Microsatellite markers reveal clear geographic structuring among threatened noble crayfish (Astacus astacus) populations in Northern and Central Europe

ARTICLE *in* CONSERVATION GENETICS · MARCH 2013

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RESEARCH ARTICLE

Microsatellite markers reveal clear geographic structuring among threatened noble crayfish (*Astacus astacus*) populations in Northern and Central Europe

Riho Gross · Stefan Palm · Kuldar Kõiv · Tore Prestegaard · Japo Jussila · Tiit Paaver · Juergen Geist · Harri Kokko · Anna Karjalainen · Lennart Edsman

Received: 28 September 2012 / Accepted: 5 March 2013

Abstract Noble crayfish (*Astacus astacus* L.), the most highly valued freshwater crayfish in Europe, is threatened due to a long-term population decline caused mainly by the spread of crayfish plague. Reintroduction of the noble crayfish into restored waters is a common practice but the geographic and genetic origin of stocking material has rarely been considered, partially because previous genetic studies have been hampered by lack of nuclear gene markers with known inheritance. This study represents the first large scale population genetic survey of the noble crayfish (633 adults from 18 locations) based on 10 newly developed microsatellite markers. We focused primarily on the Baltic Sea area (Estonia, Finland and Sweden) where the largest proportion of the remaining populations exists. To allow comparisons, samples from the Black Sea catchment (the Danube drainage) were also included. Two highly differentiated population groups were identified corresponding to the Baltic Sea and the Black Sea catchments, respectively. The Baltic Sea catchment populations had significantly lower genetic variation and private allele numbers than the Black Sea catchment populations. Within the Baltic Sea area, a clear genetic structure was revealed with population samples corresponding well to their geographic origin, suggesting little impact of long-distance translocations. The clear genetic structure strongly suggests that the choice of stocking material for re-introductions and supplemental releases needs to be based on empirical genetic knowledge.

Keywords Genetic variation · Genetic differentiation · Population structure · Microsatellite DNA · Conservation genetics

R. Gross (✉) · K. Kõiv · T. Paaver
Department of Aquaculture, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Kreutzwaldi 48, 51014 Tartu, Estonia
e-mail: riho.gross@emu.ee

S. Palm · T. Prestegaard · L. Edsman
Department of Aquatic Resources, Institute of Freshwater Research, Swedish University of Agricultural Sciences, Stångholmsvägen 2, 17893 Drottningholm, Sweden

J. Jussila · H. Kokko · A. Karjalainen
Department of Biology, University of Eastern Finland, P.O. Box 1627, 70211 Kuopio, Finland

J. Geist
Aquatic Systems Biology Unit, Department of Ecology and Ecosystem Management, Technische Universität München, Mühlenweg 22, 85354 Freising, Germany

Introduction

Freshwater biodiversity is globally declining with invertebrate species such as mussels (Geist 2011) and crayfishes (Reynolds and Souty-Grosset 2012) being particularly affected. This loss of biodiversity comprises multiple levels of biological organization, from habitat diversity to communities and species, to the level of intra-specific genetic diversity. Genetic variation is essential for securing the evolutionary potential of a species and its preservation is a prerequisite for effective conservation and sustainable harvesting (e.g. Allendorf et al. 2008). For threatened and simultaneously exploited species, knowledge of intra-species genetic diversity is particularly important. In such cases identification of genetic structuring may yield important information on existence of unique or vulnerable subpopulations, which can simultaneously be utilized when planning management actions like re-establishment or enhancement programs.

Published online: 14 March 2013

The noble crayfish (*Astacus astacus* L.) is the most common and the most highly valued freshwater crayfish species in Europe. Its native range extends from Russia and Ukraine in the east, to Finland, Sweden, Norway in the north, to Greece in the south, and France in the west. In Northern Europe, the noble crayfish traditionally has formed the basis for culturally and economically important fishery (Jussila and Mannonen 2004; Souty-Grosset et al. 2006). For example, in Sweden, the current per capita consumption of freshwater crayfish (noble crayfish and other species combined) amounts to 0.5 kg per year (Gren et al. 2009). At the same time, the noble crayfish has suffered from a serious and long-term population decline (Souty-Grosset et al. 2006; Edsman et al. 2010) mainly due to crayfish plague which is caused by an oomycete (*Aphanomyces astaci* Schikora) and has been spread all over Europe by the introduced North American signal crayfish (*Pacifastacus leniusculus* Dana) (Bohman et al. 2006; Diéguez-Uribeondo et al. 2006; Wutz and Geist 2013).

Threats of lower or local importance include habitat destruction, watercourse alteration, acidification, pollution, predation and sometimes overfishing and pouching (Füreder et al. 2006; Paaver and Hurt 2009; Tulonen et al. 2010; Edsman et al. 2010). As a result, the noble crayfish has been classified as vulnerable by the IUCN Red List of Threatened Species (Edsman et al. 2010) but for instance in the national Red Lists of Norway (Oug et al. 2010) and Czech Republic (Farkac et al. 2005) it is classified as endangered while in the Swedish Red List (Gärdenfors 2010) it is listed as critically endangered. Action plans for the conservation of the noble crayfish have been produced in several countries, e.g. Sweden (Edsman and Schröder 2009), Finland (Jussila and Mannonen 2004) and Estonia (Tuusti et al. 1998). In these plans, re-introduction of the noble crayfish into waters that used to hold populations and which were restored through habitat modification and liming, or became crayfish plague free through eradication of the signal crayfish are the most common conservation measures. Supplemental release into existing populations, aimed at supporting local fisheries, is also a common practice. So far, however, the geographic and genetic origin of crayfish has rarely been considered when selecting material for re-introductions or supplemental stocking. The main reason for this is a very limited knowledge of intra-species genetic diversity in the noble crayfish.

