Importance of Determining Genetic Code In Crayfish and Methods Used

Zübeyde HANOL BEKTAŞ iD

Fisheries Research Institute,32500, Eğirdir, Isparta, Turkey

ABSTRACT

Crayfish are one of many freshwater organisms that are subject to biodiversity changes. The crayfish stocks in the world and Turkey have been damaged due to the crayfish plaque caused by *Aphanomyces astaci*, overfishing water pollution and other causes. For the continuation of endangered stocks of these species, it is necessary to protect the populations and restocking into apropriate environment. Environmental factors have an vital role in the genetic structure of a population. So the genetic diversity between population is vital because the information of the genetic construction of population is curical due to the ensuring sustainability and conservation of these species when evaluated with other factors but, unfortunately, still restricted. With this review, the genetic study conducted so far will be discussed to try getting important information for preservation and management of crayfish, because preservation of genetic diversity is very important for survival or persistence of these species such a long time.

Keywords: Genetic diversity, fisheries, protection, biodiversity, molecular identification

Kerevitlerde Genetik Şifre Belirlemenin Önemi ve Kullanılan Yöntemler

Öz: Kerevitler biyoçeşitlilik değişimlerine maruz kalan birçok tatlı su organizmalarından biridir. Dünyada ve Türkiye'de kerevit stokları *Aphanomyces astac*i fungusunun neden olduğu hastalık, aşırı avcılık, su kirliliği ve diğer nedenlerden tükenme tehlikesiyle karşı karşıya kalmıştır. Stokları tehlikede olan bu canlıların devamı için populasyonların korunması ve uygun çevrelere yeniden stoklanması gerekmektedir. Çevresel faktörler bir populasyonun genetik kompozisyonunda çok önemli rol oynar. Bu yüzden populasyonlar arasındaki genetik çeşitlilik büyük öneme sahiptir çünkü populasyonların genetik yapılarının bilinmesi bu türün devamının sağlanması ve korunması açısından hayatidir fakat ne yazıkki bu konuda yapılan çalışmalar hala sınırlıdır. Bu derlemeyle şimdiye kadar yapılan genetik çalışmalar tartışılarak kerevitlerin korunması ve yönetimi için önemli bilgiler elde edilmeye çalışılacaktır çünkü genetik çeşitliliğin korunması bu türlerin uzun süre yaşaması ya da varlığını sürdürmesi için çok önemlidir.

Anahtar kelimeler: Genetik çeşitlilik, su ürünleri, koruma, biyoçeşitlilik, moleküler tanılama

Introduction

Importance of crayfish

In this review, the situation of the freshwater crayfish group, a water organism found in every continent, is discussed, 59% of these species found in North America, 23% of in Oceania (Southeast Asia Islands), 10% in South America, 5% in Europe and 1.1% in Asia with only 7 species. Also 1.4% in Madagascar with 9 species. But the other phylogenetic studies argues that there are only 4 species in Asia (Koizumi et al. 2012) and are widely regarded as keystones of habitats which they found. There are more than 600 crayfish species belong to

the three family that have different distribution over the world. In Turkey the crayfish species which have natural distribution in natural lakes, reservoirs, dam lakes, ponds and rivers is *Astacus leptodactylus* Esch., 1823. It is reported that this species represented by a single and two sub-species in Turkey. While *A. leptodactylus leptodactylus* Esch., 1823 have distribution in the Black Sea, North Marmara and Thrace Regions; Iznik and Terkos Lake with the Maritsa and the Danube River and Gelemen stream *A. leptodactylus salinus* Nordman, 1842 in South Marmara, Aegean and Central Anatolia Regions; Manyas, Eğirdir, Beyşehir, Uluabat,

Akşehir, Eber, Gölcük and Miliç lakes (Geldiay and Kocataş 1970; Harlıoğlu and Harlıoğlu 2006). Also Harlıoğlu and Güner (2007) had reported *Austropotamobius torrentium* (Shrank, 1803) presence in Madara Brook.

Crayfish are valuable animals economic and ecologically. They have a significant conneciton in the aquatic food chain in many streams and lakes. (Helfrich and DiStefano 2009). Crayfish have an important environmental role in many freshwater habitats and play an important economic and cultural role in many communities. Unfortunately, in many parts of the world, some crayfish species in freshwater ecosystems are under pressure and efforts should be made to protect them. Owen et al. (2015) reported that crayfish is a highly endangered component of these freshwater ecosystems and that more than 30% of the world's species are at risk of extinction.

