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MOLECULAR APPROACHES TO BIVALVE POPULATION STUDIES: A REVIEW

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Abstract: This paper presents a literature review concerning the importance of molecular approaches in bivalve's population study. The class Bivalvia counts more than 20,000 species with a wide distribution both in freshwater and marine environment. Given their importance especially in aquaculture as a source of food, they have also a strong economic impact upon human society. This review encompasses best practices in bivalve studies from field sampling to laboratory analyses, addressing questions about molecular methods and tools commonly used by specialized researchers. Molecular tools specifically deals with phylogeography, population genetics, biology, ecology and taxonomy. In all these fields, molecular markers play an important role by completing some unanswered questions such as the role of the bivalves in the ecosystems in relation to anthropogenic and global change issues. Numerous genetic markers were developed for specific problems, thereferore we identify as a major issue the absence of uniform and universally recognized methods. The various sections of the paper emphasize from peer reviewed literature literature which are considered the most useful markers, costs and benefits of different methodology, major gaps of knowledge.in bivalve population studies. By reviewing virtually all genetic markers employed during nearly half a century of bivalve molecular research, in our opinion two are the best option "tools: the mitochondrial COI (cytochrome oxidase subunit I) and nuclear ITS2 (internal transcribed spacer 2).

INTRODUCTION

Once Darwin's work was published (especially 'On the origin of species by means of natural selection') (Darwin 1859) it was a *desideratum* for most biologists to reconstruct the evolutive history of Earth's organisms and express it as phylogenetic trees (Haeckel, 1866 in Nei and Kumar, 2000). The most obvious method for obtaining phylogenetic trees is the reconstruction of paleontological data. Although it is recognized that this types of data are fragmented and incomplete, therefore most researchers use morphological and physiological comparative methods in order to fill the gaps of knowledge. Historically, these classic techniques illustrated major aspects of the evolution of organisms. Morphological and physiological transformations during the evolution of living beings are so complex that often classic techniques are unable to depict the evolutional history of a given taxon group in a satisfactory way. Furthermore, details of classic phylogenetic trees were often controversial (Nei and Kumar, 2000). Nowadays phylogenetic trees can achieve a better resolution due to the development of molecular techniques that allow new insights into the diversity of life and the way it evolved. This can be accomplished by studying the organisms at a different level by combining both classical and modern techniques in a way that evolutionary relationships between organisms can be easily inferred. The main cause of evolution is the transformation (modification) of genes through mutations (substitutions, insertions/deletions, recombination, gene conversions etc.). Mutations settle in and affect populations because of natural selection and/or gene dispersion (Nei and Kumar, 2000; Nei, 1987; Hartl and Clark, 1997). Gene mutations produce new morphological and physiological features, which are inherited by descendants, if the gene does not additional modifications (mutations) later on. Thus, by completing a valid phylogenetic tree belonging to a certain group of species, it is possible to identify liniation (i.e. genealogy) based on specific phenological features due to gene mutations (Nei and Kumar, 2000). In addition, information concerning the environmental conditions to which the genealogic line is exposed (i.e. presence/absence of target features in relation to known environmental conditions) may help to assess if peculiar features evolve under the influence of natural selection and/or of gene dispersion (Nei and Kumar, 2000). Genetically speaking, the majority of natural populations are extremely variable. In a population that reproduces sexually, any pair of individuals is different from a genetic point of view, with the exception of identical twins (monozigous). When establishing the direct relationship between a genetic locus and a protein, usually the locus will have two or even more allele (Nei and Kumar, 2000). At a different level, these structural modifications of the genetic material are mainly governed by two simultaneous and opposed processes which are at the basis of evolution at a molecular level: natural selection and gene dispersion. Natural selection is a process through which, at a certain point in time, advantageous mutations are selected at population level, while gene dispersion is a random process through which aleatory mutations get fixed inside the population, being either beneficial or detrimental. In time, these two processes shape the population. The importance of the two processes in evolution has been supported or infirmed by various opinions. Recent progress in the domain of molecular biology has led to a meaningful change of this situation. The basic information that stands at the basis of life in any of its forms is encoded in the nucleic acids: DNA (deoxyribonucleic acid) and RNA (ribonucleic acid).