Previous studies of genetic diversity in this species have been hampered by lack of variable nuclear genetic markers with known inheritance. Early work on isozymes found very few variable markers (Agerberg 1990; Fevolden et al. 1994). Edsman et al. (2002) revealed microsatellite-like variation within the rDNA-ITS1 region and presented evidence for genetic differences between populations. The ITS-linked microsatellites have found some practical use (e.g. Edsman et al. 2002; Alaranta et al. 2006) and observed length fragments show inheritance from parents to offspring (Alaranta et al. 2011). However, the ITS1 region is part of a multicopy gene family, and the ITS-linked microsatellites cannot be treated as discrete co-dominant Mendelian markers (Harris and Crandall 2000), which has precluded standard population genetic analyses. Similar drawbacks are associated with another PCR-based finger-printing approach (multi-locus ISSRS markers) which has been used to infer genetic relationships among populations (Schulz et al. 2004).

The first large-scale study of genetic diversity of the noble crayfish in the Black Sea, North Sea and Baltic Sea basins was published only recently (Schrimpf et al. 2011) and was based on mtDNA COI gene analysis. A single common haplotype was found across the whole study area but high frequencies of private haplotypes in all major catchment areas indicated differentiation of populations throughout Europe despite extensive human translocations. Higher haplotype diversity and number of private haplotypes in the Black sea basin compared to the North Sea and the Baltic Sea basins further suggested a glacial refuge in the Balkan area. Although the above mentioned studies have provided the first evidence for presence of genetic variation and population structuring at both local and larger geographic scales within the species, there is need for comprehensive surveys based on standard nuclear marker systems.

For the noble crayfish, the first conventional microsatellites were developed only recently (Kõiv et al. 2008, 2009) and the aim of the present study was to assess genetic diversity and to reveal population genetic structure of the noble crayfish in Northern and Central Europe using these markers. Also, we aimed to detect the genetic footprints of possible long-distance or between-country translocations. We focused primarily on the Baltic Sea area (Estonia, Finland and Sweden) where the largest portion of the remaining populations exists (Holdich et al. 2009) and where a main responsibility for the species' conservation thus resides. To allow comparisons on a larger geographical scale, samples from Germany and the Czech Republic (the Danube drainage, Black Sea catchment) were also included.

Methods

Sample collection and DNA analyses

Samples from a total of 630 adult noble crayfish from 18 locations in Northern Europe and four locations in Central Europe were collected during years 2000–2008 (Fig. 1,

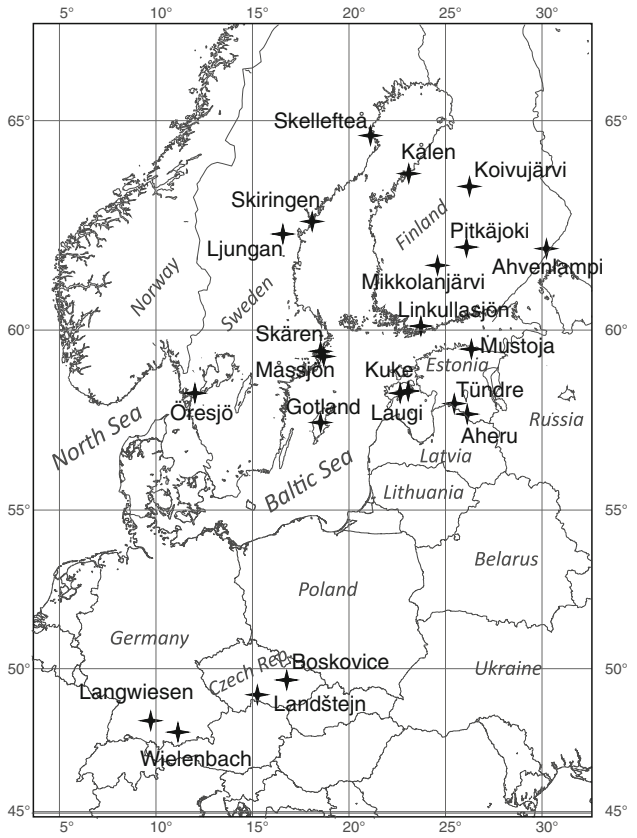

Fig. 1 Sampling locations of the noble crayfish in Northern and Central Europe

Table 1). Most samples represent wild populations in lakes, streams or rivers, but some samples were taken from crayfish farms, artificial ponds or reservoirs. The degree of knowledge regarding sample origin is variable, ranging from "native" (presumably not introduced or stocked) and "uncertain" (probably stocked, possibly introduced, etc.) to cases where a population is known to have been stocked or introduced (Table 1).

Genomic DNA was isolated from muscle tissue according to the simplified method of Laird et al. (1991). A total of 10 microsatellite loci were analysed: *Aas2*, *Aas6*, *Aas7*, *Aas8*, *Aas11*, *Aas766*, *Aas1198*, *Aas3040*, *Aas3666*, and *Aas3950* (Kõiv et al. 2008, 2009). All forward primers were 5′-tailed with the 19 bp M13 sequence (5′-CACGACGTTGTAAAACGAC-3′) and were used in combination with the 5′ end-labeled universal primers of identical M13 sequence (fluorescent dyes TET, HEX or 6-FAM; Table 2) to generate labelled amplification products for automated analysis of fragments. The PCR reaction (total volume 10 µl) contained 1 × PCR buffer with $(NH_4)_2SO_4$, 100 µM dNTP, 1.5–2.5 mM $MgCl_2$, 20 to 100 nM forward primer, 200 nM of reverse and M13 primers, 0.2 U *Taq* DNA polymerase (Fermentas UAB, Vilnius), and ~10 ng of DNA template (Table 2).

Amplifications were performed using the following protocol: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at variable temperatures (Table 2) for 40 s, and extension at 72 °C for 60 s, which were followed by a final extension at 72 °C for 30 min. The amplification products were separated by capillary electrophoresis on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) and the sizes of the microsatellite alleles were determined using Genotyper 2.0 software (Applied Biosystems, Foster City, CA).

Statistical analyses

Data were assessed for potential genotyping errors, such as null alleles, large allele dropout or scoring errors, using the programme MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2006). FSTAT v. 2.9.3.2 programme package (Goudet 2001) was used for calculating allele frequencies, F_{IS} and pair-wise F_{ST} values (as estimated by Weir and Cockerham's θ), for estimating expected and observed heterozygosities (H_E, H_O) and allelic richness (A_R), and for testing the significance of differences in average values of A_R, H_E and H_O among groups of populations (1,000 permutations, two-sided tests). GENEPOP v. 3.3 (Raymond and Rousset 1995a) was used to test genotypic distributions for conformance to Hardy–Weinberg (HW) expectations and to test the loci for genotypic disequilibria. All probability tests were based on the Markov chain method (Guo and Thompson 1992; Raymond and Rousset 1995b) using 1,000 de-memorization steps 100 batches and 1,000 iterations per batch. Sequential Bonferroni adjustments (Rice 1989) were applied to correct for the effect of multiple tests.

