

Introduction

The *Perccottus glenii* species – nowadays a representative element of the Odontobutidae family due to its high invasion capacity – is also known as the Amur sleeper. The species represents a fresh water fish which gained the scientists' interest even from the beginning of the 20th century, for its status of non-native species (Terlecki and Pałka, 2012). Later, a special attention was given to its invasion within a wide area in a very short period, the features which facilitated its spread being particularly investigated.

Up to present, the majority of the studies destined to the invasive *Perccottus glenii* monitoring focused on the geographic, biologic and ecologic aspects, in order to build an image as clear as possible about the *Perccottus glenii* potential of representing a risk factor for the environment. Its fast spread to the West of Europe drove the emergence of some prophylactic programs in countries where the species has not even been identified yet (Verbrugge et al., 2012; Verreycken et al., 2013). In spite of the danger it represents for the fresh water ecosystems and the significant volume of morpho-physiological and ecologic data obtained so far for the *Perccottus glenii* species, its genetic profile is a subject scarcely approached.

Research purpose

This paper approaches for the first time the genetic aspects regarding the invasiveness of *Perccottus glenii* species and its main purpose is to investigate the adaptability and spread molecular mechanisms of *Perccottus glenii* invasive populations within catchment basins on Romanian territory.

Objectives

1. To estimate the genetic variability level within and between populations for *Perccottus glenii* individuals from the catchment basins of Siret and Tisa rivers;
2. To estimate the phylogeographic origin of *Perccottus glenii* invasive populations from the Siret and Tisa catchment basins;
3. To quantify the gene flow between the *Perccottus glenii* populations analysed.

THE INTRODUCTORY PART of the present paper covers two chapters, which aimed at familiarizing the readers with few details regarding both the ecology and morphology of *Perccottus glenii* species, and the meaning and use of molecular markers and statistical analysis for the results discussion.

Chapter 1. The biologic profile of *Perccottus glenii* species – a threat for fresh water ecosystems

1.1. General overview of the invasion process

The biotic invasions represent the result of a process which comprises several steps, and which starts once the organisms are transported from their native area into new habitats.

When a species reaches a non-native area, through deliberate or non-deliberate introduction, it becomes an introduced or non-native species (Keller et al., 2011).

A second step in the invasion process occurs when a species survives in the new habitat and manages to reproduce without human control, becoming a stable species (Keller et al., 2011).

Finally, the sum of more events, connected particularly to the impact of non-native species both on the habitat and the local fauna, as well as on the human communities, but also their wide spread make the non-native species become invasive (Iacob and Petrescu-Mag, 2008).

1.2. *Perccottus glenii* – a fast colonizing species on a wide area

The main area the species *Perccottus glenii* was introduced from is the Amur River, in the East of Asia, covering part of Russia, China, and North Korea (Bogutskaya and Naseka, 2002). The native area comprises also the streams of some Yellow Sea tributaries, extending in the East up to the North-West of the Shakalin Island (Reshetnikov, 2013). The oldest record of *Perccottus glenii* introduction in the East of Europe dates from 1912.

After 2005, the presence of the Amur sleeper was recorded in Romania in the Siret river basin (Nalbant et al., 2004), the Danube Delta (Năstase, 2008), in the Danube river, at the Iron Gates (Popa et al., 2006), and in the catchment basins of the Bega and Mureş rivers (Covaciu-Marcov et al., 2011; Copilaş-Ciocianu and Pârvulescu, 2011). It was also pinpointed in other European countries: Moldova (Mosu et al., 2007), Croatia (Caleta et al., 2010), Latvia (Pupiņa and Pupiņš, 2012), Estonia, Finland, Bosnia and Herzegovina, and Slovenia (Reshetnikov, 2010).

