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Mitochondrial cytochrome oxidase I gene analysis indicates a restricted genetic background in Finnish noble crayfish *(Astacus astacus)* stocks

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ABSTRACT

Key-words: *noble crayfish, native species, population decline, genetic diversity, cytochrome oxidase I*

The IUCN Red List indexes the noble crayfish *(Astacus astacus)* as vulnerable, with a declining population trend. The main threats to the species are the crayfish plague caused by the oomycete *Aphanomyces astaci* and the introduced North American crayfish that act as the carriers of this disease. In Finland, the noble crayfish is considered as a native species, which original distribution area covers the southern part of the country, but the species distribution has been dispersed to cover almost the whole country. The aim of this study was to survey the genetic diversity among the Finnish noble crayfish populations. The mitochondrial cytochrome oxidase I (COI)-gene was sequenced from 742 individuals representing 59 populations from Finland and Estonia. As a result, only a single haplotype was found. Based on these results, the genetic diversity of noble crayfish in its Northern distribution range is remarkably low. The observed lack of variation can result from several mechanisms including small size of the founder population and the intense spreading of the species by manmade stockings. The restricted diversity can also be caused by eradication of the original populations due to crayfish plague epidemics and spreading of the invasive crayfish species carrying the crayfish plague. It is also possible that all contemporary Finnish noble crayfish populations originate from stockings with no variation in respect to COI-gene.

RÉSUMÉ

L'analyse du gène mitochondrial du cytochrome oxydase I indique un fond génétique restreint dans les populations finlandaises de l'écrevisse à pattes rouges (*Astacus astacus*)

Mots-clés : *l'écrevisse à pattes rouges, espèces indigènes,*

La liste rouge de l'UICN inscrit l'écrevisse à pattes rouges (*Astacus astacus*) comme vulnérable, avec une tendance à la baisse de la population. Les principales menaces pesant sur l'espèce sont la peste de l'écrevisse, causée par l'oomycète *Aphanomyces astaci*, et les écrevisses nord-américaines introduites qui agissent comme porteurs de cette maladie. En Finlande, l'écrevisse à pattes rouges est considérée comme une espèce indigène, dont l'aire de répartition d'origine couvre la partie sud du pays, mais la répartition de l'espèce a été étendue et couvre presque tout le pays. Le but de cette étude était d'étudier la diversité génétique parmi les populations finlandaises d'écrevisses à pattes rouges. Le gène mitochondrial du cytochrome oxydase I (COI) de 742 écrevisses a été séquencé,

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déclin de population, diversité génétique, cytochrome oxydase I

représentant 59 populations de Finlande et d'Estonie. Finalement, un seul haplotype a été trouvé. Basé sur ce résultat, la diversité génétique de l'écrevisse à pattes rouges dans son aire de distribution septentrionale est remarquablement faible. Le manque observé de variation peut résulter de plusieurs mécanismes, dont la petite taille de la population fondatrice et l'intense propagation des espèces par déversements. La diversité restreinte peut aussi être causée par l'éradication des populations d'origine en raison de l'épidémie de peste des écrevisses et la propagation des espèces d'écrevisses invasives transportant la peste de l'écrevisse. Il est également possible que toutes les populations d'écrevisses à pattes rouges contemporaines finlandaises proviennent de repeuplements sans variation par rapport au gène COI.

