PT | FI | SW |
| LPL6 | 7.8 | 6.2 | 6.8 | 5.8 | 0.853 | 0.844 | 0.817 | 0.739 | 1.000 | 0.933 | 0.842 | 0.762 | −0.180 | −0.108 | −0.032 | −0.032 | 0.102 | 0.00 | 0.00 | 0.00 | 0.00 |
| LPL22 | 4.0 | 5.9 | 4.0 | 1.0 | 0.714 | 0.810 | 0.549 | – | 0.154 | 0.750 | 0.000 | – | 0.791 | 0.075 | 1.000 | – | 0.246 | 0.32 | 0.00 | 0.36 | 0.00 |
| LPL26 | 2.3 | 3.3 | 2.3 | 1.8 | 0.191 | 0.422 | 0.246 | 0.150 | 0.200 | 0.433 | 0.182 | 0.080 | −0.050 | −0.027 | 0.266 | 0.473 | 0.027 | 0.00 | 0.00 | 0.07 | 0.10 |
| LPL40 | 3.0 | 2.0 | 3.4 | 3.3 | 0.545 | 0.452 | 0.525 | 0.610 | 0.667 | 0.467 | 0.273 | 0.680 | −0.233 | −0.033 | 0.487 | −0.118 | 0.253 | 0.00 | 0.00 | 0.17 | 0.00 |
| LPL15 | 7.8 | 6.3 | 7.9 | 6.8 | 0.853 | 0.847 | 0.858 | 0.811 | 1.000 | 0.966 | 0.909 | 0.880 | −0.180 | −0.143 | −0.061 | −0.088 | 0.073 | 0.00 | 0.00 | 0.00 | 0.00 |
| LPL32 | 2.3 | 2.7 | 2.7 | 2.8 | 0.191 | 0.344 | 0.320 | 0.318 | 0.200 | 0.300 | 0.273 | 0.080 | −0.050 | 0.129 | 0.152 | 0.752 | −0.006 | 0.00 | 0.02 | 0.07 | 0.20 |
| LPL45 | 2.0 | 1.8 | 2.0 | 1.3 | 0.480 | 0.135 | 0.132 | 0.040 | 0.333 | 0.143 | 0.136 | 0.040 | 0.314 | −0.059 | −0.033 | 0.000 | 0.184 | 0.09 | 0.00 | 0.00 | 0.00 |
| Scop1 | 4.9 | 6.1 | 6.0 | 5.5 | 0.749 | 0.824 | 0.807 | 0.775 | 0.467 | 0.690 | 0.773 | 0.500 | 0.386 | 0.165 | 0.043 | 0.360 | 0.069 | 0.14 | 0.07 | 0.00 | 0.15 |
| Scop31 | 7.4 | 7.4 | 8.2 | 6.3 | 0.729 | 0.566 | 0.479 | 0.530 | 0.467 | 0.600 | 0.136 | 0.320 | 0.479 | 0.298 | 0.473 | 0.087 | 0.102 | 0.21 | 0.14 | 0.20 | 0.02 |
| Scop9 | 3.9 | 2.8 | 2.8 | 2.3 | 0.880 | 0.879 | 0.804 | 0.831 | 0.467 | 0.621 | 0.429 | 0.760 | 0.368 | −0.062 | 0.720 | 0.401 | 0.035 | 0.13 | 0.00 | 0.24 | 0.13 |

$N_a$ allelic richness; $H_E$ expected heterozygosity; $H_O$ observed heterozygosity; $F_{IS}$ inbreeding coefficient; $F_{ST}$ fixation index;
Population codes: *GB* Great Britain; *PT* Portugal; *FI* Finland; *SE* Sweden

using GENEPOP v.4.1 (Raymond and Rousset 1995) with 10,000 iterations.

The model-based approach implemented in STRUCTURE v.2.3.3 (Pritchard et al. 2000) was used to assign individuals to genetic clusters and identify the most likely number of populations (K). Assignment is conducted in ways that minimise deviations from HWE and linkage equilibrium within each cluster. No particular population structure was assumed a priori and ten independent runs were carried out for each value of K (1–5). The length of the burn-in period was set to $1 \times 10^5$, followed by $5 \times 10^5$ Markov chain Monte Carlo (MCMC) iterations. Correlated allele frequencies and admixed populations were assumed. The most likely value for K was selected based on the posterior probability of the data for a given K (Pritchard et al. 2000).