In this way, the evolutional relationships between organisms can be studied by comparing DNA differences (Nei and Kumar, 2000). Molecular phylogeny is a relatively new field of interest which benefits from a wide range of modern molecular tools in order to reconstruct the phylogenetic and phylogeographic relations between organisms. Recently, molecular biology has provided new methods for evolutionary investigations. These methods are based on DNA sequencing which can offer information about the evolutional modifications of species through their structure. Among these tools we can assume that molecular markers are frequently used, especially mitochondrial and nuclear markers. New molecular approaches had an important role both in solving phylogenetical problems and evolutionary conflicts.

The purpose of this review is to report the use of mitochondrial and nuclear molecular markers in phylogenetic and phylogeographic studies of bivalves as a target taxon. Bivalves are commercially important species in aquaculture or wild harvest. There are 386 bivalves species from 4 orders: Arcoida (2), Ostreoida (1), Unionoida (308) and Veneroida (75) which are included into the IUCN red list (IUCN, 2010) while some other species are problematic alien pests (DAISIE, 2011). Studying bivalves is a challenging scientific exercise, while understanding their ecological roles can be very useful for conservation problems. First of all it is fundamental to clarify their taxonomical position, phylogeny and phylogeography and this result can only be achieved through an approach that combines classical morphological and more modern molecular studies. Nowadays, many studies involving biodiversity are in fact omitting the classical approaches in identifying the taxonomy of a species. For example, the modern molecular approaches tend to transform the classical morphological identifications of species (phenotypes) in identifications at a molecular level (genotypes), only by implying the reading of the DNA which is the molecule of life. This also leads to very accurate obtained sequences that may be attributed to an incorrectly identified organism, since molecular taxonomist's knowledge about the phenotypes is very basic. What this modern identification of organisms is actually lacking is in fact the morphological based identifications (Boero, 2010). The class Bivalvia is yet one of the most targeted taxon in molecular phylogeny studies, especially for the orders Unionoida, Veneroida, Ostreoida and Mytiloida, as many different species belonging to theses orders have economical importance for fishery and aquaculture (Klinbunga et al., 2003; Pie et al., 2006; Baker et al., 2008). Some new farmed species like *Nodipecten nodosus* (Linnaeus, 1758) become very important for commercial purposes due to their relative ease of culture and the decline of other fisheries (Petersen et al., 2008). The Japanese oyster *Crassostrea gigas* (Thunberg, 1793) became a leader of the fish market for exactly the same reasons and nowadays is harvested worldwide (Cardoso et al., 2007). Other commercially important or potentially important species are cryptogenic (sensu Carlton, 2009) and can only be discriminated by using molecular markers (Kong and Li, 2009). Moreover, Carstensen et al., 2009, demonstrated on the basis of mitochondrial markers that the two commercial species of *Donax* (*Donax marincovichi* and *Donax obesulus*) were in fact the same, *Donax obesulus* (Reeve, 1854). Recently, the consumption of commercial food coming from canned or frozen bivalve posed an identification problem due to the lack of morphological criteria, thus the only way for identifying the species was by molecular means, which is the case of *Cerastoderma edule* (Linne, 1758) and *C. glaucum* (Poiret, 1789) (Freire et al., 2010). Molecular studies are also important to understand processes of bioinvasion and pathways of introduction of alien bivalve species. Studies involved inferring the phylogeography of Ponto-Caspian originating alien species like *Mytilopsis leucophaeata* (Conrad, 1831) (Therriault et al., 2004) or *Dreissena bugensis* (Andrusov, 1897) (Gelembiuk et al., 2006; May et al., 2006; Grigorovich et al., 2008; Quaglia et al., 2008) in order to explain their expansion into new territories. There is also the case of the introduced species as *Crassostrea gigas*, which after being introduced for aquaculture, has acclimated and colonized wild areas hybridizing with native oysters (Cardoso et al., 2007). Molecular approaches were also used for detecting larvae of some alien bivalves as *Xenostrobus securis* (Lamarck, 1819) (Santaclara et al., 2007) or *Limnoperna fortunei* (Dunker, 1857) (Pie et al., 2006). These methods also helped in solving questions concerning vectors of NIS bivalve introduction – i.e the case of the "lessepsian migrant" *Brachidontes pharaonis* (Fischer P. 1870) which was proved to be transferred by ballast water (Shefer et al., 2004).