INTRODUCTION

In the IUCN (International Union for the Conservation of Nature) Red List the noble crayfish *(Astacus astacus)* has been listed as vulnerable, with a declining population trend all over Europe (IUCN, 2014). The main threats to the species are the crayfish plague, the alien crayfish species and the modification and pollution of its natural environment (Souty-Grosset *et al.*, 2006). Due to the high amount of manmade crayfish translocations and stockings, the natural genetic structure of the species has been efficiently shattered, mixed and diminished in large parts of the Europe (Souty-Grosset and Reynolds, 2009; Schrimpf *et al.*, 2011, 2014). The narrow diversity may have affected its ability to survive in the changing environment. Nowadays, the determination of the evolutionary significant units (ESU's), including the maintenance of genetic variability and the possible locally adapted traits has been raised as key issues in the species conservation (Crandall *et al.*, 2009; Kozák *et al.*, 2011; Schrimpf, 2013). The maintenance of the variability improves the species possibilities to adapt to the changing environmental conditions (Kozák *et al.*, 2011) including the possibilities to resist new diseases. Recently, it has been recommended that the re-establishment programs which aim to restore the native crayfish in certain areas, should also take into account the genetic diversity and variability of the donor population (Souty-Grosset and Reynolds, 2009), but also, whenever possible, to use the locally adapted populations from a close geographic distance to retain the local diversity of the species (Souty-Grosset and Reynolds, 2009; Kozák *et al.*, 2011; Schrimpf *et al.*, 2011, 2014).

The crayfish plague *(Aphanomyces astaci)* has been reported to have entered the Finnish waters in the year 1893 (Järvi, 1910; Alderman, 1996). Soon after that, the lively crayfisheries were quickly destroyed along with the eradication of numerous productive noble crayfish populations from Southern Finland (Järvi, 1910; Kilpinen, 2003; Jussila and Mannonen, 2004). Following the epidemics, noble crayfish were intensively restocked to the collapsed lakes, but when the population densities reached the exploitable levels, the disease often hit again for unknown reasons (Fürst, 1995; Westman, 2000; Erkamo *et al.*, 2010). Therefore, it seemed that most of the commercially productive noble crayfish stocks were lost and the introduction and the dispersal of the signal crayfish *(Pacifastacus leniusculus),* with a suspected better disease resistance (Unestam, 1972, 1975), were initiated in Southern Finland during the 1960's (Westman, 1973, 2000). Nowadays, the signal crayfish inhabits the original distribution area of the noble crayfish, which in its part is being pushed towards the Eastern and Northern parts of Finland (Jussila *et al.*, 2015a).

On the basis of the fossilised findings (Souty-Grosset *et al.*, 2006), the noble crayfish is considered native species in Finland (Järvi, 1910; Lehtonen, 1975; Kilpinen, 2003). The original distribution area of the species covers the southern and central parts of the Finland (Järvi, 1910; Cukerzis, 1988; Skurdal and Taugbøl, 2002), the area being connected to the large freshwater basin, Lake Ancylus, after the first ice age (Sauramo, 1954). During the intense period of the noble crayfish trade and export in 1800's (Järvi, 1910), and due to its high economic value (Jussila and Mannonen, 2004), the species distribution was expanded to cover the whole country by manmade introductions (Westman, 1973, 1991).

Previous studies concerning the noble crayfish diversity in Finland (Alaranta *et al.*, 2006, 2011) have been based on the microsatellite-like repeat located in the internal transcribed spacer 1-region (Edsman *et al.*, 2002), or on the microsatellites on a limited number of populations (Gross *et al.*, 2013). On the European wide scale, also mitochondrial cytochrome oxidase I (COI) gene and 16S rDNA-gene have been utilized to explore the biogeography and diversity of the species (Schrimpf *et al.*, 2011, 2014). The main aim of this study was to determine the genetic diversity among the Finnish noble crayfish populations on a wider scale and to compare the results to the published data from Europe. The second aim was to find out, if the repeated crayfish plague epidemics and subsequent crayfish stockings have affected the noble crayfish diversity among the wild populations in this region. The mitochondrial DNA (mtDNA) was chosen, since it has been already shown to encompass diversity (Schrimpf *et al.*, 2014) and perhaps the results would also allow tracing the dispersal of the noble crayfish in this region.

MATERIALS AND METHODS

> *SAMPLING*

We sampled 57 noble crayfish populations from Finland and two populations from Estonia (Table I). The crayfish were provided by the local crayfishermen as part of their commercial catch and the collection was organised in collaboration with the local Fisheries Advisory Centers. Among the Finnish populations, there were 17 populations located in the original distribution area of noble crayfish below the 61st latitude (Figure 1). The area is nowadays mainly colonized by the signal crayfish. The rest of the populations were collected from eastern and northern parts of Finland, where the noble crayfish populations have been established by the stockings. From each population, 10–30 individuals with equal number of females and males when possible were collected for the analyses (Table I). The walking legs from each individual were stored into 70% ethanol for the further DNA extractions.