The factors threatening the crayfish populations

People have been changing the dispersion of species over the years, they not only causing extinctions, but also causing species to migrate through biogeographic obstacles and into new environments regardless of the geographic and genetic resources of stock material (Clavero et al. 2015). The population is declining and the extinction of species takes place at an unprecedented rate all over the world (May 2010). There are more than 79,800 species on The IUCN Red List, and more than 23,000 are danger of extinction, including 41% of amphibians, 34% of conifers, 33% of reef building corals, 25% of mammals and 13% of birds. The extinction occurs in marine, freshwater and also in terrestrial systems. Numbers of described threatened species by big groups of organisms were given in Table 1. (Cook et al. 2008).

Table 1. Numbers of threatened species by big groups of organisms (IUCN Red List version 2017-1)

	Estimated Number of described species	Number of species evaluated by 2017
VERTEBRATES		
Mammals	5,560	5,560
Bird	11,121	11,121
Reptiles	10,450	5,473
Amphibians	7,635	6,533
Fishes	33,500	16,134
INVERTEBRATES		
Insects	1000000	6,912
Molluscs	85.000	7.276
Crustaceans	47.000	3.177
Corals	2.175	864
Arachnids	102.248	249
Velvet Worms	165	11
Horseshoe Crabs	4	4
PLANTS		
Mosses	16.236	102
Ferns and Allies	12.000	417
Gymnosperms	1.051	1.011
Flowering Plants	268.000	20.725
Green Algae	6.050	13
Red Algae	7.104	58
FUNGI AND PROTISTS		
Lichens	17.000	8
Mushrooms	31.496	25
Brown algae	3.784	15

Researches shows that freshwater environments occupy only 0.8% of the Earth, with about 6% of species identified and under pressure from water pollution, flow modification, habitat fragmentation and exotic species. These habitat breaks cause high susceptibility to speciation, habitat destruction and limited disintegration capacity. The combination of these factors increases the rate of rapid disappearance. So, freshwater ecosystems are vital in accordance to biodiversity, but are under severe pressure and therefore are in need of conservation efforts (Ricciardi and Rasmussen 1999; Owen et al. 2015). The freshwater species that in particular risk in many part of world are crayfish.

In the 19th century the natural crayfish have been depleted dramatically rapidly due to the emergence of crayfish plaque and led to the extinction of natural species in many parts of Europe. All domestic European crayfish species have experienced strong declines over the last century, mainly due to the outbreak of crayfish plague, a disease caused by oomycete *Aphanomyces astaci* (Clavero et al. 2015). It is clear that the population of the world has decreased and the extinctions of species have occurred in an unprecedented proportion. Despite the fact that extinction is a natural phenomenon, evidence has shown that the rates of extinction at present are greater than in the past and possibly attributable to human actions. Since we can not fully understand the current and past biodiversity levels, it is difficult to predict the rates of extinction accurately (Furse et al. 2012). In Turkey, the crayfsh population are also affect from crayfish plaque. Crayfish plaque disease problem is true for Turkish population. Harlıoğlu (2004) had reported that after 1985, the yield of *A. leptodactylus* decreased tragically in many Turkish populations as a result of crayfish plague disease. In 1991, the harvest was only 320 tonnes.

In 2010, freshwater crayfish were evaluated in the IUCN Red-Threatened Species List and evaluating 528 of them, indicating that crayfish were among the 5 most threatened groups of animals in the world.

The crayfish species under risk of extinction are *Austopotamobius pallipes* (Lereboullet), *Astacus astacus* (Linnaeus), *A. leptodactylus* (Eschscholtz), *Austropotamobius torrentium* (Shrank) (Fetzner 2011). Recent threats include global climate change results increased environmental temperature (Furse et al. 2012), and rising density and compactness of variable weather events.

Low capacity in the natural distribution of crayfish and inhibition of gene flow through habitat alteration results strong/severe fragmentation of remaining populations. Therefore to prevent destruction of natural stocks, it is required to take measures such as protection of natural habitats and stocking of appropriate water resources.

The other major causes in the endangered of crayfish are environmental factors, low population numbers, small geographic limits and habitat loss.

Our objective in this review is to examine, present and discuss the applications of existing molecular genetic methods to protect economically valuable freshwater crayfish. Examples will be given of how these techniques are applied in freshwater crayfish, particularly in determining the levels of genetic diversity and how the obtained data can be used to maintain these very important species. Because the first thing to consider in order to protect species is to know the genetic structure or diversity. That is, higher genetic diversity means more survival, and more successful results can be obtained if protection methods are taken in this direction.

Genetic Methods Used To Determining The Genetic Differences of Crayfish

Many national and international organisations and states make investment for the protection of this important aquatic organism. Although this organism studied many years, in genetic sence the obtained data from study which has started recently may have an critical role for the protection of crayfish (Gouin et al. 2006; McKniff 2012).