Phylogeography can provide further information which may complete the evolutional history of closely related species. Genetic analysis combined with geographical distribution of genes was effective for the identification and revision of three cryptic species of *Brachidontes*: *B. pharaonis* from Mediterranean and Red Sea, *B. variabilis* from Indian Ocean and *B. variabilis* from western Pacific Ocean. Although a morphological differentiation has never been successful (Sirna-Terranova et al., 2007), results led to reconsider the taxonomical position of the whole family Mytilidae. A similar case was reported for *Pisidum* sp. in lakes Prespa and Ohrid (Balkan Peninsula) where the speciation process led to almost morphologically similar species (Schultheiß et al., 2008). Phylogeographical studies can be very useful for conserving the genetic integrity of some endangered freshwater mussels as in the case of the genus *Lampsilis* from southeastern United States (Roe et al., 2001) or, for explaining population history, vicariant[1] processes and population expansions (Plouviez et al., 2009).

This paper will present a critical review of the most and less common molecular techniques for studying bivalves. The aim of the work is to identify and present major technical issues arising from sampling to data analysis and interpretation of results in this particular field of molecular biology. The authors intend to answer to FAQ such as: which are the

[1] ex. in marine organisms the rise of Isthmus of Panama led to evolution of related species in Atlantic and Pacific sides

cost/benefit of molecular approaches to bivalve population studies and their major constraints? What will be the future development of this Science? What are the main ecological questions which could find an answer by using this specific approach? Which are the major gaps of knowledge that need to be filled in order to increase confidence in these methodologies? Is the available literature and scientific experience enough developed to establish "best practices" in bivalve molecular studies?

Sampling and bivalve's tissue preservation issues

The first step in every population, species or individual study, no matter of its nature (morphological, biochemical, molecular, etc), implies sampling of biological material. Besides sampling for morphological data analysis that involves collecting the whole individual (Krogmann and Holstein, 2010) there is another approach to sampling, in order to obtain data for molecular/genetic analysis. Sampling for morphological analysis represents an invasive method leading to damage of individuals by killing and storing them in fixation fluids (Krogmann and Holstein, 2010). Sampling methods for molecular analysis that rely on non-invasive methods have been employing only recently. Non-invasive methods have appeared as a response to investigate the species and population without causing any harm to individuals. In the case of vertebrates this methods make use of hairs, feathers, faeces, urine, moulted skin, bucal or skin cells (swabs) which permit analysis of individuals even without interacting with them (Beja-Pareira et al., 2009). A little different is the approach in the case of invertebrates, where their diversity makes harder to generate tissue sample and extraction methods. If for small invertebrates there is need to use the whole individual, in bigger ones legs, abdomens, feet or muscle biopsy can be used (Gemeinholzer et al., 2010). As en example in polyplacophoran molluscs there has been successful amplification of DNA from foot tissue mucus (Palmer et al., 2008). The use" of PCR based techniques allow rapid processing of samples (Taberlet, 1999; Waits and Paetkau, 2005). Although in the past these methodologies were subjected to some genotyping errors (Taberlet et al., 1999), at present they have been using with more efficiency for many type of organisms (Gemeinholzer et al., 2010). Bivalve specimens can be collected from rivers, lakes, lagoons, seas and oceans, practically from every hydrological region of the world, especially during the spring –summer period when the favourable ecological and biological conditions for many species are meet. The best sampling practice involves dredging (Dreyer et al., 2003) or hand collection by snorkelling or Scuba diving (Elderkin et al., 2007; Arnaud-Haond et al., 2005). When sampling of the whole specimen is not allowed for conservation reasons, i.e. critically endangered (CR), endangered (E), vulnerable (VU), or near threatened (NT) (IUCN, 2010) species, at least three non-destructive tissue-sample methods can be applied: 1)mantel biopsy (Berg et al., 1995; Buhay et al., 2002; Grobler et al., 2005; Kochzius and Nuryanto, 2008); 2) ligament biopsy (Doherty et al., 2007); 3) swabbing the foot and viscera of bivalves (Henley et al., 2006). This third procedure was found to be more successful both for obtaining DNA and guaranteeing the bivalve survival.