> *MOLECULAR ANALYSES*

The DNA from the samples collected in 2004 (Table I) was extracted as described by Alaranta *et al.* (2006). For the latter part of the sample set, EZNA Insect DNA Isolation Kit (Omega Bio-Tek) or Insect DNA Kit (Zymo Research) were applied. The DNA extractions were made from the proximal joints of walking legs containing both cuticle and muscle tissue. The tissue was disrupted with ceramic beads in TissueLyser II (Qiagen) in presence of the lysis buffer and the DNA extractions were then made according the manufacturers' protocols.

PCR was carried out using primers ASTCOIf and ASTCOIr (Schrimpf *et al.*, 2011, 2014) in 25 μL reaction volume containing 1 U of Dream*Taq* DNA polymerase (Thermo), 2X Dream*Taq* Green master mix (Thermo), 10 mM of both primers and 10–100 ng of template DNA. The reaction volume was filled with PCR-grade water. The amplification was conducted in PTC-200 thermal cycler (MJ Research) in following conditions: 95 °C, 3 min, 35× (95 °C, 30 s; 53 °C, 30 s; 72 °C, 30 s) and 72 °C 10 min. Each 96-well plate contained a positive control (crayfish DNA) and a blank reaction without a template.

The amplification was randomly checked in 1.5% agarose gel containing 0.5 μM EtBr and the samples were then sent for ExoSAP-IT purification and sequencing to the GATC Biotech, Germany, where the Sanger sequencing reactions were performed with the primer ASTCOIf.

> *DATA ANALYSIS*

The resulting sequence data were manually checked and edited in Geneious version 7.4 (Kearse *et al.*, 2012). A consensus sequence of the observed haplotype was entered into the

Table I

The sampled noble crayfish populations, their locations, sampling years and the years of crayfish plague epidemics, if known ([1] NR = no reported cases of the crayfish plague or population collapses).

Location (Lake, Town)	Sampling Year	Crayfish Plague History[1]	*n* of individuals sequenced
Pond Ahvenlampi, Kitee	2004	NR	30
Lake Ala-Kintaus, Petäjävesi	2004	1964–1969	10
Lake Ala-Siili, Pieksämäki	2004	1950	10
Crayfish farm Angla, Estonia	2004	NR	9
Lake Eväjärvi, Längelmäki	2013	1960, 1970	10
Lake Horonjärvi, Vesanto	2004	NR	30
Lake Hosusjärvi, Valkeala	2004	1965	10
Lake Ilvesjärvi, Keuruu	2004	NR	10
Lake Iso-Lauas, Kuopio	1995	1996, 2000	10
Lake Jokijärvi (Murhinniemi), Taivalkoski	2004	NR	3
Lake Jokijärvi (Romppasensalmi), Taivalkoski	2004	NR	10
Lake Jokijärvi (Suojala), Taivalkoski	2004	NR	9
Lake Jokijärvi (Yläniva), Taivalkoski	2004	NR	22
River Karvianjoki, Kankaanpää	2014	NR	10
River Kasijoki, Kuopio	2004	NR	10
Lake Kitere, Ristiina	2004	2000	9
Lake Koantaus, Simpele	2013	1970	8
Lake Koivujärvi, Kiuruvesi	2004	NR	10
Lake Kärkjärvi, Rapattila	2013	NR	10
Lake Köyliönjärvi, Köyliö	2004	1909-1939	30
Pond Likolampi, Parikkala	2013	NR	10
Pond Linkullasjön, Inkoo	2004	NR	25
Lake Luvanjärvi, Hyrynsalmi	2009	NR	6
Lake Mikitänjärvi, Hyrynsalmi	2011	Several	10
Lake Mikkolanjärvi, Orivesi	2004	NR	10
Lake Mäntyjärvi, Kaavi	1997	2009	10
River Nuottijoki, Hyrynsalmi	2011	NR	8
Lake Oulujärvi (Kaivannonsalmi), Oulu	2009	Several	10
Lake Oulujärvi (Vaala), Oulu	2009	Several	9
River Pajakkajoki, Kuhmo	2004	2009	30
River Pajakkakoski, Kuhmo	2009	2009	7
Lake Peipsi, Estonia	2004	NR	10
River Perhonjoki, Kokkola	2004	1962, 1963, 1968, 1977	25
Lake Pienrautjärvi, Parikkala	2013	1990	9
River Pisankoski, Nilsiä	2004	1972, 1976	9
River Pitkäjoki (Leppävesi), Toivakka	2004	NR	10
Pond Pohjanlampi (Jännevesi), Jännevirta	2004	NR	10
Lake Pyhtäänjärvi, Laukaa	2004	NR	9
Lake Pyhäjärvi, Pyhäjärvi	2004	1956, 1998	10
Lake Riihijärvi, Toivakka	2004	NR	10
Lake Rytky, Kuopio	2004	NR	10
Lake Saarinen, Tervo	2004	NR	10