Molecular methods have been used to determine the levels of genetic diversity in a species, data requirements in stocking studies, estimation of effective population sizes, and determination of disease susceptibility (DeSalle and Amato 2004; Furse et al. 2012). Potential uses of molecular methods encompasses a wide range of applications, and the methods used to determine the genetic diversity of crayfish have been discussed under the following headings.

Since the mid of 1980s, the scientist have tried to determine the genetic structue of crayfish. Different methods have been used for this. The first methods were depend on protein electrophoresis that revealed a low variation between Europen crayfish (Fevolden et al. 1994). In recent years, the molecular methods have been applied in crayfish successfully and revealed high diversity.

For determining genetic differentiation of populations, there are several studies such as mitochondrial DNA (in genetic analysis the mitochondrial genoms are perfect targets because they have no introns, reduce the recombination and in the haploid mode of inheretence), ISSR, ITS1(Largiader et al. 2000; Li et al. 2012; Matallanas et al. 2012) RAPD PCR (Schulz 2000) and microsatellites (Gouin et al. 2002).

Mitochondrial DNA markers

Because of the maternal mode of heredity and lack of recombination, mtDNA was a commonly used genetic methods for investigation of population structure (Wilson et al. 1985; Avise et al. 1987).

Koizumi et al. (2012) investigated the genetic structure of Japon crayfish. They determined that the most of populations composed of 16S mtDNA haplotypes and showed important genetic divergence (Fst=0,96) and also the nuclear DNA sequences showed deep seperation between strains. However the mtDNA has advantages in population genetic structure study, it has some disadvantages also. The

first represents only one locus, and the deduced gene trees may be incompatible with organism phylogeny (Avise 1994). Resistance to a single genetic locus greatly reduces the significant spatial or temporal detection power. Second, mtDNA allows only reconstruction of maternal lines. Thus, in species-based populations, the population structure of maternal hereditary mtDNA may be different from the biparental heritable nuclear energy.

Inter simple sequence repeats (ISSR)

From the used genetic methods the inter-simple sequence repeats (ISSRs) are a new molecular marker of PCR amplification of DNA by a single primer consisting of a repeating sequence fixed by 2-4 arbitrary nucleotides at the 3 'or 5' end, and have been used successfully many in populations genetic studies. Schulz et al. (2004) have been investigated 8 *A. astacus* stock using ISSR-PCR in Germany and Poland. They have been search 22 ambigious and polymorphic markers using statistical analysis. They found that the polymorphic loci in a population ranged from 4-19. The determined relative genetic diversity between population ranged from 0.6-0.1 by Shannon index. For this reason, it has proven that ISSR markers are appropriate for assessing DNA variations between and within the population and for producing a significant distinction in most of the stocks.

ITS1 fragment analysis

ITS1 fragment analysis, one of the other molecular methods, is a relatively simple tool to study genetically distinct crayfish populations and to determine the origin of these populations. Despite that, the ITS1 fragment variation can be used to investigate differences between populations, as documented in a study carried out by Liu et al. (2013) investigated the population genetic structure of *Pracambarus clarkii* in China using ITS1. They reported that diversity within population (%95.26) was higher than diversity between population (%4.74) using AMOVA. Genetic differences between Taiwan and the environment ($F_{ST} = 0.160$) was medium while difference between China and America populations ($F_{ST} = 0.682$ and 0.977) were significantly higher. Gene flow between China and American populations ($N_m = 0.006$ and 0.117 respectively) were significantly lower than China and the environment (1.536). In another study, Edsman et al. (2002) investigated 15 *A. astacus* population with ITS1 markers and they found that different population had different form of division.

RAPD (Random amplified polymorphic DNA) PCR

RAPD-PCR has some advantages over other techniques used for population analysis. (RFLPs and microsatellites). For example, RAPD-PCR is relatively faster, simpler and cheaper to conduct. Besides, it requires a small amount of DNA to form and provide a large number of polymorphic loci; Both are particularly valuable when working with populations of species that are danger of extinction or low genetic diversity RAPD-PCR has many advantages as well as some disadvantages. Of course, the most important shortcoming of RAPD-PCR is the dominant nature of the bands created by this method. In relation to this, Zhang et al. (2015) indicated that it is impossible to distinguish a heterozygous locust from a dominant RAPD marker in a DNA segment amplified from a homozygous locust (Zhang et al. 2005). Schulz (2000) examined the genetic structure of 5 *A.astacus* stock using RAPD-PCR. Although geographic proximity of these 5 stock less than 20 km the genetic structure of all of them were different.