## Results

Of the 42,238 reads obtained from Genoscreen, 475 contained microsatellite inserts with perfect motifs (only one motif) and simple repeats only, from di- to pentanucleotide; however, only 72 were suitable for primer design. Of these, the amplicon sizes varied from 90 to 316 bp; 75 % were dinucleotides (AC repeats were the most common dinucleotide repeat), 17 % trinucleotides (ACA the most common trinucleotide repeat), 4 % tetranucleotides and 4 % pentanucleotides.

From the 30 pairs of primers tested, seven loci were successfully combined in two multiplexed PCR reactions (Table 1) and yielded very clear and balanced electrophoresis profiles that matched the peaks obtained from single-locus PCRs. Two populations (Portugal and Great Britain) were then successfully scored using the multiplex approach and the other two (Sweden and Finland) were analysed using the traditional simplex approach, due to the poor DNA condition of these population samples. The number of alleles ranged from 3 to 15 per locus, with a mean of eight (Table 1).

From the five previously described microsatellites (Azuma et al. 2011), two of the markers (i.e. Scop 13 and 19) did not amplify at all in any of the samples; however, the remaining three loci were also successfully combined in one multiplexed

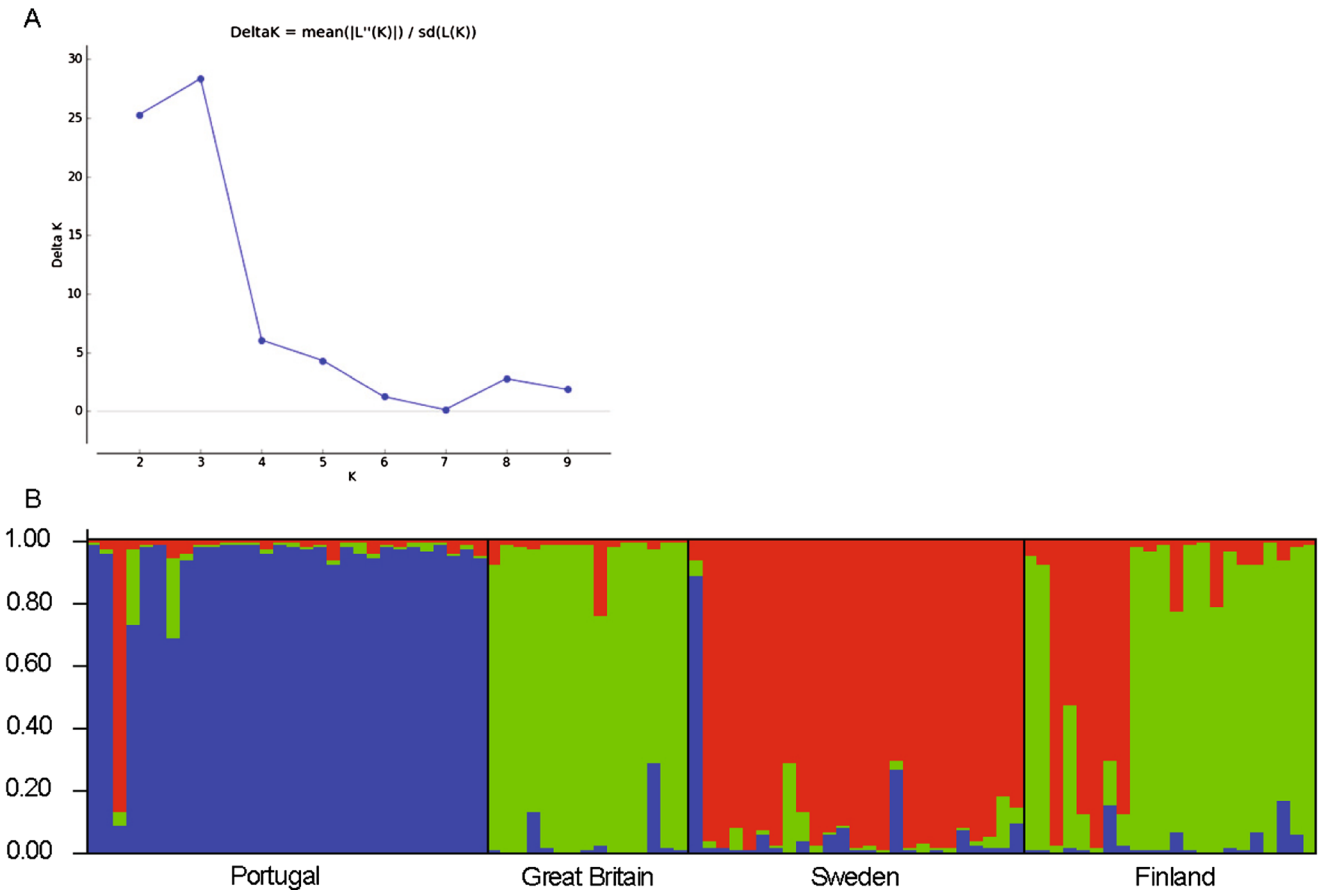


**Fig. 1** STRUCTURE analyses in *Pacifastacus leniusculus*. **a** The graph indicates Evanno's ΔK over ten runs for each of the K genetic clusters (K [1, 10]). Maximal value of ΔK=28.340164 for K=3. **b** K=3 using all populations; each individual is represented by a vertical bar in K coloured segments, with the length of each bar being proportional to the estimated membership coefficient. The *black lines* separate individuals from different geographic regions, i.e. Portugal, Great Britain, Sweden and Finland

PCR (Table 1). Moreover, in the four European populations scored in the present study, the alleles sizes had a different range from the previously described Japanese populations, ranging from 4 to 22 per locus, with a mean of 14 (Table 1). The results of $H_E$ and $H_O$ are shown in Table 2, as well as the *p*-values for HWE in Table 1.

Finally, the plots of ΔK (Evanno et al. 2005) based on the STRUCTURE analysis indicated that three is the most likely number of clusters present in the full dataset, corresponding to Portugal, Sweden and Great Britain+Finland (Fig. 1a, b).

## Discussion

Our study considerably increases the number of available microsatellites markers needed for studying the population genetic structure of *P. leniusculus*. We successfully scored seven new microsatellites in two multiplex PCR reactions. Moreover, our results indicate that, from the five previously published markers (Azuma et al. 2011), only three were able to be used in European populations, which were also combined here in a third