The best fixative for the conservation of bivalve tissue samples for molecular analysis is 90%-95% ethanol (Araujo *et al*., 2009; Arruda *et al*., 2009; Fernández-Tajes and Méndez 2009; DeBoer et al., 2008). It is known that mollusc tissues are especially rich in mucopolysaccharides, up to 90% of the total GAG (glycosaminoglycans) of the mollusc body (Arumugam et al., 2009) and this is very often a problem for DNA isolation, independently of the storage agent (Sokolov, 2000). Other conservation methods imply tissue frosting by liquid nitrogen at -80°C (Machordom *et al*., 2003; Elderkin et al., 2006; Sokolov, 2000, Baker et al., 2004; Zanatta & Murphy, 2008). This method is probably the best way of preserving tissues but in some cases it decreases the yield of DNA or it may raise problems during the field work. Due to these inconveniences conservation in ethanol is preferred. Moreover ethanol guarantees lower cost and better manoeuvrability on the field (Gemeinholzer et al., 2010). Formalin is the less effective fixative for molecular studies. It was used in the past for specimen storage from archival collection (Schander and Halanych, 2003) that were firstly fixed in formalin for 24-48 h, then transferred and stored in alcohol vials (Boyle et al., 2004). This compound degrades DNA (Sturm et al., 2006), although very particular methods of DNA extraction from deep-sea bivalve specimens conserved in formalin were successfully developed (Chase et al., 1998; Boyle et al., 2004; Zardus *et al*., 2006).

An important aspect of bivalve sampling for molecular studies is the correct determination of the sampling effort, i.e. the number of specimens that need to be collected, in relation to the aim of the research. Some Authors suggested a collection of up to 30 individuals for getting a sufficient picture of the genetic structure of endangered species, such as *Pinna nobilis* (Linne, 1758) (Katsares et al., 2008). Generally, in case of endangered species when it is not possible to sample only part of the animal, thus the survival of the samples is not guaranteed, different Authors (Roe et al., 2001) used 1 up to 5 individuals and when possible up to 414 tissue samples (DeBoer et al., 2008). In the case of non endangered species up to over 500 individuals from multiple populations can be collected for a more accurate estimate (Diaz-Almela et al., 2004) (Tab. 1)

DNA extraction, amplification and sequencing

Following the tissue sample collection laboratory work consists in three major processing steps: DNA isolation (Chomczynski and Sacchi, 1987), PCR amplification (Mullis and Faloona, 1983) and sequencing of the amplified products (Fig.1) (Sanger et al., 1977).

A major technical issue of bivalve DNA isolation is the difficulty of obtaining a high quality or pure DNA due to the general high concentration of mucopolysaccharides in mollusk tissues. These compounds are usually isolated along with the DNA, inhibiting the activity of multiple enzymes such as ligases, polymerases, restriction enzymes. Sokolov (2000)

proposed an inexpensive method of DNA extraction that yielded high-quality DNA suitable for different molecular analysis. The Author used for this method mollusc species from 3 orders: Polyplacophora, Gastropoda and Bivalvia (Tab. 2) considering muscle tissue as the most appropriate source for DNA in molluscs

A short description of the protocol is as follows:

Sample preparation and lysis :50 -70 mg of sliced muscle →2 ml plastic tube with 1 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% SDS (sodium dodecyl sulphate), 0.2-0.4 mg/ml proteinase K) → vortex → incubation at 55°C until complete digestion + 100 µl saturated KCl for precipitation of mucolpolysaccharides and some proteins along with the insoluble potassium dodecyl sulphate + 15 min centrifugation maximal speed + supernatant collection in another clean tube and extract twice with phenol/chloroform/isoamyl alcohol (25:24:1).

DNA binding: Transfer of supernatant in another tube + isopropanol + incubation 10-15 min at room temp + 20 min centrifugation + discard the supernatant + wash DNA pellet in 70 % alcohol + drying the pellet and dissolve in 100 µl of TE buffer + RNAse A + incubation at 37°C for 30-60 min.