Cytochrome oxidase subunit I (COI)

The cytochrome oxidase subunit I (COI), one of the maternal mitochondrial genes, has been used extensively to investigate the population structure (Schrimpf et al. 2011; Matallanas et al. 2012), phylogenetics, phylogeny and systematics of different crayfish species or groups.

Li et al. (2012) investigated distribution model after establishment and population genetic structure of *P. clarkii* in China. In this study they analyzed genetic structure and diversity of populations from 37 sampling region area using COI 16S rRNA mitochondrial gene sequences and 12 nuclear microsatellite. They reported that from the phylogenetic analysis, bayesian study and isolation between distance, the population located in same area have similar genetic composition and the population in China were high genetic diversity but they didn't show expansion. Their study revealed that the COI 16S rRNA mitochondrial gene sequences is good tool to evaluating population genetic structure and genetic diversity of *P. clarkii*. In another study, Schrimpf et al. (2011) investigated the haplotype diversity of *A. astacus* by analyzing the partial sequences of cytochrome oxidase subunit I of 416 sample from 92 crayfish stock in Black Sea, North Sea and Baltic Sea. From the defined 22 haplotype the one of them was common in whole working area. The high haplotype diversity ($HD = 0.94 \pm 0.24$) was found in Balkans while the low haplotype diversity ($HD = 0.299 \pm 0.038$ and $HD = 0.163 \pm 0.058$) were found in Central Asia. And also this study show to us the COI is useful in the investigation genetic diversity of *A. astacus*.

Schrimpf et al. (2014) studied a portion of the mitochondrial cytochrome oxidase subunit and 16S rRNA from 540 noble crayfish samples taken from 156 sites spread around five sea basins in Europe. Also, they carried out a microsatellite analysis of 289 individuals from 22 sites. Both mitochondrial and nuclear markers have been implicated in anthropogenic translocations in central Europe, resulting in genetically relatively homogeneous populations. Whereas, some areas showed a distinct genetic structure with endemic haplotypes and private alleles indicating that these areas were refugia for *A. astacus* in central Europe and that these populations have not been subject to anthropogenic translocations. Furthermore, researchers have found the highest genetic diversity in the Black Sea basin and especially in the Western Balkans and other Black Sea populations. And also, Akhan et al. (2014) investigated the genetic differentiation among Turkish populations of the narrow-clawed crayfish using a partial sequence of cytochrome oxidase subunit I gene (585 bp) of 183 specimens from 17 different crayfish populations. From this study they disclosed a strong haplotype structure with three prominent clades diverged by a range between 20 and 50 mutations and substantial inter-group pairwise sequence divergence (5.19–6.95 %), They determined the high level of genetic variability (Hd = 95.8 %, p = 4.17 %) and numerous private haplotypes. Helms et al. (2015) examined the genetic structure and morphology between three populations of 3 catchments of the drainage using mitochondrial COI gene sequences and geometric morphometry, and multiple populations of *Cambarus englishi* Hobbs and Halland *Cambarus halli* both of them endemic to the Tallapoosa River.

Bernini et al. (2016) described the genetic structure of *A. italicus* populations in northern Italy (Lombardy Alpine foothills and northern Apennines) by using the cytochrome c oxidase subunit I, to assess the present evolutionary diversity and phylogenetic history from a conservation perspective. As a result they proposed to consider these two clades as distinct molecular operational taxonomic units for the conservation of this endangered crayfish.

Microsatellites

The another genetic method microsatellites or variable number of tandem repeats (VNTRs) are a class of nuclear DNA with repeating units of 1-6 nucleotides, usually formed in a cluster called a locus. A microsatellite locus typically varies between 5 and 40 repeats; the most common forms of repeats are dinucleotide, trinucleotide, and tetranucleotide repeats. Two desirable features of microsatellites are its easy sample preparation and high information

content. In terms of preparation, microsatellite studies use small tissue samples that can be preserved for later use, e.g. freshwater crayfish samples in 95% ethanol, due to the stability of the DNA as compared to enzymes that degrade over time. This also allows the microsatellites to still be amplified during the process of the polymerase chain reaction (PCR). Because small samples are used, microsatellites are optimal for their use of fast and cheap DNA extractions. In addition, since microsatellites are species-specific markers, there is less likelihood that cross-contamination by non-target organisms is a problem (Selkoe and Toone 2006). In terms of high information content, microsatellites as single-locus, co-dominant markers can efficiently be combined in the genotyping process, which allows for fast and inexpensive replication (Selkoe and Toone 2006). Microsatellite markers are utilized in population genetics studies interested in population structure and the relatedness of individuals, migration (gene flow) rates, changes in the past ten to one hundred generations, and present-day demography or connectivity patterns (Selkoe and Toone 2006). Due to the relatively high mutation rate, microsatellites are useful for their large amount of genetic variability and rapid evolution rate for detecting more recent changes in population structure.

