Knowledge and Management of Aquatic Ecosystems (2016) 417, 13
© L. Skuza et al., published by EDP Sciences, 2016
DOI: 10.1051/kmae/2015046

www.kmae-journal.org

Knowledge & Management of Aquatic Ecosystems

Journal fully supported by Onema

Research Article Open Access

Molecular characterization of the noble crayfish (*Astacus astacus* L.) population from Pomeranian lakes (north-western Poland) based on mitochondrial DNA

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Received June 15, 2015 – Revised December 27, 2015 – Accepted December 31, 2015

Abstract – The genetic variability between individuals from five crayfish (*Astacus astacus* L.) populations was determined. The analysis was based on sequences variations of mitochondrial DNA (cytochrome oxidase subunit I (*COI*) and 16S ribosomal DNA. Mitochondrial DNA sequences are widely used to detect genetic variation within and between populations. Data analysis revealed the existence of two COI haplotypes – most common haplotype Hap01 and one new haplotype, differed only in one substitution. Analysis of the 16S rDNA sequences obtained showed no differences in nucleotide composition. The results of the analysis are important part of project "Active protection of noble crayfish in lakes of Pomeranian Complex Landscape Parks" financed by Financial Mechanism of the European Economic Area 2014–2016.

Key-words: *Astacus astacus* / genetic diversity / mtDNA / haplotype / Pomerania lakes

Résumé – Caractérisation moléculaire de la population écrevisse à pattes rouges (*Astacus astacus* L.) des lacs de Poméranie (nord-ouest de la Pologne) à partir de l'ADN mitochondrial. La variabilité génétique entre les individus de cinq populations d'écrevisses à pattes rouges (*Astacus astacus* L.) a été déterminée. L'analyse a été basée sur les variations de séquences de l'ADN mitochondrial (sous-unité de la cytochrome oxydase I (*COI*) et l'ADN ribosomal 16S. Les séquences d'ADN mitochondrial sont largement utilisées pour détecter une variation génétique au sein et entre les populations. L'analyse des données a révélé l'existence de deux haplotypes COI – l'haplotype le plus commun Hap01 et un nouvel haplotype, différant en une seule substitution. L'analyse des séquences d'rDNA 16S n'a montré aucune différence dans la composition des nucléotides. Les résultats de l'analyse sont une partie importante du projet « Protection active de l'écrevisse à pattes rouges dans les lacs des Parcs Paysagers Poméraniens » financé par le Mécanisme Financier de l'Espace Economique Européen de 2014 à 2016.

Mots-clés : *Astacus astacus* / diversité génétique / ADNmt / haplotype / lac de Poméranie

1 Introduction

The freshwater crayfish in Poland are represented by two crayfish species, the noble crayfish, *Astacus astacus* Linnaeus 1758, and the narrow-clawed crayfish, *Astacus leptodactylus* Eschscholtz 1823. It is the largest freshwater invertebrate (Holdich, 2002) and very sensitive bioindicator of changes in the water environment (Gondko *et al.*, 1992). Unfortunately in over the last two decades they have diminished in numbers in Polish lakes and rivers (Śmietana, 2001; Śmietana *et al.*, 2004; Schulz *et al.*, 2006a). An essential prerequisite for the protection of species is the knowledge of genetic variations within and between remaining populations (Avise, 2004; Riffel and Schreiber, 1995; Haig, 1998) and the protection should be carried out by restocking the species (Strużyński and Śmietana, 1999; Śmietana, 2001; Schulz *et al.*, 2006a).

For describing the genetic structure of noble crayfish a variety of methods have been applied: allozymic variation (Fevolden *et al.*, 1994), RAPD (Schulz, 2000), microsatellite length variation in the rDNA ITS1 region (Edsman *et al.* 2002), ISSR (Schulz *et al.*, 2004) as well as mtDNA *COI* gene analysis (Schrimpf *et al.*, 2011, 2014).

Mitochondrial DNA is powerful instrument to detect genetic variation within and between populations. For this reason

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two most popular mitochondrial region were used as a phylogeographic markers: cytochrome oxidase subunit I (*COI*) and 16S ribosomal DNA. Mitochondrial cytochrome oxidase I (*COI*) gene has recently gained more attention in developing DNA barcodes for species identification and biodiversity analysis, while the 16S rDNA is often used for studies at middle categorical levels such as in families or genera (Gerber *et al.*, 2001).