Population structure was studied using two methods. First, we obtained an unrooted neighbour-joining cladogram (Saitou and Nei 1987) based on the D_A genetic distance (Nei et al. 1983) matrix between populations, using the DISPAN software (Ota 1993). Bootstrapping 1,000 times over loci assessed the strength of the support for each node in the tree. The second approach utilized the program STRUCTURE v. 2.3.2, which is based on a Bayesian, model-based algorithm and identifies clusters of related individuals from multilocus genotypes (Pritchard et al. 2000; Falush et al. 2003). We used a model assuming admixture and correlated allele frequencies between K population groups (burn-in 50 000 replications, simulation 100 000 Markov chain Monte Carlo (MCMC) replicates), and no prior information on sample location for individuals (USEPOPINFO = 0). Separate sets of runs with STRUCTURE were carried out without or with the assistance of sample group information (LOCPRIOR = 0 or 1, respectively) (Hubisz et al. 2009). As recommended

Table 1 Characterisation of the studied noble crayfish populations

Country, population	Type of habitat	Catchment	Coordinates	Year of sampling	Sample size	Status, notes
Estonia						
Kuke	Stream	Baltic Sea	N58°26′44″; E23°2′18″	2005	32	Native, Saaremaa island
Laugi	Stream	Baltic Sea	N58°19′35″; E22°35′28″	2007	29	Native, Saaremaa island
Mustoja	River	Baltic Sea	N59°34′56″; E26°10′32″	2007	30	Native
Tündre	Lake	Baltic Sea	N57°57′27″; E25°36′06″	2006	24	Re-established based on R. Õhne (L. Peipsi drainage) stock
Aheru	Lake	Baltic Sea	N57°40′58″; E26°21′07″	2005	24	Mixed origin, enhanced by L. Kallijärv stock (L. Peipsi drainage)
Finland						
Linkullasjön	Lake	Baltic Sea	N60°5′49″; E23°52′60″	2003–2005	20	Uncertain
Mikkolanjärvi	Lake	Baltic Sea	N61°36′53″; E24°35′31″	2003–2005	20	No information available
Kålen	Pond	Baltic Sea	N63°49′12″; E23°13′24″	2003–2005	20	Re-established based on R. Perhonjoki stock
Koivujärvi	Lake	Baltic Sea	N63°28′58″; E26°15′49″	2003–2005	20	Uncertain
Pitkäjoki	River	Baltic Sea	N62°7′18″; E26°3′11″	2003–2005	20	Uncertain
Ahvenlampi	Lake	Baltic Sea	N62°4′19″; E30°21′51″	2003–2005	23	Uncertain
Sweden						
Gotland	Pond	Baltic Sea	N57°36′28″; E18°32′2″	2008	47	Farmed stock with mixed origin, Gotland island
Ljungan	River	Baltic Sea	N62°29′12″; E16°22′6″	2008	43	Uncertain
Skären	Lake	Baltic Sea	N59°32′34″; E18°38′8″	2000	24	Uncertain
Måssjön	Lake	Baltic Sea	N59°30′44″; E18°35′60″	2000	24	Uncertain
Skellefteå	River	Baltic Sea	N64°44′22″; E21°1′17″	2001	39	Uncertain
Skiringen	Lake	Baltic Sea	N62°41′54″; E17°56′41″	2007–2008	48	Introduced (Ljungan origin; see above)
Öresjö	Lake	North Sea (Skagerrak)	N58°16′11″; E12°8′20″	2004–2007	48	Mixed origin
Czech Republic						
Boskovice	Water reservoir	Danube, Black Sea	N49°29′44″; E16°41′56″	2008	30	Native
Landštejn	Water reservoir	Danube, Black Sea	N49°1′16″; E15°14′36″	2008	30	Native
Germany						
Langwiesen	Pond	Danube, Black Sea	N48°29′3″; E9°43′35″	2007	15	Stocked by native stocks of local origin
Wielenbach	Pond	Danube, Black Sea	N47°53′2″; E11°9′22″	2007	20	Stocked by native stocks of local origin

by the authors, we adopted a hierarchical approach starting with the full set of sampling sites ($n = 22$) and further analysed each proposed cluster until no further subdivision was indicated in each cluster (i.e. $K = 1$). Runs were replicated three times at each K to confirm consistency of log-likelihood probabilities. True population number (K) estimates were inferred based on the rate of change in the log probability of data between successive K values (ΔK) according to Evanno et al. (2005) who demonstrated a significant advantage of their method for correct estimation of the number of clusters K in situations with a hierarchical pattern of population structure. Populations were assigned to the group in which the inferred average ancestry of individuals was higher than 0.5.

Results

Genetic diversity

A total of 103 alleles were observed across the 10 microsatellite loci with an average of 10.3 alleles per locus, ranging from 7 alleles at *Aas2* and *Aas11* to 19 alleles at *Aas3950* (Table 2). The average observed heterozygosity

Table 2 Characterisation of the studied microsatellite loci in the noble crayfish (*Astacus astacus*)