Up to 2008, the Amur sleeper was already registered in over 36 Russian provinces, 12 European countries (Latvia, Lithuania, Estonia, Byelorussia, Ukraine, Moldova, Poland, Slovakia, Hungary, Serbia, Bulgaria, Romania) and three Asian countries (Kazakhstan, Uzbekistan and Mongolia), (Reshetnikov, 2010).

1.3. *Perccottus glenii* – the morphophysiological traits favouring the establishment and spread of the species in non-native habitats

Perccottus glenii is a small fish, the mature individuals reaching about 6-8 cm in length. The maximum weight and length of this species – 250 g, respectively 25 cm, – was recorded in a non-native area, at the age of about 10 years (Reshetnikov, 2004). Due to these features, the fish can be easily transported by mistake, alongside other species, for aquaculture purposes (Reshetnikov and Schliewen, 2013).

Perccottus glenii reaches sexual maturity very early, after the first year of life, and males offer parental care to their offspring, which, according to some scientists, is another strategic behaviour for better survival of an invasive species (Grabowska et al., 2011).

The species' resistance to a wide temperature spectrum gives the Amur sleeper the possibility to survive to higher water temperatures, while at low temperatures the fish manages to hibernate.

Perccottus glenii lives mainly in waters with low oxygen concentration, where other predator species, like perch, bleak, roach, goldfish or carp cannot survive (Caleta et al., 2010). This way, the number of natural enemies decreases significantly.

THE EXPERIMENTAL PART of this thesis comprises two wider chapters, 3 and 4, which describe the biologic material and the methods used for the analysis carried out within this study, as well as the results and discussions.

Chapter 3. Material and methods

3.1. The biologic material analysed in this study

Within this study, a number of 125 *Perccottus glenii* individuals were subjected to DNA analysis, the majority from the Siret catchment basin. Apart from this area, the study also included individuals captured from the Tisa River, near the Tiszafüred locality, on the Hungarian territory, as well as individuals from the Mureş catchment basin, from the Silvia Channel.

To isolate and purify the genomic DNA, muscular tissue was sampled for all *Perccottus glenii* individuals.

The sample preservation affected significantly the efficiency of the DNA extraction, irrespective of the protocol used. Thus, three kinds of samples were analysed:

1. Individuals fixed and preserved in ethanol shortly before the DNA extraction;
2. Individuals preserved in ethanol over five years before the DNA extraction;
3. Individuals preserved in formalin between 1 and 13 years.

3.2. Analysis methods

Before being subjected to molecular analysis, the *Perccottus glenii* individuals belonging to the two populations (Siret and Tisa) were morphometrically investigated. Several phenotypic parameters were considered: Lt = total length, Ls = standard length; C = circumference; Hd = maximum height; Ha = minimum height; Lc = the length of the head; Gr = thickness; g = weight.

According to the preservation method (formalin or ethanol), different protocols were used for the DNA isolation and purification, based on phenol: chloroform: isoamyl alcohol extraction, and based on the DNA cohesion to the resin particles or silica membrane, respectively.

After DNA extraction, sample processing continued with the PCR reaction for the mitochondrial control region and the *cyt b* gene, using primers particularly designed in the laboratory. The PCR results were tested through the agarose gel electrophoresis, and the successfully amplified samples were then purified and sequenced.

Furthermore, the data was statistically interpreted using DNA-SP 5, Arlequin, STRUCTURE v.2.3.4, CLUMPP v1.1, Distruct v1.1 and Network.

Chapter 4. Results and discussion

4.1. Analysis of phenotypic parameters

The statistical analysis of the phenotypic parameters indicated different values between the Siret and Tisa populations overall.

According to the discussions presented in the 3.3.2 chapter, the formalin and the ethanol, when used as preservation liquids, have certain effects on the biologic material, both molecularly and morphometrically. The morphologic changes are caused by the ethanol-induced desiccation of tissues.

4.2. DNA quantification for the samples chosen for the genetic analysis

During the first step of molecular analysis, DNA extraction one, I noticed – according to the literature (Carter, 2003) – that using the same extraction protocol, the final DNA concentration is lower for the formalin preserved samples than for the ethanol preserved ones.