Śmietana (2013) in his research indicates that the populations studied belong to the strongest populations (evaluated by population density – catch per unit effort (CPUE) – Table 1) of the species found in the waters of the Polish lakes (Figure 1). Due to the dramatic rate of extinction the species in the recent years on this area (Śmietana, 2013) the results of the research are of essential importance for the development of strategy restitution of noble crayfish in Polish lakes. At present in this area a species restoration programme entitled "Active protection of the noble crayfish in lakes of the Pomeranian Complex of Landscape Parks" financed by Financial Mechanism of the European Economic Area 2014–2016 is being implemented. The project focuses on a detailed inventory of water bodies that meet the requirements of effective restitution of noble crayfish and restocking new populations with the use of semi-natural breed YOY.

Taking into account the above, the aims of the present work were: the study of the current genetic structure of Polish populations of noble crayfish through the use of a fragment of mitochondrial *COI* and 16S rDNA genes and propose, based on the results, areas for conservation of *A. astacus* in Poland.

2 Materials and methods

2.1 Sample

Specimens of *Astacus astacus* L. (N = 50) were collected from 5 locations from NW Poland: Babinki (BA), Biwakowe (BI), Graniczne (GR), Kwisno (KW) and Sęki (SE) situated in the Bytów Lakeland using the free diving method in the autumn period in 2014 (Figure 1). The samples (the fifth pair of walking legs) were stored in 75% ethanol until DNA extraction and the animals were returned to the place where they were caught.

2.2 DNA isolation

DNA was extracted from the muscle tissue using High Pure PCR Template Preparation Kit (Roche Diagnostics). Both quality and concentration of the DNA were assessed by agarose gel electrophoresis and spectrophotometry (NanoDrop 2000; Thermo Scientific).

2.3 Amplification and sequencing

A fragment from the mtDNA *COI* and 16S rDNA gene were amplified using the same composition of the reaction tubes in a final volume of 25 µL with 1× FastStart PCR Master (Roche), 0.2 µM of each primer and 40 ng template DNA. The primers used for amplification of *COI* were COI-F ('GCGGGGATAGTAGGAACCTC') and COI-R (5'ATTTACCGCCCCTAAAATCG') (Schrimpf *et al.*, 2011), while the primers used for amplification of 16S rDNA were 16S-F (5'CCTGTTTANCAAAAACAT3') and 16S-R 5'AGATAGAAACCAACCTGG3') (Crandall *et al.*, 1996).

The optimal PCR programme for *COI* amplification included an initial denaturation (at 95 °C for 2 min), followed by 35 cycles of denaturation at a temperature of 95 °C (for 45 s), annealing primers at 50 °C (for 45 s) and extension at 72 °C (for 1 min) and a final extension step of 72 °C for 5 min.

The optimal PCR programme for 16S rDNA amplification included an initial denaturation (at 95 °C for 3 min), followed by 30 cycles of denaturation at a temperature of 95 °C (for 1 min), annealing primers at 42 °C (for 1 min) and extension at 72 °C (for 1.5 min) and a final extension step of 72 °C for 1.5 min. PCR products were checked by electrophoresis in 1.8% agarose gel containing ethidium bromide and a TBE buffer (pH 8.0); the gels were visualized under UV.

The PCR products were purified and sequenced on both strands by Genomed (Poland) using the PCR primers. The sequence of the new haplotype reported in this paper has been deposited in the GenBank nucleotide sequence database with the accession number KT072746.

2.4 DNA alignment and sequence analysis

The nucleotide sequences were aligned using CLUSTALW software (Thompson *et al.*, 1994). After alignment, the final sequence length used was 352 bp for *COI* gene and 490 bp for 16S rDNA gene. The genetic diversity estimates (haplotype diversity, H; nucleotide diversity, π) were calculated using DnaSPv5.10 programme (Rozas *et al.*, 2008).

The sequences obtained from this study were analyzed in the same data set with other sequences deposited in GenBank (*Astacus astacus* KF888296 - KF888325 (*COI*), *Pontastacus leptodactylus* KC789393 (*COI*) and a sequence of *Pacifastacus leniusculus* AY151519 (*COI*) as a outgroup.

Neighbour-Joining (NJ) method was used for phylogenetic reconstructions using the "*p*" distance. The NJ analyses were conducted on MEGA version 6.06 (Tamura *et al.*, 2013).