Locus	Primer sequence 5′–3′	Repeat motif[a]	T_a (°C)	$MgCl_2$ concentration (mM)	Forward primer concentration (nM)	M13 primer label	Allele size range[b] (bp)	*A*	H_o
Aas2	F: M13-GGGAATGCAAGTGTGAGTGTT	$GT_{(23)}$	60.0	1.5	20	TET	147–177	7	0.156
	R: TTTTGCGTCATCCGTATGTG								
Aas6	F: M13-AGACACAAACGCACATGGAA	$GA_{(26)}$	60.0	1.5	20	HEX	154–188	12	0.340
	R: GTGTCTGGCAGGCGTATGAT								
Aas7	F: M13-ACATTGCCAAGTTTGTTTCC	$CT_{(24)}$	56.5	1.5	20	FAM	254–276	11	0.456
	R: CATCCTCTTTGCCTTCCATTT								
Aas8	F: M13-GGGCAACAGACATACAACGAT	$CA_{(29)}$	56.5	1.5	20	TET	185–205	9	0.075
	R: TTTCTGCTGTTTTTCGCTCA								
Aas11	F: M13-CTAGGAGCCATTTGGTGGAC	$GT_{(20)}$	60.0	1.5	20	FAM	185–197	7	0.097
	R: CTGTAGCGAACACAGCAACC								
Aas766	F: M13-CGTACTGCCTCTCTGCCACT	$GT_{(29)}$	55.5	2.5	100	TET	229–311	11	0.470
	R: AACACACTCCACACCATTCG								
Aas1198	F: M13-CGTTTATTTCCTTTCCCCTTCA	$CT_{(24)}$	55.5	2.5	100	HEX	163–205	9	0.394
	R: TGCATTGAGGTGGTGTCACT								
Aas3040	F: M13-GTTGTGTGGTAACTCCTGACGA	$TA_{(20)}$	55.5	2.5	100	FAM	251–275	8	0.242
	R: CAATCGTATCCCACATGCAG								
Aas3666	F: M13-TTTTGCATCGTCAGCGAACAT	$GT_{(27)}$	56.5	1.5	20	HEX	233–253	10	0.148
	R: TCTCAGCGACAAGGTACTGGAAGC								
Aas3950	F: M13-ATCTAAGTCGTTCTCCTGAAGACC	$CT_{(42)}$	55.5	2.5	100	FAM	180–216	19	0.517
	R: TACTGGTGATTGTGGGTGGTACG								
Average								10.3	0.289

[a] Longest uninterrupted repeat

[b] Size including a 19 bp M13 tail

T_a annealing temperature, *A* number of observed alleles, H_o observed heterozygosity

of the studied loci was 0.289 and varied from 0.075 (*Aas8*) to 0.517 (*Aas3950*) (Table 2).

All microsatellite loci in studied crayfish populations were in linkage equilibrium (data not shown). Only three populations (Gotland and Öresjö from Sweden, and Langwiesen from Germany) displayed significant deviations from expected HW proportions (deficit of heterozygotes) after applying sequential Bonferroni correction (Table 3). MICROCHECKER software provided evidence for putative null alleles at 6 out of 10 microsatellite loci in 8 populations (one to three loci per population, Table 3). However, as only 13 out of 220 tests were significant (5.9 %, i.e. close to the expected Type-I error level), we decided not to exclude any loci from further analysis. Moreover, omitting some loci with putative null alleles (e.g. *Aas7*) had only negligible effect on the results of genetic analyses (data not shown).

Genetic variation, expressed as the mean allelic richness and observed heterozygosity, was significantly higher ($P < 0.05$) in the populations from the Black Sea catchment than in the Baltic Sea catchment (Table 3). Within countries, the level of genetic diversity among populations varied relatively little, except in Estonia ($A_R = 1.7$–1.8 and $H_o = 0.193$–0.195 in Laugi and Mustoja vs. $A_R = 3.0$, $H_o = 0.303$ in Tündre) and in Sweden ($A_R = 1.7$, $H_o = 0.184$ in Skären vs. $A_R = 2.7$, $H_o = 0.375$ in Gotland) (Table 3).

Of the total 103 alleles, only 47 were shared by the Baltic Sea and the Black Sea catchment populations, 18 alleles were confined to the Baltic Sea catchment and 38 alleles were found only in the Danube drainage of the Black Sea catchment. Among countries, a total of 39 private alleles were detected with 8, 1, 2, 15 and 13 alleles confined to the Estonian, Finnish, Swedish, Czech and German populations, respectively (Table 3).

Genetic differentiation and population structure

Differences in allele frequencies were significant ($P < 0.05$) for most pairwise comparisons of populations except for those between Kålen, Koivujärvi and Pitkäjoki populations in Finland, and those between Måssjön and Skellefteå, Skellefteå and Skiringen, and Skiringen and Öresjö population pairs in Sweden (Table 4). The overall level of genetic differentiation between all studied samples was high (global $F_{ST} = 0.264$) with pairwise estimates of F_{ST} ranging from 0.018 (between Kålen and Pitkäjoki populations in Finland) to 0.500 (between Finnish

Table 3 Genetic diversity indices of the noble crayfish (*Astacus astacus*) populations

Population	N	A	A_R	A_{pr}	H_E	H_O	F_{IS}	P_{HW}	Loci with putative 0-alleles
Estonia	139	5.2	3.5	8	0.244	0.221			
Kuke	32	2.2	1.8	0	0.216	0.208	0.037	n.s.	
Laugi	29	2.2	1.7	0	0.167	0.195	−0.172	n.s.	
Mustoja	30	2.1	1.8	1	0.207	0.193	0.070	n.s.	
Tündre	24	3.8	3.0	4	0.311	0.303	0.029	n.s.	*Aas1198, Aas7*
Aheru	24	2.7	2.3	1	0.237	0.229	0.035	n.s.	
Average		2.6	2.1[a]	1.2	0.228[a]	0.225[a]	0.010[a]		
Finland	123	3.3	2.8	1	0.279	0.211			
Linkullasjön	20	1.8	1.7	0	0.194	0.202	−0.042	n.s.	
Mikkolanjärvi	20	2.6	2.2	0	0.217	0.220	−0.013	n.s.	
Kålen	20	2.3	2.1	0	0.222	0.220	0.009	n.s.	
Koivujärvi	20	2.3	2.1	0	0.295	0.245	0.174	n.s.	*Aas1198, Aas7*
Pitkäjoki	20	2.3	2.1	0	0.228	0.190	0.169	n.s.	
Ahvenlampi	23	1.7	1.6	1	0.214	0.193	0.101	n.s.	*Aas7*
Average		2.2	2.0[a]	0.2	0.228[a]	0.212[a]	0.076[a]		
Sweden	273	3.7	2.9	2	0.333	0.289			
Gotland	47	3.1	2.7	0	0.385	0.375	0.026	**	*Aas3950*
Ljungan	43	2.2	2.0	0	0.300	0.305	−0.016	n.s.	*Aas8*
Skären	24	1.8	1.7	1	0.215	0.184	0.147	n.s.	*Aas3040, Aas1198*
Måssjön	24	1.7	1.7	0	0.198	0.238	−0.207	n.s.	
Skellefteå	39	2.5	2.2	0	0.310	0.304	0.019	n.s.	
Skiringen	48	2.1	1.9	0	0.290	0.308	−0.065	n.s.	
Öresjö	48	2.6	2.0	0	0.272	0.240	0.120	*	*Aas3040, Aas7, Aas2*
Average		2.3	2.0[a]	0.1	0.281[a]	0.279[a]	0.011[a]		
Czech Republic	60	6.2	5.4	15	0.514	0.474			
Boskovice	30	4.3	3.5	7	0.513	0.506	0.015	n.s.	
Landštejn	30	4.3	3.3	6	0.450	0.439	0.025	n.s.	
Average		4.3	3.4[b]	6.5	0.481[b]	0.472[b]	0.020[a]		
Germany	35	6.1	6.0	13	0.612	0.538			
Langwiesen	15	4.6	4.2	3	0.619	0.500	0.198	***	*Aas8*
Wielenbach	20	5.0	4.1	3	0.578	0.567	0.020	n.s.	
Average		4.8	4.2[b]	3.0	0.598[b]	0.533[b]	0.096[a]		