4.3. Discussions upon the sequencing results of the mitochondrial control region and *cyt b* gene

4.3.1. The genetic profile of *Perccottus glenii* individuals from the catchment basins of the Siret and Tisa rivers, for the mitochondrial control region

For the mitochondrial control region, a total sequence length of 904 bp was obtained. These included the total D-loop region and a small fragment of the ARNt. Within the 51 analysed individuals, two haplotypes, differentiated through one transversion, were identified.

4.3.2. Estimate of D-loop haplotypes divergence for the invasive *Perccottus glenii* individuals from the Siret and Tisa catchment basins, compared to native individuals

The three nucleotide sequences which can be found so far on the internet database described two different haplotypes, which also differ from the haplotypes identified for invasive *Perccottus glenii* individuals from the Siret and Tisa catchment basins.

The two haplotypes described for the native individuals are separated by six mutations, three of which are transitions and the other three – transversions.

4.3.3. The genetic profile of *Perccottus glenii* individuals from the catchment basins of Siret and Tisa, for the *cyt b* gene

The analysis of the *cyt b* gene, with a total length of 1173 bp, also described two haplotypes for all of the invasive *Perccottus glenii* individuals, sampled from Siret and Tisa catchment basins. The two haplotypes differ through seven transitions.

4.3.4. Estimate of the *cyt b* haplotypes divergence for the invasive *Perccottus glenii* individuals from the Siret and Tisa catchment basins, compared to native individuals

To investigate the origin and the potentially different genetic evolution of the invasive *Perccottus glenii* analysed in this study, from a native source population, the analysis proceeded with testing the origin of the two non-native *cyt b* haplotypes, identified in the Siret and Tisa basins, to the native haplogroups. Thus, a haplotypes network was built, based on a 112 sequences data set, which included, apart from the 61 haplotypes identified for the native *Perccottus glenii* individuals, the two haplotypes described for the invaded Siret and Tisa catchment basins. The result showed that the non-native *cyt b* haplotypes form two separate haplogroups, with an independent evolution compared to the five native haplogroups.

4.3.5. The efficiency of molecular markers chosen to quantify the genetic variability of *Perccottus glenii* species by taxonomic level

The relevance of genetic variability on a lower taxonomic level is better highlighted through a comparative analysis, which includes the higher taxonomic levels for a certain taxon analysed.

The analysis carried out for the Perciformes order shows the fact that the *cyt b* gene has a greater discrimination capacity for the lower taxa, starting with the species and continuing with the family, and even the suborder. Instead, the mitochondrial control region is a better molecular marker for the genetic differences quantification within the order.

4.3.6. Genetic diversity of *Perccottus glenii* invasive population from the Siret basin compared to the one from the Tisa basin

The *cyt b* haplotypes were identified for the same individuals for which the D-loop haplotypes were described. That is why the two DNA fragments were merged, to obtain in the end two different haplotypes, with a total length of 2077 nucleotides. The two haplotypes were described for the entire set of individuals sampled from the Siret and Tisa catchment basins. Even if they can be reached in both populations, their frequency per population differs, mainly due to significant differences in the number of individuals from the two analysed populations.

4.3.7. Genetic structure and gene flow between the *Perccottus glenii* populations, from the Siret and Tisa catchment basins

The graphic output of genetic clusters admixture for the two invasive populations, Siret and Tisa, shows the absence of any different genetic structure between them. Two genetic clusters were identified for both populations, in a different percentage, proportional with the two haplotypes frequency described for the Siret and Tisa populations.