3 Results

Amplification with the *COI* and 16S rDNA primers pairs produced, in all the samples, 352 bp size products and 490 bp size products, respectively. Sequence analysis within 50 individuals of *A. astcus* from 5 Polish populations revealed 1 substitution in *COI* fragment. Only two haplotypes were detected in the *COI* gene region – the most common haplotype Hap01 and one unique haplotype (NH) at low frequency (accession number KT072746), which was very similar to haplotype Hap01 and differed only in one position (221), were a substitution (G to A) occurs.

The species *A. astacus* show a haplotype diversity of 0.3333 and a nucleotide diversity of 0.00095. The highest haplotype and nucleotide diversity were found in the population Babinki (BA), where two different haplotypes were

Table 1. Samples of *A. astacus* (10 individuals per population) studied in the present work. Columns show respectively: code, population, locality and region, haplotypes found, number of polymorphic sites (S), haplotype diversity (Hd) and nucleotide diversity (π) for *COI* analysis.

	Code	Population size CPUE	Lake name	Haplotypes found	Number of sites polymorphic (S)	Haplotype diversity (Hd)	Nucleotide diversity (π)
1	**BA**	2.14	Babinki	2	1	0.3556	0.00091
2	**BI**	0.94	Biwakowe	1	0	0.0	0. 0
3	**GR**	3.99	Graniczne	1	0	0.0	0.0
4	**KW**	1.45	Kwisno	1	0	0.0	0.0
5	**SE**	0.39	Sęki	1	0	0.0	0.0