N Sample size, A average number of alleles/locus, A_R mean allelic richness, A_{pr} number of private alleles, H_E expected and H_O observed heterozygosity, F_{IS} inbreeding coeficient and P_{HW} probability of deviations from expected Hardy–Weinberg proportions after sequential Bonferroni adjustments (10 simultaneous tests per population)

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

[a,b] Different letters in superscript indicate that the corresponding average parameter values differ significantly ($P < 0.01$)

Ahvenlampi and Swedish Skären population) (Table 4). The level of differentiation between populations from different Northern European countries (Estonia, Finland, Sweden) was relatively high (average pair-wise F_{ST} ranging from 0.243 between Finland and Sweden to 0.328 between Estonia and Finland) but generally lower than their differentiation from Central European populations (average pair-wise F_{ST} from 0.285 between Finland and Germany to 0.365 between Estonia and Czech Republic). Within countries, populations were more differentiated in Finland and Sweden (average pair-wise F_{ST} 0.201 and 0.161, respectively) than in Estonia, the Czech Republic or Germany (average pair-wise F_{ST} 0.104, 0.113 and 0.056, respectively).

The pair-wise D_A genetic distance matrix-based NJ cladogram clearly separated all Baltic catchment populations from the Black Sea catchment populations (bootstrap support 95 %). Within the catchments, most of the populations were clustered according to their country of origin (bootstrap support from 59 to 68 %) with the exception of

Table 4 Pairwise estimates of F_{ST} between populations of the noble crayfish (above diagonal) and significance values of allelic differentiation for each population pair (below diagonal) after sequential Bonferroni adjustments (231 simultaneous comparisons)

	Population	Estonia					Finland						Sweden							Czech Rep.		Germany	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	Kuke	0.000	0.077	0.050	0.113	0.195	0.292	0.387	0.352	0.336	0.322	0.441	0.202	0.294	0.362	0.328	0.265	0.246	0.249	0.322	0.455	0.358	0.353
2	Laugi	*	0.000	0.077	0.124	0.147	0.326	0.371	0.343	0.337	0.301	0.459	0.208	0.309	0.396	0.321	0.250	0.250	0.270	0.332	0.465	0.371	0.350
3	Mustoja	*	*	0.000	0.085	0.101	0.356	0.335	0.314	0.316	0.279	0.437	0.195	0.312	0.397	0.323	0.235	0.261	0.292	0.304	0.443	0.334	0.333
4	Tündre	*	*	*	0.000	0.070	0.261	0.283	0.246	0.233	0.223	0.377	0.139	0.246	0.319	0.324	0.209	0.214	0.241	0.244	0.377	0.247	0.253
5	Aheru	*	*	*	*	0.000	0.362	0.283	0.306	0.291	0.269	0.390	0.201	0.324	0.428	0.365	0.263	0.293	0.335	0.294	0.417	0.277	0.283
6	Linkullasjön	*	*	*	*	*	0.000	0.321	0.328	0.245	0.308	0.306	0.134	0.116	0.292	0.322	0.246	0.127	0.123	0.323	0.439	0.346	0.311
7	Mikkolanjärvi	*	*	*	*	*	*	0.000	0.119	0.106	0.123	0.249	0.120	0.176	0.460	0.264	0.148	0.199	0.305	0.258	0.363	0.301	0.252
8	Kålen	*	*	*	*	*	*	*	0.000	0.051	0.018	0.323	0.099	0.212	0.433	0.356	0.163	0.192	0.297	0.246	0.340	0.312	0.264
9	Koivujärvi	*	*	*	*	*	*	*	n.s.	0.000	0.060	0.163	0.090	0.146	0.381	0.330	0.137	0.136	0.233	0.218	0.291	0.275	0.228
10	Pitkäjoki	*	*	*	*	*	*	*	n.s.	n.s.	0.000	0.288	0.084	0.237	0.394	0.372	0.186	0.214	0.305	0.209	0.305	0.279	0.223
11	Ahvenlampi	*	*	*	*	*	*	*	*	*	*	0.000	0.176	0.232	0.500	0.425	0.285	0.247	0.306	0.320	0.386	0.327	0.301
12	Gotland	*	*	*	*	*	*	*	*	*	*	*	0.000	0.112	0.258	0.204	0.107	0.106	0.150	0.178	0.294	0.194	0.183
13	Ljungan	*	*	*	*	*	*	*	*	*	*	*	*	0.000	0.266	0.153	0.088	0.024	0.064	0.298	0.403	0.333	0.315
14	Skären	*	*	*	*	*	*	*	*	*	*	*	*	*	0.000	0.410	0.314	0.228	0.203	0.343	0.453	0.393	0.364
15	Måssjön	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0.000	0.108	0.164	0.212	0.351	0.476	0.378	0.355
16	Skellefteå	*	*	*	*	*	*	*	*	*	*	*	*	*	*	n.s.	0.000	0.063	0.133	0.257	0.372	0.300	0.277
17	Skiringen	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	n.s.	0.000	0.020	0.292	0.398	0.340	0.319
18	Öresjö	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	n.s.	0.000	0.330	0.441	0.365	0.357
19	Boskovice	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0.000	0.113	0.129	0.100
20	Landštejn	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0.000	0.202	0.129
21	Langwiesen	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0.000	0.056
22	Wielenbach	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0.000