There are also several drawbacks of microsatellites. First, microsatellites require species-specific isolation (Selkoe and Toone 2006). Because the DNA sequences of the primers are highly conserved within a particular species, one has to make new primers for each experiment when working with different species. It is difficult to isolate new primers and the failure rate is high. Yue et al. (2008) studied 4 clone of *P. clarkii* with 5 microsatellite in 120 individuals. They reported that each clone contained identical individual as genetic and found as heterozygous in majority of the microsatellites and it was confirmed that all of them were belonged to *P. clarkii*. In another study, Yue et al. (2010) studied genetic diversity and population structure of *P. clarkii* using 9 polymorphic microsatellites. A significant heterozygote deficiency was observed in the studied population. And also, Ahn et al. (2011) studied population analysis of *Cambaroides similis* in Korea and developed and identified 8 microsatellite locus from 49 sample in 4 location (one population from Mt. Bukhan (BH), 3 of from Mt. Gwanak GA). The locus identified per locus was ranged between 2-12. Investigated heterozygosity and expected heterozygosity were found as 0.00-0.33 and 0.25-0.43 respectively. The genetic difference between GA and BH population was 0.789 and within GA population was 0.454. It was thought this high difference to be related from

geographic distance. Gross et al. (2013) developed 10 new microsatellite markers for reveal geographic structiring of *A. astacus*. It is a first large scale study for this species. They studied with 633 species and 18 locations. They identified two highly differentiated population groups along Baltic and Black Sea respectively. The Baltic Sea populations had lower genetic variations and private allele numbers than Black Sea. These studies show us the utility of microsatellites for determining genetic difference of crayfish. Also, Coleman et al (2013), Vorburger et al (2014), Blaha et al (2016), have studied with microsatellite markers in different species and demonstrated that the genetic markers so powerfull and useful to define and manage populations of threatened species based on the notion that populations with unique lineages of mtDNA.

Next generation sequencing method

The another and new genetic method which is used in genetic analysis is next generation sequencing method. Miller et al. (2013) were made genetic analysis of *Euastacus bispinosus* using Next generation sequencing method. In this study 10 out of 15 identified polymorphic loci were characterized using 22 individual in the Glenelg River. Number of locus per alleles and expected heterozygosity was determined as 2,80 and 0,36 respectively. These results are considered to be low for genetic diversity. Also in Crawford River 10 locus were genotyped and genetic diversity and population structure was investigated. As a result of analysis high genetic diversity were identified between species ($F_{ST}=$ 0,49). The all of the methods which mentioned above are useful for the determination of genetic analysis and diversity of crayfish species. For the sustainable of crayfish in the future these genetic methods and conservation strategies must be evaluated together.

Results

From the mt DNA markers, ISSR-PCR, COI, ITS1, RAPD-PCR, next generation sequencing and microsatellites techniques the Next generation sequencing and microsatellites have been introduced recently but give good results. Each methods have advantages and disadvantages but the important one is which is the most useful. For understanding of that we need to compare these method with each other or have to use different gene sequences in the mitochondria. But we can say that the molecular techniquies used so far for investigation the population structure of crayfish species are useful. But these studies is not enough alone for the protection of these species in the strict sense. For the sustainability of these species in the future years firstly the genetic structures of all populations must

be reveal and than aquaculture studies must be conducted according to the results of these molecular studies. Otherwise the futurity of these species will be in threat.

Discussion

Conservation strategies can be achieved by understanding the genetic make-up because the endangered species are spatially fragmented. When there is a effort involving restocking and reintroduction we need a detailed information about population genetics of related species (Bernini et al. 2016). However, the information of genetic difference within and between remaining populations is a prerequisite for the continuation of species by many authorities (Avise 1994; Riffel and Schreiber 1995; Haig 1998). During stock measurement, if these factors are ignored, a hazard such as neglected geographic variation of species which have evolutional importance is occurs (Schulz et al. 2004). For this reason, Moritz (1994) put forward that animal populations have significant genetic diversity could be considered as separate inventories and managed as different units.

The management of genetic diversity and, ultimately, the survival of a population or an entire species depends on several factors. In the short term, it can be said that the genetic variation is insignificant, but in the long run, genetic diversity is required for the ability of the population to adapt to changing environmental conditions. For this reason, a follow-up management should include protection of genetic diversity. Choosing appropriate donor populations is easier and healthier when the estimated stock size and the quality of the disease quality together are assessed. Strong populations which have high polimorfizm or genetic diversity are preferred for determine the minimum genetic diversity, so that Moritz (1994) reported that the populations have important genetic difference might be managed as seperate stocks and different protection units (Fevolden et al. 1994).