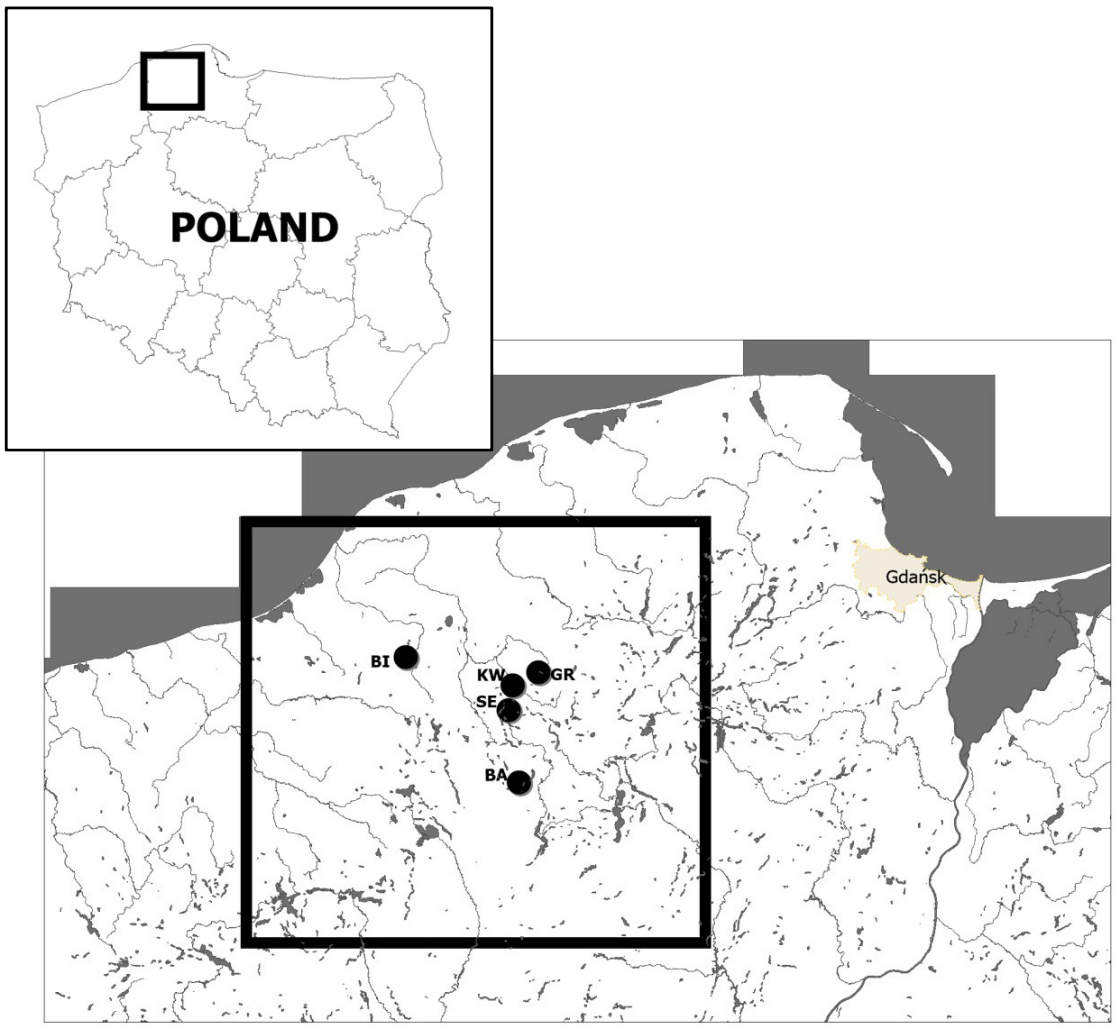

Fig. 1. Sampling locations of Polish noble crayfish included in this study. BA – lake Babinki, BI – lake Biwakowe, GR – lake Graniczne, KW – lake Kwisno, SE – lak Sęki.

detected-Hap01 and NH (only in 2 of 10 individuals) (Table 1). The remaining 4 Polish populations were monomorphic.

Cluster analysis of *COI* indicated a division of the studied species in two groups (Figure 2). The first group includes all analysed populations, while the second group corresponds to individuals from BA population carrying the new haplotype (NH) described.

Analysis of the 16S rDNA sequences obtained showed no differences in nucleotide composition. Only one haplotype across all analysed crayfish populations was found, 100% homologous with Aas01 16S ribosomal RNA gene (GenBank: KF888279).

To show the position of NH among other described haplotypes, sequences deposited in GenBank of *COI A. astacus* were included (Figure 3). Cluster analysis of 30 *COI* haplotypes described by Schrimpf *et al.* (2014) indicated a division of the studied species in two groups. One includes 5 Croatian haplotypes (Hap41, Hap43-46), while the second

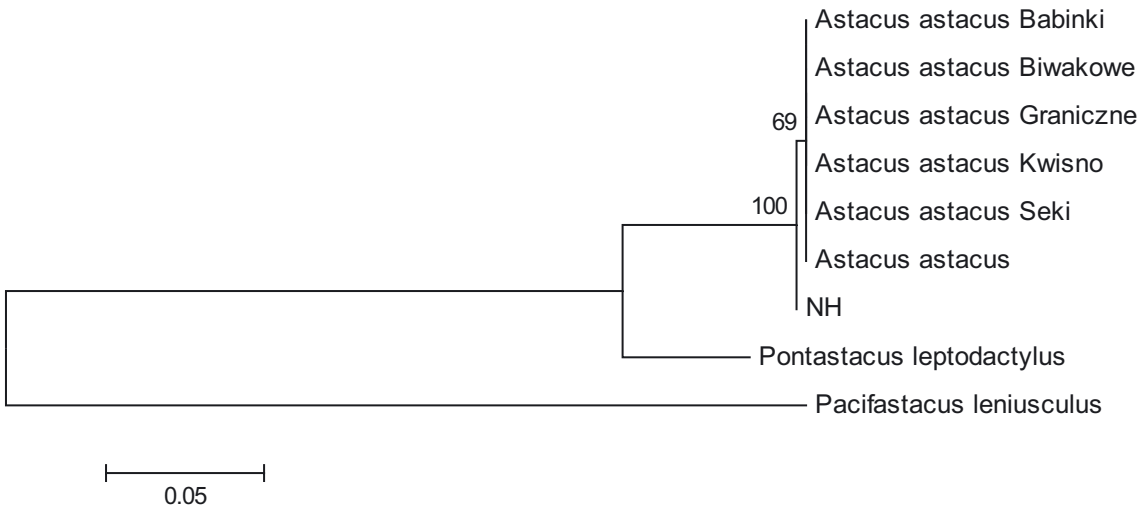

Fig. 2. Neighbor-Joining tree based on "*p*" distances showing populations studied based on the COI data, constructed using MEGA 6.06 software. NH – new haplotype.