n.s. not significant

* $P < 0.05$

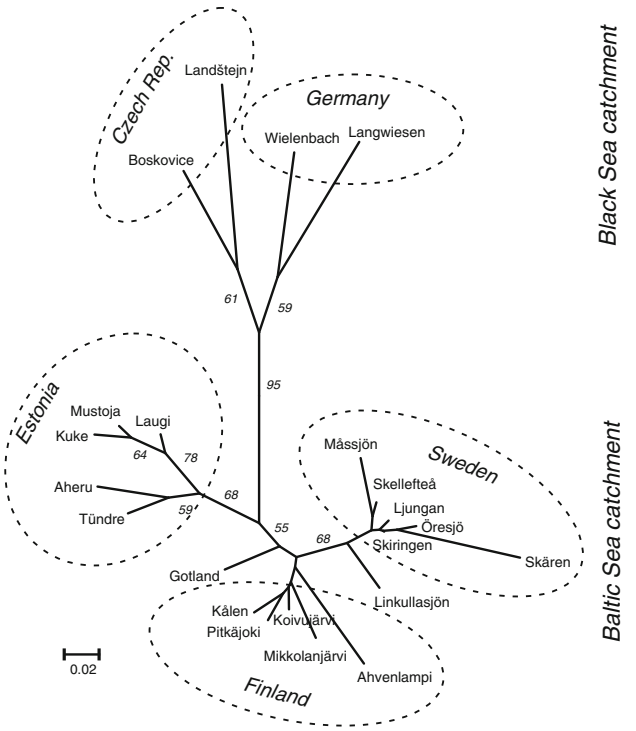

Fig. 2 Unrooted neighbour-joining cladogram based on D_A genetic distances (Nei et al. 1983) between the noble crayfish populations. Major geographical groupings of populations are indicated by *dotted circles*. *Numbers* indicate branches with bootstrap support above 50 % in 1,000 replicates

the Swedish Gotland and the Finnish Linkullasjön samples (Fig. 2). Sub-structuring within a country was observed only in Estonia where populations from inland lakes in southern Estonia (Aheru and Tündre) and from coastal streams in western/northern Estonia (Kuke, Laugi and Mustoja) formed two moderately supported subclusters (Fig. 2).

The first round of hierarchical STRUCTURE analysis with the full set of studied noble crayfish populations inferred three genetic clusters ($K = 3$) both without or with the assistance of sample group information: one including exclusively the individuals from Estonia, the second including the individuals from Finland and Sweden, and the third including the individuals from Central Europe (the Czech Republic and Germany) (Fig. 3).

In the second round, when the three clusters were analysed separately, the Estonian cluster was subdivided into two groups ($K = 2$): group (i) included the individuals from western and northern Estonia (Kuke, Laugi and Mustoja populations) and group (ii) included the individuals from southern Estonia (Tündre and Aheru populations) (Fig. 3). The Finnish-Swedish cluster was also subdivided into two groups ($K = 2$), one mostly including individuals from Finnish populations (except Linkullasjön) and another from the Swedish populations (except Gotland) (Fig. 3). The central European cluster was subdivided into three groups ($K = 3$) with the German populations forming a single group and the Czech populations forming two distinct groups (Fig. 3).

In the third round, the Finnish group was further subdivided into three groups ($K = 3$) corresponding to Gotland, Ahvenlampi and the rest of the Finnish populations, respectively, and the Swedish group was subdivided into two groups ($K = 2$; Skären and Linkullasjön *versus* the rest of Swedish populations) (Fig. 3). At each hierarchical level of STRUCTURE analysis, individuals from some populations displayed a low proportion of ancestry in another genetic cluster (Fig. 3). Altogether, the first two rounds of STRUCTURE analysis inferred genetic groups highly similar to the results of the distance-based cladogram analysis. However, in addition to that in Estonia, STRUCTURE analysis revealed sub-structuring of populations within country also in Finland and in Sweden which was not evident in the distance-based analysis.

Discussion

This is the first population genetic survey of the noble crayfish on a large geographical scale based on microsatellite markers. Previous studies using nuclear genetic markers were limited both in geographical scope and by use of much less variable allozyme (Fevolden et al. 1994) or nDNA markers, such as ITS-linked microsatellites (Edsman et al. 2002; Alaranta et al. 2006), RAPDs (Schulz 2000) and ISSRs (Schulz et al. 2004) that are known to have several disadvantages (e.g. non-codominant inheritance, possible nonhomology of similarly sized fragments, reproducibility problems etc.). Microsatellite markers, in contrast, are highly polymorphic, codominant, abundant and randomly distributed throughout the genome and highly reproducible, and their application allowed us to gain substantial new information about population genetic structure and genetic variation of this highly valued and vulnerable species in Northern and Central Europe.

Large scale patterns of genetic population structure

Both cladogram and STRUCTURE analyses revealed a similar and clear structuring of the studied populations into two highly differentiated groups (between group $F_{ST} = 0.251$, average inter-group pair-wise $F_{ST} = 0.323$) according to their catchment of origin: the Baltic Sea (Swedish, Finnish and Estonian populations) and the Black Sea (the Czech and southern German populations of the Danube drainage). This is in accordance with the recent study on mtDNA COI gene haplotype frequencies, which revealed significant differentiation of the noble crayfish