multiplex PCR reaction. This is a striking and unexpected result, as those markers have been developed for *P. leniusculus*, and even cross-species amplifications are often successful and have been used for crayfish (e.g. Hulák et al. 2010). The fact that it was only possible to score three markers, together with the very different allele sizes retrieved in the European populations, leads us to believe that a rather different stock is present in Japan. This is worth investigating for sure in future studies, and highlights the possibility that transfer of markers, even in the same species, might fail.

Regarding Europe, the results of the STRUCTURE analyses suggest that Finland and Great Britain may have been introduced with crayfish from the same original source. Indeed, the Swedish hatchery Simontorp supplied juvenile signal crayfish for stocking to many European countries (Henttonen and Huner 1999), and it is likely that the origin of a substantial part of European populations might be traced back to this source. Moreover, the fact that both the Swedish and the Portuguese populations cluster separately indicates possible different independent original sources. The main sources of large-scale introductions to Sweden and Finland in the late 1960s were partly the same: Swedish signal crayfish originated from Lake Tahoe in California, USA (Fürst 1977), and those first introduced to Finland were imported from Californian lakes Tahoe and Hennessey, but large-scale imports from Sweden followed (Henttonen and Huner 1999; Skurdal et al. 1999). The Swedish population in this study, however, belongs to the first introductions into the country in the early 1960s, and originates from Lake Natoma in California. This may explain the marked genetic differentiation between the Finnish and Swedish samples. The distinct position of the Portuguese population might be either due to different origin or, more likely, due to serial founder effects and genetic drift associated with signal crayfish spread through the Iberian Peninsula.

In sum, the overall analysis of the ten microsatellites markers used in the present study can be used for characterising population genetic variation and tracking it in the future, as well as for determining phylogeographic relationships, which may help us elucidate the major invasion corridors and retrace source populations.

**Acknowledgements** Financial support was provided by the Portuguese Foundation for Science and Technology (FCT) project PTDC/AAC-AMB/117688/2010. LF was supported by Office National de l'Eau et des Milieux Aquatiques (ONEMA), AP by the Czech Science Foundation (project no. P505/12/0545) and LE by the Swedish Research Council FORMAS and SwAM. We would like to thank Stephanie Peay for collecting the UK samples and to Susana Lopes for the help with fragment analysis.

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