The amplification of heavy DNA sequences is done by primers of different genetic markers. These molecular markers have the potential information to evaluate the processes that are at the basis of population and evolutional studies (Mulvey et al., 1998). There are two major categories of molecular markers used for studying bivalves: mitochondrial (16S rDNA, 12S rDNA, COI) and nuclear (ITS1, ITS2, 18S rDNA). For all organisms mitochondrial DNA is a small circular molecule implicated in respiration processes. It consists of two genes that codify the rRNA, 22 genes that codify tRNA, 12 genes that codify for proteins and a control region. The control region is an area of non-coding DNA from mitochondrial genome. This particular region is subjected to rapid evolution in many species (Freeland, 2005). Because it is very easy to work with, mtDNA is used in population genetic studies. Although it has a small size and the sequences are conserved its mutation rate is generally high and recombination is missing. Thus, the offsprings have the same mt DNA genome as their mother's (Freeland, 2005). Mitochondrial DNA evolves faster than nuclear DNA and has a higher nucleotide mutation rate, excelling the evolution rate of the nuclear genome (Brown et al 1979). Although mtDNA is transmitted uniparentally, there are some exceptions where a double uniparental transmission (DUI) occurs, like in the case of *Mytilus* sp. (Zouros, 1992). DNA polymorphism should be studied in order to understand the phylogeographical relations between populations or species. This can be realized by analyzing haplotypes which present unique genetic marker combinations in a chromosome (Hartl and Clark, 1997). Haplotypes are different from each other for one or more nucleotides due to substitution, insertion or deletion phenomena. It has been proven that the presence of nuclear pseudogenes of mitochondrial origin (also named numts) should be taken into consideration in mtDNA study (Ballard and Whitlock, 2003). Because they are present in the majority of eukaryotes, numts shouldn't be interpreted real mitochondrial genes. Another disadvantage of mitochondrial markers is that they may behave as a single molecule with a unique evolutionary history, leading to an overestimation of the evolution parameters (Ballard and Whitlock, 2003). In consequence, these markers are not representative for the evolutionary history of a species.

Some of the most frequently used regions of the mtDNA is cytochrome b (cytb) and subunit I of the cytochrome c oxidase (COI) (Feral, 2002). Cytochrome c oxidase represents the most used marker in molecular studies and barcoding (Hebert et al*.,* 2003). This marker is a phylogenetical signal stronger than any other mitochondrial marker. The evolution of this gene is rapid and can discriminate not only amongst strongly related species but also amongst phylogroups belonging to the same species (Hebert et al*.*, 2003).

Nuclear markers such as the Second Internal Transcribed Spacer (ITS2) represent a region of the nuclear ribosomal gene group which is not codified. The ribosomal gene group consists of 3 genes (small nuclear 18S rDNA, 5.8S rDNA and large nuclear 28S rDNA) which are transcribed into RNA but translated into proteins. These three genes are transcribed in RNA and are separated by two regions: ITS1 and ITS2. After transcription, these regions are eliminated being of any other use. Consequently, these regions can rapidly accumulate substitutions due to the weak pressure of selection. This can be useful in discriminating species that are closely related (Cruickshank, 2002). ITS2 is situated between the 5.8S and 28S nuclear ribosomal genes and constitutes a DNA fragment which evolves rapidly and which has proven to be very useful in the analysis of phylogenetically relations between closely related species, such as some plant and fungi species (Coleman, 2007) or bivalve mollusks (Freire *et al.,* 2010). The way this marker is used in interpreting phylogenetics of bivalves raises a question on which method is the best in analyzing ITS sequences: cloning or direct sequencing? The majority of the ITS1 and ITS2 studies in bivalves usually involves cloning of the ITS2 region in order to separate two or more haplotypes/individual (Freire et al., 2010; Salvi et al., 2010; Vierna et al., 2010), while other Authors choose direct sequencing (Flot et al., 2006; Ladhar – Chaabouni et al., 2010). Despite the fact that direct sequencing may results in doubtful base-calls and superimposed peaks in the chromatogram (Wilkerson et al., 2004) suggesting presence of two sequences in the same individual (FIG. 2), this method is much faster and less expensive than cloning (Flot et al., 2006).

Which are the cost/benefit of molecular approaches to bivalve population studies and their major constraints?

In order to answer the question we need to go back in the early stages of molecular studies when genetic structure of bivalve populations was inferred manly by protein electrophoresis (Hubby and Lewontin, 1966). This technique consisted of enzyme extraction from different tissues followed by gel electrophoresis (using starch, acrylamide or cellulose gels) after which they were stained with chemical agents and finally bands running on the gel were interpreted (Micales and

Bonde, 1995). This practice was used successfully to distinguish between two closely related species *Cerastoderma edule* and *C. lamarcki* but failed in distinguishing between *C. lamarcki* and *C. glaucum* (Brock, 1987). Although protein electrophoresis is considered an old molecular tool, which nowadays has been substituted by DNA sequencing, enzymes can be still useful molecular markers. González-Wangüemert et al. 2009 demonstrated on the basis of PGI (phosphoglucose-isomerase) the variability of the *Cerastoderma glaucum* populations from the Mar Menor lagoon (Mediterranean Sea). This variability was found to be influenced by the environmental conditions in which the bivalve populations thrive, determining also the distribution patterns of the species in the basin. The major advantages of enzyme based techniques can be summarised as follow: homozigote and heterozigote genotypes can be distinctly separated due to the codominant expression of enzymes; zymograms can be easily inferred; analysis of isoenzymes is easy to do; monitoring populations can be very rapid as great number of loci and individuals can be analysed at the same time; genetic polymorphism can be easily studied with a high benefit/costs ratio (Manchenko, 2003).