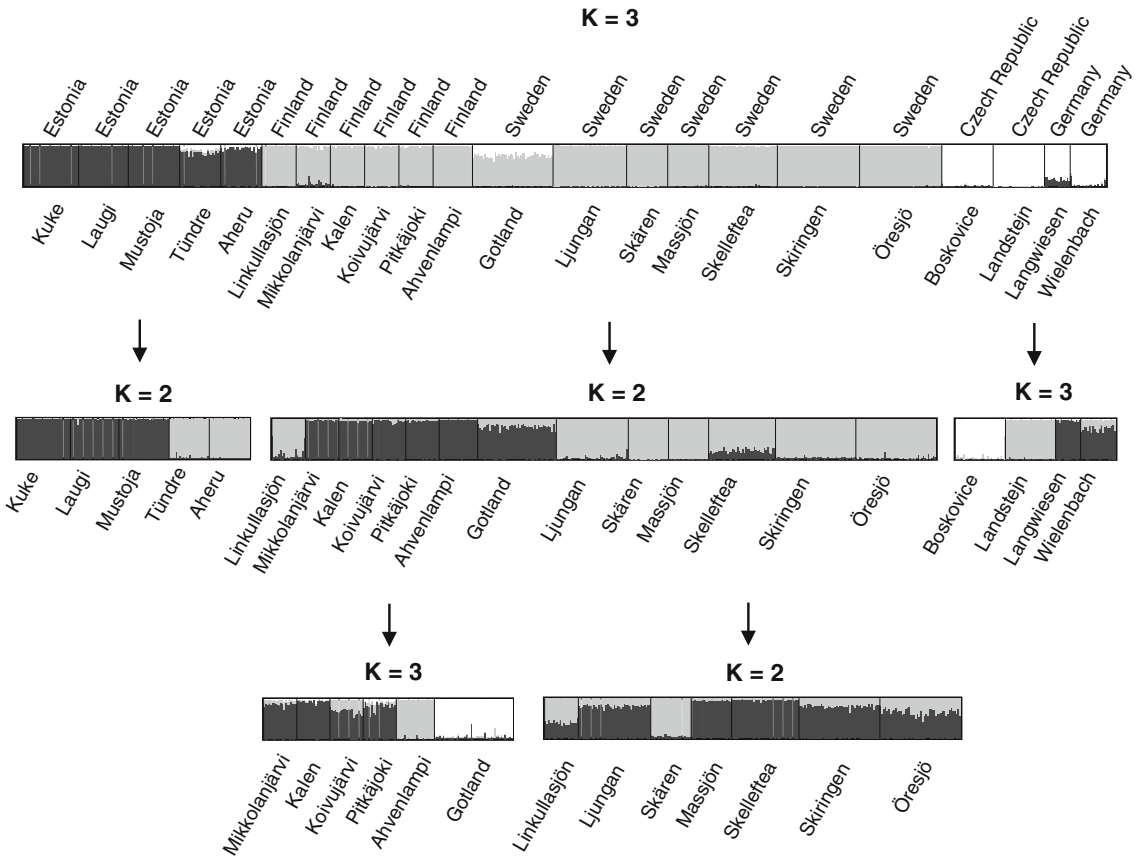

Fig. 3 Results of the hierarchical STRUCTURE analysis of the studied noble crayfish populations. *K* refers to the number of inferred genetic clusters at each hierarchy level (see text for explanations). Ancestry of individuals is indicated by *bars* of different colour (*dark grey*, *light grey* or *white*), each corresponding to a different genetic cluster

populations from the North Sea, the Baltic Sea and the Black Sea catchments (Schrimpf et al. 2011). Schrimpf et al. (2011) also reported much higher haplotype diversity and private haplotype numbers in the Black Sea catchment than in the North Sea and the Baltic Sea catchments. Likewise, we found that the Black Sea catchment populations had significantly higher genetic variation and private allele numbers than the Baltic Sea catchment populations. Comparable latitudinal shifts in intraspecific genetic diversity have been observed for many terrestrial and freshwater species and are generally explained by founder effects and population size bottlenecks associated with rapid post-glacial expansion from southern refugia northward (e.g. Hewitt 1999; Bernatchez and Wilson 1998). Schrimpf et al. (2011) speculated that the results of their study could also be explained by post-glacial re-colonization of formerly glaciated areas in Central Europe from a putative common glacial refuge in the Balkan area, but did not exclude the effect of human translocations. Our sampling of the Black Sea catchment is not representative enough, but nevertheless, we would not exclude the possibility of re-colonization of the northern areas from more than a single refugium, since the Baltic catchment populations in our study possessed a total of 18 private alleles that were not found in the Danube drainage samples. Multiple glacial refugia have been suggested also for other native species of freshwater crayfish in Europe, e.g. the white-clawed crayfish, *Austropotamobius pallipes* Lereboullet (Santucci et al. 1997; Grandjean and Souty-Grosset 2000; Gouin et al. 2001, 2006). A more representative sampling of the whole distribution area in Europe is warranted for a comprehensive evaluation of large-scale population structure in the noble crayfish. Also, use of higher number of microsatellite loci or other types of nuclear DNA markers (SNPs, single copy sequences) in combination with longer mtDNA sequences would help to trace lineage histories with a higher confidence.

Population structure in the Baltic Sea catchment

Both approaches of population structure analysis (cladogram and STRUCTURE analyses) revealed structuring of the studied Baltic Sea catchment populations into three distinct genetic groups that corresponded generally well with their geographic origin (Figs. 2, 3). Despite several uncertainties regarding past stocking events (Table 1), the

overall correspondence between genetic and geographic distances in the Baltic Sea area suggests little impact of long-distance or between-country translocations. Some exceptions exist, however. The sample from Linkullasjön in Southern Finland, for instance, tends to be genetically more similar to Swedish rather than to the other, more northern, Finnish populations (Figs. 2, 3). It seems likely that the Linkullasjön population has a mixed or translocated origin because Finland has maintained close connections to Sweden for centuries and transfers of crayfish are known to have occurred across the Baltic Sea in both directions (Alm 1929; Edsman 2004). Another outlier from the general pattern of population structure was the sample from the Swedish island of Gotland in the Baltic Sea, which was markedly distinct in comparison to the other Swedish samples. It is unclear whether or not the noble crayfish is a native species to Gotland, the only translocations to the island that we are aware of came from the Swedish mainland during the 1800s (Skurdal et al. 1999). Our results suggest an admixed origin for this particular population. It is least differentiated from the Finnish Pitkäjoki and Koivujärvi populations and the Swedish Skellefteå and Skiringen populations (Table 4), it has an intermediate position between the Finnish and Estonian population groups on the genetic distance based cladogram (Fig. 2), and the STRUCTURE analysis suggested even a low proportion of the Central European gene pool while confirming the general affinity of the Gotland sample to the Finnish genetic cluster (Fig. 3). It is quite possible that the studied samples did not represent all of the potential source populations or regions for the Gotland stock and, for example, that the populations on the southernmost part of the Swedish mainland are more closely related to crayfish in Estonia or Finland. Additional screening of the noble crayfish populations in the Baltic Sea area will be needed to test this hypothesis. A scenario of more than one genetic lineage existing within Sweden cannot be excluded, however. Post-glacial re-colonization of the Baltic Sea area from multiple refugia has been suggested for several fish species (e.g. Nesbø et al. 1999; Nilsson et al. 2001; Koskinen et al. 2002; Säisä et al. 2005, 2010).