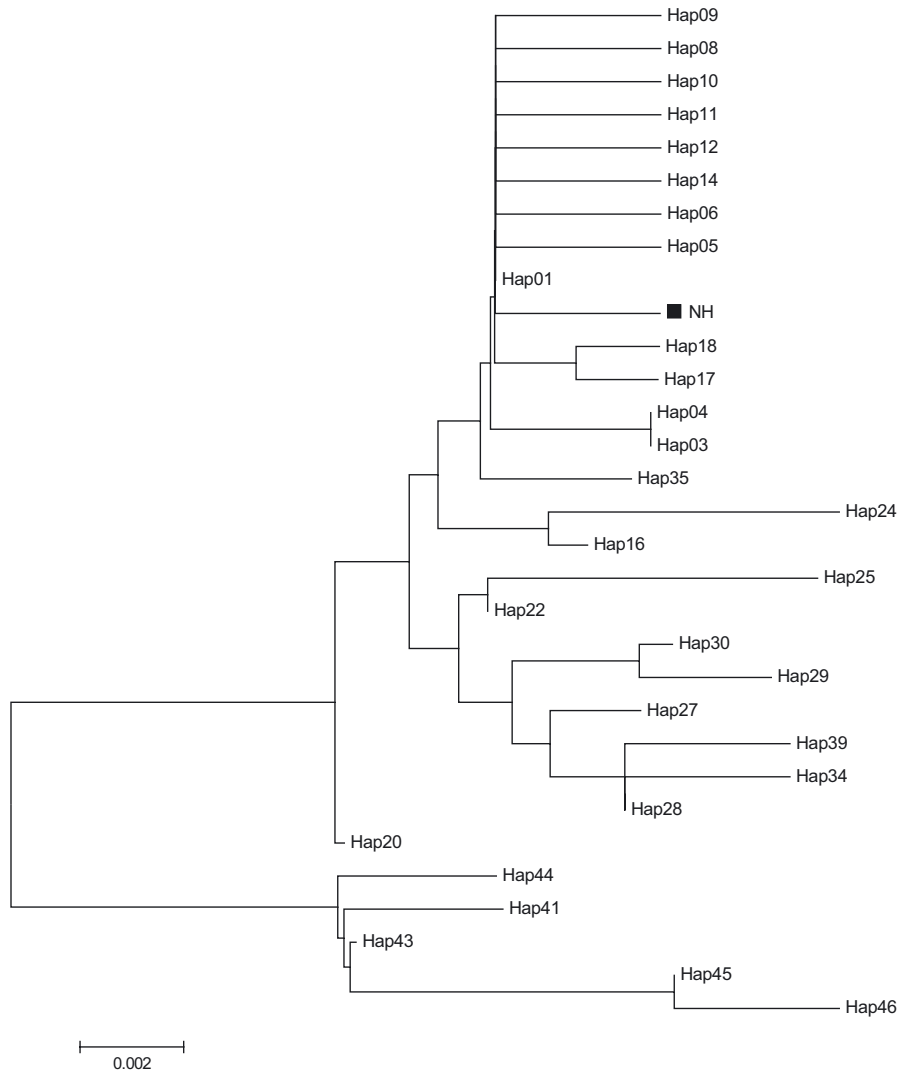

Fig. 3. Neighbor-Joining tree based on "*p*" distances showing *COI*-haplotypes of *A. astacus* (Schrimpf *et al.*, 2014) along with New Haplotype (NH) generated using MEGA 6.06 software.

group the remaining haplotypes.A separate subgroup formed Hap20 from Romania. New hyplotype is grouped together with haplotypes from Germany (Hap05-06, Hap08), Bulgaria (Hap11-12, Hap14), Romania (Hap10) and Hungary (Hap09). This group includes also the most common haplotype Hap01.

4 Discussion

Protection of the native biodiversity is the major aim of conservation biology (Frankham *et al.*, 2004). The knowledge of genetic variations within and between remaining populations is an essential prerequisite for the protection of species (Avise, 2004; Riffel and Schreiber, 1995; Haig, 1998). The genetic analysis of the surviving populations in terms of their internal diversity, illustrating the level of adaptation to the environment and also is necessary to choose best parents for crayfish which become restocking material.