Major disadvantages includes: only nucleotide substitutions that modifies the electrophoretic mobility of the molecules are easy to track; there is a risk on data interpretations due to the fact the same band of the isoenzyme representing two different alleles bears identical mobility; this techniques cannot distinguish evolutionary relations and null alleles (Muller-Starck, 2001).

Before the PCR techniques have been developed, population genetics of bivalves was strongly limited from a sequencing point of view. Since PCR became common, numerous DNA markers allowing genetic comparisons at population level have been proposed. DNA markers can be divided in two categories: PCR based (RAPD, AFLP) and non PCR based (RFLP). All methods use gel electrophoresis and represent a precious source of information on bivalve biology, ecology and conservation.

RAPD (Random Amplified Polymorphic DNA) is a technique that amplifies DNA sequences using singular primers (Williams et al., 1990). This technique does not necessitate DNA cloning or sequencing, can detect many loci simultaneously and do not require knowledge of the target sequence (Williams et al., 1990). The RAPD has been successfully applied in the differentiation of the *Cerastoderma edule* and C. *lamarcki* larvae (Andre et al., 1999).

Major advantages are: the high sensibility for detecting a wide array of polymorphisms; the possibility of automatization; low costs; the detection methods which can be based on fluorescence instead of radioactivity; the opportunity of analysing a large number of samples per day (Kumar et al., 2009).

Disadvantages are: RAPD are dominant markers, for example in population genetics allele frequency cannot be expressed since the homozigotes cannot be separated from heterozigotes; the high sensibility of this technique (reproducibility is high in a given laboratory, but results are inconclusive when the data are repeated in another laboratory) (Semagn et al., 2006). By comparing polymorphisms between two oat cultivars by RAPD in six laboratories with five primers, Penner at al., 1993 showed that the reproducibility was affected in two ways:1) different laboratories amplified different size ranges of DNA by using the same protocol; 2) only four out of five primers succeeded in amplifying DNA.

Restriction Fragment Length Polymorphism - RFLP (non PCR based marker) is another method used in the past for genetic fingerprinting. The procedure is based on some restriction enzymes that fragment the DNA sample (Botstein et al., 1980).

It has been succesfully employed for detecting larvae of *Xenostrobus securis* and *Mytillus galloprovincialis* (Lamarck, 1819) (Santaclara et al., 2007) and for distinguishing cryptic genus such as *Cerastoderma* sp.(Freire et al., 2010) and *Ensis* sp.(Fernadez-Tajez and Mendez, 2007). RFLP was also used to to detect the differences between species of Dreissenidae and to identify invasive species, such as *Mytilopsis leucophaeata* in Europe (Therriault et al., 2004).

Unlike RAPD, RFLP needs an accurate characterisation of the target genome sequence for this reason at present it has been substituted by more modern methods (e.g. microsatellites).

Major advantages are: it is a robust methodology with high reproducibility between laboratories; it can estimate heterozigosity; sequence information is not necessary as the main difference between sequences lies not in the succession of the nucleotides but in the length of digested fragments with the help of restriction enzymes. Restriction enzymes are used to specifically cut the DNA molecule at certain recognised places; it is based on the sequence homology, thus it is recommended for phylogenetic studies; it provides good discrimination both at population as well as individual level (Semagn et al., 2006; Kumar et al., 2009).

Disadvantages are: big quantities of DNA are needed; automatisation is not possible, few loci detected/analysis, time consuming, expensive, different sample/enzymes combinations needed (Kumar et al., 2009).

Amplified Fragment Length Polymorphism -AFLP is a very useful technique in case of insufficient characterised genomes. This technique was firstly used in criminology investigations while at present are also used in population genetic studies (Kumar et al., 2009). Mock et al., 2004 applied the AFLP in order to demonstrate the lack of nucleotide diversity in some *Anodonta* populations, a consideration that led to the assumption of long term isolation between populations belonging to this genus.