Large-scale releases are known to have occurred in the Skellefteå area during 1940–1960 using crayfish from northern Finland (Gydemo and Gydemo 1990). At the same time, almost all populations in northernmost Sweden and Finland are believed to be introduced (Gydemo and Gydemo 1990; Jussila and Mannonen 2004). Hence, the clear genetic difference observed between Skellefteå and the two samples from northern Finland (Kålen and Koivujärvi) is interesting. One explanation may be that the Skellefteå population is of Swedish origin (translocated or native) and that the subsequent stocking using Finnish crayfish has resulted in little or no genetic impact. Another possibility may be that genetic heterogeneity among the noble crayfish populations within Finland is more widespread than indicated in this study (cf. above), and that the Finnish stocking material used in the Skellefteå area was in fact genetically similar to the Swedish samples in the present study. To resolve this issue, screening of additional populations appears warranted.

Estonia was the only Northern European country in which sub-structuring of populations was revealed by both methods of data analysis: the populations clustered into southern (lakes Aheru and Tündre) and western/northern (streams Kuke, Laugi and Mustoja) Estonian groups (Figs. 2, 3). This within-country differentiation is probably due to limited gene flow and random genetic drift since these two population groups belong to different drainages (Fig. 1). Lakes Aheru and Tündre belong to the Gulf of Riga drainage (but are also known to be stocked by few crayfish from the Lake Peipsi drainage), while the Kuke and Laugi populations originate from island Saaremaa which was a crayfish plague free area without known introductions of crayfish from the mainland until just recent years (Paaver and Hurt 2009). River Mustoja population belongs to the Gulf of Finland drainage. Thus, a few known translocations of the noble crayfish in Estonia (Skurdal et al. 1999) seem not to have had any significant effect on the structure of wild populations.

Implications for conservation

Stocking of the noble crayfish for re-establishing extinct populations and supporting local fisheries is widespread. Currently, little or no attention is paid to the origin of the stocked individuals especially in respect to their genetic background. Factors such as geographic origin, levels of admixture, domestication, and inbreeding among the stocked crayfish would be important to know. For several reasons, the choice of stocking material may be highly relevant for management and conservation.

Following supplemental release using exogenous crayfish, hybridization among introduced and native individuals may result in reduced fitness and productivity caused by outbreeding depression (e.g. Tallmon et al. 2004). To date, however, virtually nothing is known regarding the links between genetic differention and local adaptation of the noble crayfish. Descriptive studies of morphological variation in the noble crayfish are surprisingly few, and allele frequency differences at (presumably neutral) microsatellites do not *per se* indicate presence of adaptive genetic differences, although reproductive isolation is a basic prerequisite for such differences to evolve. Future studies of local adaption are clearly needed, either using common garden studies of fitness-related traits, candidate gene approaches (e.g. Fitzpatrick et al. 2005; Hemmer-Hansen et al. 2007; Jeukens and Bernatchez 2012), large-scale

genomic surveys (e.g. Kawecki and Ebert 2004; Storz 2005; Hansen et al. 2010), or a combination of them.

Based on the results of this study, however, we strongly advise against translocations of the noble crayfish between different catchments (e.g. between the Baltic and the Black Sea catchments) and also between different countries (at least in Northern Europe where populations are well structured according to the country of origin). National restocking programmes which are initiated in response to widespread local extinctions of noble crayfish populations due to outbreaks of crayfish plague should be based on stocks from unaffected, nearby sources (with sufficient level of genetic variation) to ensure genetic similarity.

The largest threat to genetic diversity in the noble crayfish is the on-going loss of local populations due to continued spread of the crayfish plague, which in combination with stocking is expected to reduce the amount of genetic variation within the species as a whole (cf. Laikre and Ryman 1996). In Sweden alone, the number of local populations has decreased dramatically—from roughly 30,000 in the early 1900s to less than 1,000 in recent years (Edsman and Schröder 2009). Although the genetic consequences of this still on-going "extinction wave" remain largely unclear, a continued erosion of the genetic structure, including loss of unique alleles and allele combinations, may significantly reduce the species' ability to adapt to future environmental changes. Hence it is vital to halt the population decline also from a genetic perspective. Future restocking programs and supplemental releases should furthermore be based on empirical genetic data, following established conservation genetics principles.

From a conservation point of view, identification of priority populations for conservation (e.g. by means of genetic methods as described herein) should be the first step, followed by avoiding the introduction of alien crayfish species into those areas. Based on the data from this study, the genetically most distinct and variable populations within each of the identified genetic clusters should be considered for conservation to retain a maximum of the species′ genetic and evolutionary potential.

Acknowledgments We express gratitude to Mr. Margo Hurt for collecting samples from Estonian crayfish populations, to Dr. Pavel Kozák for providing Czech crayfish samples and to Mr. Hans Lindell for providing background information on Finnish crayfish stocks. The work by R. Gross, K. Kõiv and T. Paaver was financed by the Estonian Science Foundation (Grant No. 7348) and the Estonian Ministry of Education and Research (targeted finance grant SF1080022s07 and institutional research funding project IUT8-2). S. Palm acknowledges funding from the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS; Grant No. 215-2010-1235). L. Edsman and T. Prestegaard acknowledge financial support from the Swedish Board of Fisheries. J. Jussila, A. Karjalainen and H. Kokko acknowledge financing from the Ministry of Agriculture and Forestry and the strategic funding of the University of Eastern Finland.

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