Table I
Continued.

Location (Lake, Town)	Sampling Year	Crayfish Plague History[1]	*n* of individuals sequenced
Lake Saimaa, Savitaipale	2004	1893	23
River Siilinjoki, Siilinjärvi	2004	NR	10
River Sinettäjoki, Rovaniemi	2013	NR	9
River Sirkkakoski, Pello	2004	NR	9
Lake Suuri-Heinäjärvi, Kitee	2004	NR	10
Lake Suuri-Pölläkkä, Heinävesi	2004	NR	9
Lake Suuri-Vahvanen, Mikkeli	2004	NR	7
Lake Säiniönjärvi, Hirvensalmi	2004	NR	10
Lake Valkeinen, Kuopio	2004	NR	10
Pond Valkeinen, Kuopio	2004	NR	10
Lake Venesjärvi, Kankaanpää	2013	1970, 1990, 2000	10
Lake Viitajärvi, Tervo	2004	NR	10
Lake Ylijärvi, Ylijärvi	2013	2000	9
Lake Ylimmäinen, Laikko	2013	2000	10
Lake Ylä-Kintaus, Petäjävesi	2004	1999	10
Lake Ylä-Luotojärvi, Kerimäki	2004	NR	30
Lake Ylä-Säynätjärvi, Mikkeli	2004	NR	10

NCBI GenBank database with access number KP892529. A maximum likelihood tree of the whole Finnish sequence set was conducted in Geneious 7.4 (Kearse *et al.*, 2012) with PhyML function (Guindon *et al.*, 2010) using HKY85 substitution model. *P. leniusculus* COI-sequence (JF437995) was selected as an outgroup.

RESULTS

The 350 bp region of mitochondrial cytochrome oxidase I (COI)-gene was sequenced from 742 noble crayfish individuals representing 59 different noble crayfish populations from Finland and Estonia. As a result, a single haplotype, which corresponds to the haplotype Aas01_COI (KF888296), was found. The maximum likelihood tree (Figure 2) and the multiple alignments both showed, that there was no variation at all in this data set. When the sequence comparisons were made with European data, it was observed, that the Aas01_COI was the most common haplotype in Northern parts of Central Europe and the only haplotype observed in Finland.

DISCUSSION

We have studied the genetic diversity of noble crayfish *(A. astacus)* in Finland, which represents the most northern distribution area of the species (Souty-Grosset *et al.*, 2006; Kouba *et al.*, 2014). The present study represents a large dataset from noble crayfish mitochondrial COI-gene, which is one of the five most commonly used coregenes employed to evaluate the diversity of crustacean species (Bybee *et al.*, 2011; Owen *et al.*, 2015). The results obtained in this study show, that the genetic variation in the northernmost distribution area of noble crayfish is either severely diminished, or never even existed. The results are in line with the previous studies conducted with smaller sample sets (Schrimpf *et al.*, 2011, 2014). Schrimpf *et al.* (2014) found two combined haplotypes, Hap01 and Hap07 from Finnish noble crayfish stocks, although the amount of the studied individuals was very small, containing ten individuals from two populations (Lake Ylä-Kintaus, Petäjävesi (Kymijoki basin) and Pond Valkeinen, Kuopio (Vuoksi basin)). The populations in question were also included in this study (Table I)