Cryptic variety can be seen in many freshwater species. Although species with similar morphology may vary from one another understanding the level of these cryptic diversity has vital prospects for the continuation of biological diversity of freshwater species, for the identification of potential resistant and vulnerable populations, and the conservation of species diversity. If these factors are ignored, the management plans will not be effective and probably will be results with species losses (McKniff 2012; Miller et al. 2013).

The heterogenity of species may be reflect the genetic adaptation of them to the specific climate or environmental condition. Crayfish have a low genetic

diversity tendency and artificial production conditions can worsen this situation by selecting storage strategies or superior broodstocks. So the genetic diversity of crayfish in culture conditions is necessary with the management and periodic monitoring of aquaculture programs (Fetzner et al. 1997). The knowledge acquired from molecular genetic studies should be used in the sustainable management of crayfish to protect it from extinction.

The estimation of genetic diversity will help to identify potential resistance and sensitive stocks and will be a pointer in the protection of resistance stock and taking precautions for the monitoring of sensitive stock. In stocking studies genetic structure within and between population is ignored and this situation leads to serious decline in stocks and contamination of natural species. Furthermore a recovery of population can perform with healthy and sufficient population magnitude to sustain genetic polimorfism in order to adapt climatic change. So the genetic diversity must be considered in governing activities.

References

Ahn DH, Park MIH, Jung JH, Oh MJ, Kim S, Jung J, Min G-S. 2011. Isolation and characterization of microsatellite loci in the Korean crayfish, *Cambaroides similis* and application to natural population analysis. Anim Cells Syst. 15(1): 37-43.

Akhan S, Bektas Y, Berber S, Kalayci G. 2014. Population structure and genetic analysis of narrow-clawed crayfish (*Astacus leptodactylus*) populations in Turkey. Genetica. 142(5):381–395.
doi: 10.1007/s10709-014-9782-5

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Neigel JE, Reeb CA, Saunders NC, 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annu Rev Ecol Syst. 18: 489-522.
doi: 10.1146/annurev.es.18.110187.002421

Avise JC. 1994. Molecular markers, Natural History and Evolution. Chapman and Hall (eds.) New York 511p.

Bernini G, Bellati A, Pellegrino I, Negri A, Ghia D, Fea G, Galeotti P. 2016. Complexity of biogeographic pattern in the endangered crayfish *Austropotamobius italicus* in northern Italy: molecular insights of conservation concern. Conserv Genet. 17(1):141-154.
doi:10.1007/s10592-015-0767-4

Blaha M, Žurovcová M, Kouba A, Policar T, Kozák P. 2016. Founder event and its effect on genetic variation in translocated populations of noble crayfish (*Astacus astacus*). J Appl Genetics. 57(1):99–106.
doi: 10.1007/s13353-015-0296-3

Clavero M, Nores C, Kubersky-Piredda S, Centeno-Cuadros A. 2016. Interdisciplinarity to reconstruct historical introductions: solving the status of cryptogenic crayfish. Biol Rev. 91(4):1036-1049.
doi: 10.1111/brv.12205

Coleman RA, Weeks AR, Hoffmann AA. 2013. Balancing genetic uniqueness and genetic variation in determining conservation and translocation strategies: a comprehensive case study of threatened dwarf galaxias, *Galaxiella pusilla* (Mack) (Pisces: Galaxiidae). Mol. Ecol. 22(7): 1820–1835.
doi: 10.1111/mec.12227

Cook BD, Page TJ Hughes JM. 2008. Importance of cryptic species for identifying 'representative' units of biodiversity for freshwater conservation. Biol Conser. 141(11): 2821–2831.
doi:10.1016/j.biocon.2008.08.018

DeSalle R, Amato G. 2004. The expansion of conservation genetics. Nature Rev. 5(9): 702–712.
doi:10.1038/nrg1425

Miller AD, Rooyen AV, Sweeney OF, Whiterod S, Weeks A R. 2013. The development of 10 novel polymorphic markers through next generation sequencing and a preliminary population genetic analysis for the endangered Glenelg spiny crayfish, *Eustacus bispinosus*. Mol Biol Rep. 40(7):4415-9.
doi: 10.1007/s11033-013-2531-5

Edsman L, Farris JS, Kallersjo M, Prestegaard T. 2002. Genetic differentiation between noble crayfish, *Astacus astacus* (L.), populations detected by microsatellite length variation in the rDNA ITS1 region. Bull. Fr. Pêche Piscic. 367 (2002): 691-706.
doi: 10.1051/kmae:2002082