While the stone crayfish and white-clawed crayfish species complex have already been subject to molecular studies little is known about molecular structure of noble crayfish. Early genetic studies using allozyme and RAPD markers showed low genetic diversity within noble crayfish populations (Fevolden, 1994; Schulz, 2000), while the genetic distances based on ISSR markers revealed significant differences between most of the populations examined (Schulz *et al.*, 2004). Research conducted in the present study indicate very low level of diversity. The 16S rDNA gene showed no difference between all the *A. astacus* from Poland, probably due to a slower rate of variation for this marker than *COI*, as has been observed in previous studies with other organisms (Trontelj *et al.*, 2005; Verovnik *et al.*, 2005; Zaccara*et al.*, 2004). Furthermore, only two haplotypes were detected based on *COI* gene analyzing.

Similar results were obtained in a study by Schrimpf *et al.* (2011). The authors showed the relatively low level of the variation in noble crayfish *COI*-sequences. The study presents the results of evidence of the genetic diversity in European populations of noble crayfish, where the highest haplotype diversity and a highest number of private haplotypes were detected for the Black Sea populations, while the central European populations are significantly less variable. Absence of mtDNA differentiation and haplotype diversity would be expected if all populations came from one recent introduction event and from a very reduced number of populations or even only one population (Miura *et al.*, 2006; Uwai *et al.*, 2006; Pedraza-Lara *et al.*, 2010).

The area of Pomerania and, in particular, the Bytów Lakeland is characterized by high heterogeneity of the land, which is one of factors which influence a high level of biodiversity (Schulz *et al.*, 2006). This area was characterized also historically low population density, which makes the anthropogenic introductions relatively rare. For this reason, despite occurring relatively close to each other, subpopulations of aqueous species may be characterized originally by high genetic diversity. This may explain the occurrence of the unique haplotype in the lake Babinki, which hasn't been described in the literature so far (accession number KT072746).

Similar conclusion reached Schrimpf *et al.* (2014), who analyzed noble crayfish from 156 sampling locations. Based on *COI* and 16S rDNA the authors indicates that some areas show a distinct genetic structure with endemic haplotypes indicating that these areas were refugia for *A. astacus* and that these populations have not been subject to anthropogenic translocations.

Unique haplotype (NH) described in this paper appeared to be very similar to 8 others from Danube and Rhine (Figure 3), also described by Schrimpf *et al.* (2014) and to most common haplotype Hap01, which indicate a common origin of these haplotypes.

Information provided previously by tests on the stability of population in the lakes under analysis partly confirmed the genetic results described in this paper. While analysing anthropogenic factors which have the greatest influence on the occurrence of crayfish in this area, Śmietana (2013) concluded that the distance of the lake location from the nearest town/village and the number of inhabitants in the direct vicinity of the body of water are directly related. The assessment of population parameters of the noble crayfish population in selected lakes expressed by average value of the CPUE (Stuecheli, 1991; Dorn and Wojdak, 2005) showed that the most numerous populations occurred in Lake Graniczne and a slightly lower density was observed in Lake Babinki (Table 1). Graniczne Lake and Babinki Lake were situated the furthest from human settlements (27.90 km and 18.8 km, respectively), thus being the least exposed to penetration (Śmietana, 2013).

Based on the results population from Babinki Lake could be proposed as a good area for conservation of *A. astacus* in Poland.

With respect to both analysed mtDNA sequences, *COI* gene resulted more sensitive for detecting genetic variability than 16S rDNA. *COI* is a powerful marker for the study of the genetic variation at the intraspecific level in crayfish (Shull *et al.*, 2005; Versteegen *et al.*, 1997) and other crustaceans (Haye *et al.*, 2004; Meyran *et al.* 1998) because its rate of molecular evolution is about threefold greater than that of 16S rDNA gene (Knowlton and Weigt, 1998).

Mitochondrial DNA is a widely used marker to reconstruct the phylogeographic history of species. Here, we show that the analysis of partial *COI*-sequences helps to understand the genetic structure of noble crayfish. However, we realize that research should be extended and additional DNA sequences and populations should be included in the analysis to increase the resolution.

The results provide information about the structure of the studied populations that allow for the selection of parents for breeding restocking material. They will be used in terms of the development of strategies aimed at restoring the distribution of the lowlands populations of an endangered crayfish species in northern Poland.

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Cite this article as: L. Skuza, S. Keszka, R. Panicz and P. Śmietana, 2016. Molecular characterization of the noble crayfish (*Astacus astacus* L.) population from Pomeranian lakes (north-western Poland) based on mitochondrial DNA. *Knowl. Manag. Aquat. Ecosyst.*, 417, 13.