4.3.8. Population diversity, demographic expansion and spatial expansion for *Perccottus glenii* individuals from the Siret and Tisa catchment basins

The greater numbers of individuals for the Siret population compared to Tisa population, as well as the presence of the same two haplotypes in both populations, induce a higher level of genetic diversity in the Tisa population. Thus, the genetic diversity parameters, like the mean value of the nucleotide pairwise differences, the nucleotide diversity or the expected heterozygosity has greater values in the Tisa populations, compared to the Siret population. The statistical AMOVA analysis also described a higher level of variation within each population (86.86 %) than between populations (13.14%). At the same time, the F_{st} value (0.13141) approaches the 0 value, which suggests a significant genetic similarity between the two analysed invasive populations.

Gemographic expansion

The graphical representation of the demographic expansion for the two analysed populations showed a bimodal observed distribution and a unimodale expected distribution. This suggests a present stability of *Perccottus glenii* populations in the non-native habitats of Siret and Tisa catchment basins, as well as a potential of increasing their number of individuals.

Spatial expansion

According to the previous studies (Ray et al., 2003), a wide spatial expansion of a population may lead to a result similar to the demographic expansion, if there is an intensive exchange of individuals between the demes of that specific population. This was also noticed in the analysis of Siret and Tisa population spatial expansion.

4.3.9. Population diversity, demographic and spatial expansion for *Perccottus glenii* individuals from the Siret and Tisa catchment basins, compared to the native ones

Heterozygosity is quantified through values ranging from 0 (when it does not exist) to 1 (in a data set with a big number of alleles with the same frequency). In this study, the bigger number of loci with different allele frequencies was identified for *Perccottus glenii* individuals from the Amur catchment basin. Nevertheless, the different allele frequencies per locus determined a mean value of expected heterozygosity much lower than the values estimated for the other catchment basins, where there are fewer polymorphic loci, with a more uniform allele frequency.

The genetic within and between populations was estimated through the AMOVA analysis, which showed variation percentages almost equal within populations (48.47%) and between populations (51.53%). These genetic differences were also confirmed by the F_{st} value (0.51529), which suggests a balance between the shared allele frequencies and the allele frequency for each population.

After comparing the graphics of the demographic and spatial expansion of the four populations, it was found that only the Korea-Coast population showed a unimodal observed distribution, while the other three populations had bimodal (Bo-Hai and Danube populations) or multimodal (Amur

population) observed distributions. The unimodal distribution for the Korea-Coast catchment basin suggests a recent demographic expansion of this population (Slatkin and Hudson 1991; Rogers and Harpending 1992). The bimodal or multimodal distributions of the three populations show the different evolution of certain mitochondrial DNA fragments in populations in a state of demographic balance.

General inferences of the thesis

The analysis carried out in this study led to emphasizing on several aspects:

➤ The sampled individuals were preserved in different liquids (ethanol and formaldehyde) which influenced later the samples analysis. Thus, was ascertained that *Perccottus glenii* individuals preserved in formaldehyde presented morphometric differences compared to the samples preserved in ethanol, as well as a damaged DNA, modified both quantitative and qualitative, and inadequate for the molecular genetic analysis.

➤ Two molecular markers were used – the mitochondrial control region and the *cyt b* gene – with a different taxonomical relevancy for describing the evolutionary genetic differences of *Perccottus glenii* species.

➤ The sequences alignment for both mitochondrial markers described two different haplotypes.

➤ The two haplotypes frequencies differ between the Siret and Tisa catchment basins.

➤ The demographic and spatial expansion of *Perccottus glenii* individuals from Siret and Tisa basins showed a bimodal distribution, suggesting a demographic balance and a stasis expansion, with a future possibility of increasing the population number of individuals and migration.

➤ The haplotypes network – which included both the *Perccottus glenii* individuals from our territory and the 61 haplotypes described on NCBI for the *cytb* gene – showed the fact that the two haplotypes identified for the invasive *Perccottus glenii* individuals formed two different haplogroups, with a separate evolution compared to the one of native individuals.

➤ The invasive *Perccottus glenii* populations present a mean heterozygosity value that is higher than the one of native populations from the Bo-Hai and Amur catchment basins.

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