Fetzner Jr J M, Sheehan R J, Seeb Wl. 1997. Genetic implication of broodstock selection for crayfish aquaculture in the Midwestern United States. Aquaculture. 154(1): 39-55.
doi: 10.1016/S0044-8486(97)00026-4

Fetzner Jr J W. 2011. The crayfish and lobster taxonomy browser. Available online via: http://iz.carnegiemnh.org/crayfish/NewAstacidea/infraorder.asp?io=Astacidea

Fevolden S E, Taugbøl T, SkurdaL J. 1994. Allozymic variation among populations of noble crayfish, *Astacus astacus* L., in southern Norway: implications for management. Aquacult Fish Manage. 25(9): 927-935.
doi:10.1111/j.1365-2109.1994.tb01354.x

Furse J M, Bone J W P, Appleton S D, Leland J C, Coughran J. 2012. Conservation of Imperiled Crayfish–Euastacus bindal (Decapoda: Parastacidae), a Highland Crayfish from Far North Queensland, Australia. J Crustacean BioL. 32(4):677–683.
doi:10.1163/193724012X633405

Geldiay R, Kocataş A. 1970. Türkiye Astacus (Decapoda) Populasyonlarının Dağılış ve Taksonomik Tespiti, Ege Üni. Fen Fak. Gen. Zool. Kürsüsü, İlmi Raporlar serisi, No:94, Biologie 63, İzmir, 11p.[in Turkish].

Gouin N, Souty-Grosset C, Ropiquet A, Grandjean F. 2002. High dispersal ability of *Austropotamobius pallipes* revealed by microsatellite markers in French brook. Bulletin Fraçais de le Peche et de la Pisciculture. 367(2002):681-689.
doi:10.1051/kmae:2002059

Gouin N, Grandjean F, Souty-Grosset C. 2006. Population genetic structure of the endangered crayfish *Austropotamobius pallipes* in France based on microsatellite variation: biogeographical inferences

and conservation implications. Freshwater Biol. 51(7):1369-1387.
doi: 10.1111/j.1365-2427.2006.01570.x

Gross R, Palm S, Kõiv K, Prestegaard T, Jussila J, Paaver T, Geist J, Kokko H, Karjalainen A, Edsman L. 2013. Microsatellite markers reveal clear geographic structuring among threatened noble crayfish (*Astacus astacus*) populations in Northern and Central Europe, Conserv Genet. 14 (4):809-821.
doi: 10.1007/s10592-013-0476-9

Haig S. 1998. Molecular contributions to conservation. Ecology. 79 (2): 413-425.

Harlıoğlu M. M 2004. The present situation of freshwater crayfish, *Astacus leptodactylus* (Eschscholtz, 1823) in Turkey. Aquaculture. 230 (1-4):181–187

Harlıoğlu M M, Harlıoğlu A G 2006. Threat of non-native crayfish species introductionsm into Turkey: global lessons. Rev Fish Biol Fish 16(2):171–181.

Harlıoğlu M M, Güner U.2007. Studies on the recently discovered crayfish, *Austropotamobius torrentium* (Shrank, 1803), in Turkey: morphological analysis and meat yield. Aquaculture Res. 37(5): 538- 542.
doi:10.1111/j.1365-2109.2006.01451.x

Helfrich LA, DiStefano RJ. 2009. Sustaining America's Aquatic Biodiversity Crayfish Biodiversity and Conservation. Virginia Tech. 420-524.

Helms B, Vaught RC, Suciu SK, Santos SR. 2015. Cryptic diversity within two endemic crayfish species of the Southeastern US revealed by molecular genetics and geometric morphometrics. Hydrobiologia. 755(1): 283-298.
doi:10.1007/s10750-015-2311-4

Koizumi I, Usio N, Kawai T, Azuma N, Masuda R. 2012. Loss of genetic diversity means loss of geological information: The endangered Japanese crayfish exhibits remarkable historical footprints. Plos One. 7(3): e33986.
doi: 10.1371/journal.pone.0033986

McKniff J. 2012. Investigation of the population genetics of crayfish (*Orconectes virilis*) using AFLP markers, Degree of Bachelor of Science. p: 1-39.