Figure 1
A map of Finland showing the original distribution area of noble crayfish (Astacus astacus) as grey, and the locations of the sampled populations. The locations with two overlapping symbols are marked with an asterisk.

However, the differences observed in Finnish populations by Schrimpf *et al.* (2014) were located on the 16S mtDNA-gene instead of COI-gene, and the results based on COI-haplotype were identical.

The COI-haplotype Aas01_COI, detected in this study, was the most common haplotype also in the whole European-wide noble crayfish material (Schrimpf *et al.*, 2011, 2014). Among the European noble crayfish populations, the haplotype diversity observed in the COI-gene was considerably higher than on the 16S mtDNA-gene, although in general, 16S mtDNA gene has been reported to be the most variable marker for freshwater crayfish, but it was also stated, that the result might be partially caused by smaller samples sizes for COI-gene (Fezner and Crandall 2001; Crandall *et al.*, 2009). In addition, the intraspecific variation in the COI-gene seems to be highly variable, between 0.0–4.6% (da Silva *et al.*, 2011). In our study, COI-gene

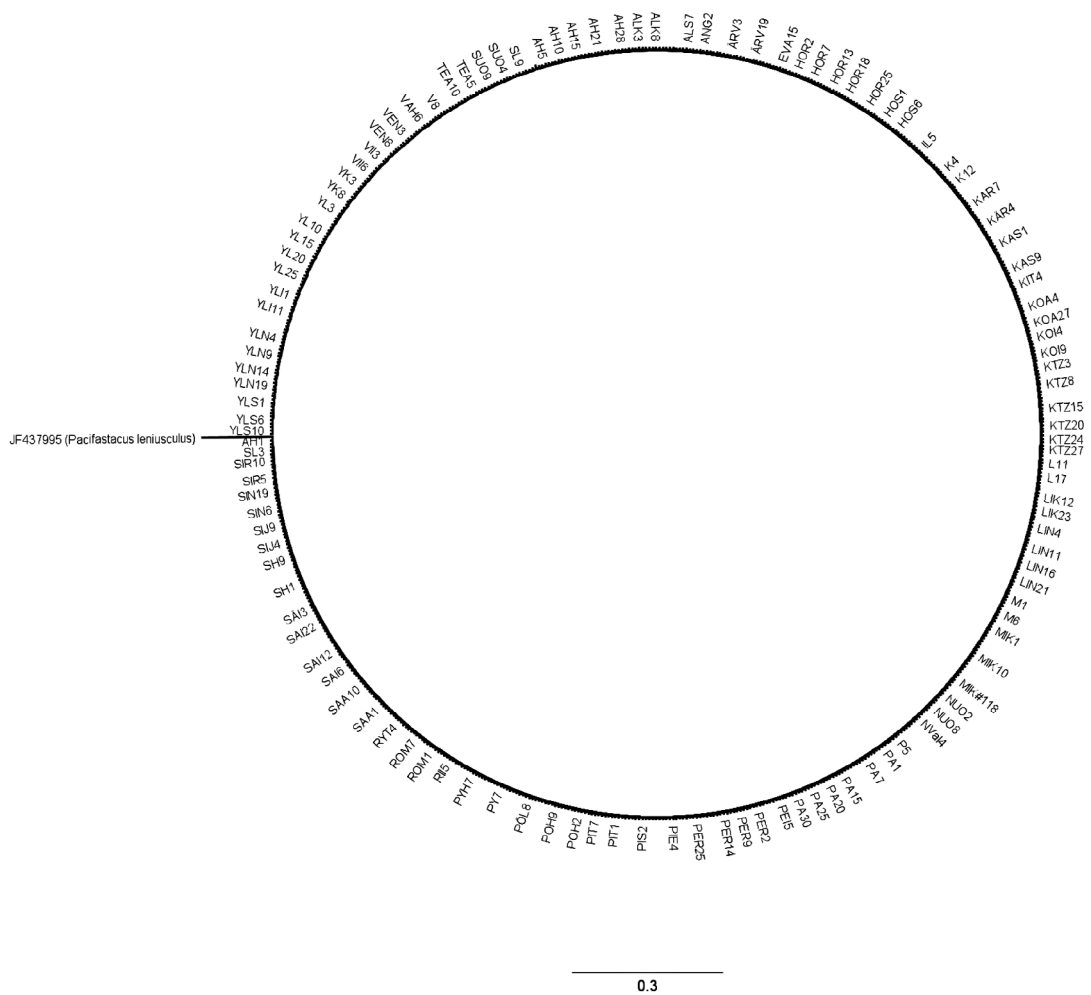

Figure 2
Maximum likelihood (ML) -tree of the sequenced COI-region showing the haplotype diversity among Finnish noble crayfish (Astacus astacus) populations. The ML-tree of the whole Finnish sequence set was conducted in Geneious 7.4 with PhyML function, using HKY85 as a substitution model. The tree was rooted with Pacifastacus leniusculus COI-gene (JF437995).

was selected as a target instead of 16S mtDNA gene based on its higher variability among the noble crayfish populations of Europe, the species at issue also in our study. Based on the previous studies, we expected it to be the more variable mitochondrial target, even though a single SNP was previously observed in 16S mtDNA data among Finnish noble crayfish populations (Schrimpf *et al.*, 2014). The zero variation detected in this study could also be a result of a short (350 bp) region sequenced in this study. However, the European data was conducted with the same primer pair and therefore, it was expected that the selected region should show some variability.