Li Y, Guo X, Deng W, Luo W, Wang W. 2012. Population genetic structure and post-establishment dispersal patterns of the red swamp crayfish *Procambarus clarkii* in China. Plos One. 7(7):e40652
doi: 10.1371/journal.pone.0040652

Liu G, Zhou L, Li X, Lu D. 2013. Population genetic structure of the invasive red swamp crayfish in China revealed by ITS1 variations. Biochem Genet. 51(11-12): 841-852.
doi:10.1007/s10528-013-9611-z

Largiader C R, Herger F, Lörtscher M, Scholl A. 2000. Assessment of natural and artificial propagation of the white-clawed crayfish (*Austropotamobius pallipes* species complex) in the Alpine region with nuclear and mitochondrial markers. Molecular Ecology. 9 (1):25-37.
doi:10.1046/j.1365-294x.2000.00830.x

Matallanas B, Callejas C, Ochando M D. 2012. A genetic approach to Spanish population of the threatened Austropotamobius italicus located at three different

scenarios, The Scientific World Journal. 2012 Article ID 975930.
doi:10.1100/2012/975930

May RM. 2010. Ecological science and tomorrow's world. Philosophical Transactions of the Royal Society B. 365:(1537): 41–47.
doi: 10.1098/rstb.2009.0164

Moritz C. 1994. Defining evolutionarily significant units for the conservation. Trends Ecol Evol. 9(10):373-375.
doi:10.1016/0169-5347(94)90057-4

Owen CL, Bracken-Grissom H, Stern D, Crandall KA. 2015 A synthetic phylogeny of freshwater crayfish: insights for conservation. Philosophical Transactions Royal Society B. 370(1662): 20140009.
doi:10.1098/rstb.2014.0009

Ricciardi A, Rasmussen JB. 1999. Extinction rates in North American freshwater fauna. Conserv Biol. 13(5): 1220–1222.
doi:10.1046/j.1523-1739.1999.98380.x

Riffel M, Schreiber A. 1995. Coarse-grained population structure in Central European sculpin (*Cottus gobio* L.): secondary contact or ongoing genetic drift? J Zool Syst Evol Res. 33(3-4):173-184.
doi:10.1111/j.1439-0469.1995.tb00970.x

Schrimpf A, Schulz HK, Theissinger K, Parvulescu L, Schulz R. 2011. The first large-scale genetic analysis of the vulnerable noble crayfish *Astacus astacus* reveals low haplotype diversity in central European populations. Knowl Manag Aquat Ec. 401:35
doi:10.1051/kmae/2011065

Schrimpf A, Theissinger K, Dahlem Jr, Maguire I, Pârvulescu L, Schulz H K, Schulz R. 2014. Phylogeography of noble crayfish (*Astacus astacus*) reveals multiple refugia. Freshwater Biol. 59(4):761-776.
doi:10.1111/fwb.12302

Schulz HK, Smietana P, Schulz R. 2004. Assessment of DNA variations of the noble crayfish (*Astacus astacus* l.) in Germany and Poland using inter-simple sequence repeats (ıssrs) Bull Fr Peche Piscic. 372-373(2004):387-399.
doi:10.1051/kmae:2004012

Schulz R. 2000. Status of the noble crayfish *Astacus astacus* (L.) in Germany: monitoring protocol and the use of RAPD markers to assess the genetic structure of populations. Bull. Fr. Pêche Piscic. 356(2000): 123-138.
doi:10.1051/kmae:2000007

Selkoe KA, Toone RT. 2006. Microsatellites for ecologists: a practical guide tousing and evaluating. Ecol Lett. 9 (5): 615-629.
doi:10.1111/j.14610248.2006.00889.x

Vorburger C, Rhyner N, Hartikainen H, Jokela J. 2014. A set of new and cross-amplifying microsatellite loci for conservation genetics of the endangered stone crayfish (*Austropotamobius torrentium*). Conservation Genet Resour. 6(3):629–63.
doi: 10.1007/s12686-014-0157-0

Wilson AC, Cann RL, Carr MG, Gyllensten UB, Helm-Bychowski M, Higushi R G, Palumbi SR, Prager EM, Sage RD, Stoneking M, 1985. Mitochondrial DNA

and two perspectives on evolutionary genetics. Biol J Linn Soc. 26(4): 375-400.
doi: 10.1111/j.1095-8312.1985.tb02048.x

Yue GH, Wang G L, Zhu B Q, Wang C M, Zhu Z Y, Lo L C. 2008. Discovery of four natural clones in a crayfish species *Procambarus clarkii*. Int J Biol Sci. 4(5): 279-282

Yue GH, Li J, Bai Z, Wang CM, Feng F. 2010. Genetic diversity and population structure of the invasive alien red swamp crayfish. Biol Invasions. 12(8): 2697-2706.
doi: 10.1007/s10530-009-9675-1

Zhang YJ, Rao GY. 2005. Genetic diversity of an amphicarpic species, Amphicarpaea edgeworthii Benth. (Leguminosae) based on RAPD markers. Biochem Syst Ecol. 33(12): 1246-1257.
doi:10.1016/j.bse.2005.07.009