Amplification of nuclear mitochondrial pseudogenes (numts) is a well-known phenomenon complicating the mitochondrial diversity analysis and COI-gene based DNA barcoding (Nguyen *et al.*, 2002; Zhang *et al.*, 2008; Schizas 2012). Numts have been observed from several crustacean species (Buhay, 2009; Bybee *et al.*, 2011; da Silva *et al.*, 2011), the main cause being false diversity in certain species. During this study, the sequencing data was manually checked and approximately 10% of the sequencing results were excluded due to low quality chromatograms that appeared randomly in all studied populations. In most of the cases the messy chromatograms appeared in the middle part of the sequence, while the

beginning and the end parts were both clear and readable. These kind of amplification errors could likely be caused by COI-like nuclear pseudogenes. However, with the zero level variation observed in this data set, numts cannot be considered as a source of false variation.

We will speculate on four possible reasons for the observed low diversity among Finnish noble crayfish populations in our study. The first possible reason could be a small founder population of noble crayfish in the Lake Ancylus (Sauramo, 1954) after the last ice age. As the mitochondrial DNA is maternally inherited, and its evolutionary rate is usually lower compared to nuclear DNA, the low diversity observed in this study may therefore reflect a low diversity, or a low number of individuals in the founder population. Furthermore, due to the low evolutionary speed of the mitochondrial DNA it may not even show the possible local adaptations which have happened after the first ice age. Although the calibrated molecular clock is yet unavailable for the noble crayfish, the rate for the mitochondrial evolution in general seems to be very similar in different species with different generation times, being roughly 1–2%/site/My (Stoeckle *et al.*, 2014). Therefore, nuclear markers, like microsatellites could provide more information of possible adaptive lineages and diversification of the noble crayfish populations in Finland (Gross *et al.*, 2013). The microsatellite markers exploited in small scale for Finnish noble crayfish populations showed, that variation in the nuclear DNA level exists also in Northern populations of *A. astacus* (Gross *et al.*, 2013).

The second reason for the limited diversity could be the effect of the crayfish stockings, since the noble crayfish was spread from its original distribution area in Southern Finland all over the country by human induced introductions (Järvi, 1910; Westman, 1973, 1991). The intense stockings were initiated when the value of the noble crayfish trade was discovered during 1800's, and obviously, during that time the genetic diversity was not taken into account when the crayfish were spread all around the country. The distribution area of the species was quickly spread towards its northern limit in the Lapland (Westman, 1973, 1991). In several cases, the basic information about the stocking events, *i.e.* the origin and the amount of stocking material, is completely lacking. Occasionally, when some information is available it often describes that a small number of stocklings, even less than 100 individuals, have been utilised as a founder of a new population (Westman, 1991; Kilpinen, 2003). A small number of stocklings have also been listed as one main reason for the low stocking success (Erkamo *et al.*, 2010). These kinds of stocking events have also likely had an impact in the intraspecific diversity.

As a third reason for the low genetic diversity we suggest the crayfish plague epidemics, which have destroyed numerous noble crayfish populations in Finland (Järvi, 1910; Jussila and Mannonen, 2004) and all over Europe (Alderman, 1996; Souty-Grosset *et al.,* 2006). The first wave of the disease, which started at 1890's, may have caused a loss of diversity and destruction of the original haplotypes that were present in the original distribution area of the noble crayfish. In addition, the destruction of the productive noble crayfish populations initiated a new wave of stockings, since the collapsed commercially valuable populations were commonly and frequently tried to re-establish (Erkamo *et al.*, 2010). Among the 59 studied populations, 19 noble crayfish populations located inside the noble crayfish original distribution area. There were 23 populations with past crayfish plague epidemics, but there were also 36 populations with no reported cases of population collapses or crayfish plague epidemics. Among the non-collapsed populations, 11 populations were located in the original distribution area of the noble crayfish and could thus be considered as autochthonous. However, the information of the possible stockings and crayfish plague epidemics of these populations was also imprecise and partially incomplete. Therefore, even those populations cannot be safely considered as original. Nevertheless, in these populations the diversity parameters based on mtDNA COI-gene were as low as they were in the any other examined populations.

Furthermore, the fourth reason for the low diversity are the large scale introduction and stockings of signal crayfish, which were started in 1970's and since then, a new wave of crayfish plague have been threating the remaining noble crayfish populations in Finland and in Europe. Nowadays the original distribution area of noble crayfish in Finland (Figure 1) is mainly inhabited with signal crayfish populations (Westman, 1991; Kirjavainen and Sipponen, 2004)

causing disappearance of the noble crayfish within that region due to the crayfish plague (Viljamaa-Dirks *et al.*, 2013), while the noble crayfish remains as stocked populations in the northern and eastern parts of Finland (Kirjavainen and Sipponen, 2004; Jussila *et al.*, 2015a, 2015b).The introduced alien crayfish species, together with the crayfish plague disease they are carrying, are acting as a constant risk factor that weakens the survival chances of the native European crayfish species. Richman *et al.* (2015) have recently stated that although approximately 30% of the world's crayfish species are threatened, the European crayfish species are facing the greatest number of risks and the invasive crayfish species are one of the main reasons for this situation.

We conclude that the genetic diversity of noble crayfish in its Northern distribution range is remarkably low. The observed low variation can be caused by several reasons, one could be the small size of the founder population, since the diversification, which happened after the last ice age, may not yet be visible in the mitochondrial DNA. The second reason is the fact that most of the noble crayfish populations existing nowadays originate from manmade stockings. The reason for the large scale stockings is the eradication of the original populations due to the crayfish plague epidemics. The crayfish plague has thereby had its effect on the noble crayfish on two ways: first, it has collapsed a huge number of noble crayfish populations, and then it also launched the stockings of the noble crayfish. The crayfish plague was also the main reason for the initiation of the signal crayfish introduction and spreading, which is today making the situation of noble crayfish even more difficult. Further studies are needed to determine the genetic regions and methods suitable to evaluate the population diversity parameters in these geographical regions in order to reach out the most suitable populations for the conservation efforts of this species in its northernmost distribution area.

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