Examination of the genetic variation of aquacultured species such as *Crassostrea virginica* (Gmelin, 1791) by using both microsatellites and AFLP encouraged the use of these latter markers when microsatellites are not available (Yu and Guo,

2005). AFLP was also used for studies aiming at clarifying the genetic control of morphological characters in bivalves, such as the colour of the shell in *Argopecten iradians iradians* (Lamarck, 1819) (Qin et al., 2007).
Major advantages are: the opportunity of assessing a large number of polymorphisms; the knowledge of sequences is not necessary; a large number of individuals per locus can be easily assessed (up to 100 individuals per100 loci.week^{-1}); a high multiplex ratio, i. e. number of different loci that can be analysed simultaneously per each experiment (Kumar et al., 2009).
Disadvantages are: high costs of primers; low reproducibility (i.e. AFLP patterns changes when reanalysed); dominance issues (i.e., up to 2-10 individuals per locus are needed for dominant markers compared to co-dominant ones); homology issues (the greatest disadvantage of AFLP states that the co-migratory bands are homologous although there is no a priori opinion to accept this statement (Robinson and Harris, 1999).
Microsatellites are short repeated DNA sequences with a length between 1-6 repeated base pairs, like dinucleotides $(CA)_n$, followed by $(AT)_n$, $(GA)_n$ and $(GC)_{n,}$ (Ellegren, 2004). They are known also as simple sequence repeats (SSR), variable number tandem repeats (VNTR), short tandem repeats (STR) and they represent nowadays the most used molecular markers in addressing questions about ecological research (Selkoe and Toonen, 2006).
Microsatellites are successfully employed in the population genetics of bivalves (Tarnowska et al., 2010) due to their resolution at interspecific and intraspecific level, in characterizing relict bivalve species (i.e. *Hypanis colorata* (Eichwald, 1829), Popa et al., 2011), revealing bottleneck effects (Launey et al., 2001), changes in effective population size (Appleyard and Ward, 2006), genetic drift (Ni et al., 2011) and parentage analysis (Wang et al., 2010).
Advantage: codominant alleles; very abundant and random distribution in the genome of eukaryotes; high reproducibility; does not require high quantities of template DNA.
Disadvantage: the main problem regarding the microsatellites is their specific nature, their laborious characterisation that involves cloning, PCR and selection; high costs, if primers are unavailable and need to be developed; there are null alleles (unamplified DNA) resulting from mutations occurring at sites where primer anneals (Kumar, 2009).

What are the main ecological questions which could find an answer by using these specific approaches?

When referring to ecology of bivalves we automatically think about interactions between organisms and their environment (Gosling, 2003). In the past, these relations were studied by means of morphological studies, direct observations and statistical interpretation of data. Despite the great quantity of information obtained by ecologists, there were some questions, and still there are many, left unanswered due to the lack of proper tools. The recent discovering and use of molecular markers has greatly enhanced the opportunity to fill gaps of knowledge in bivalves' ecology. Generally speaking, molecular methods succeeded in providing information on relationship between species (Huff et al., 2004; Mahidol et al., 2007; Espineira et al., 2009; Vierna et al., 2010), on their evolutionary history (Stepien et al., 1999; Cunha et al., 2011; Etter et al., 2011), on genetic variation within species populations (Luttikhuizen et al., 2003; Zardus et al., 2006), on population size o, migratory events, and biodiversity conservation issues such as hybridization events (Westfall and Gardner, 2010)

Which are the major gaps of knowledge that need to be filled in order to increase confidence in these methodologies?

Regarding phylogenetic connections in Bivalvia, a number of molecular studies have been focused on answering questions on the taxonomic position of protobranchiate bivalves, such as Arcoida Mytilidae, Heterodonta and Anomalodesmata. From an evolutionary point of view, other aspects of bivalves have been investigated, among which is the carnivory in septibranchs, colonisation of freshwater environments or deep-sea, habits of wood or rock–boring and even symbiosis with algae or bacteria (Ponder and Lindberg, 2008). Despite this, there are still many aspects of the bivalves that need to be investigated, especially due to the large number of species (aprox. 7500 Gosling, 2003) belonging to this order many different taxa still need to be properly characterised from a taxonomical point of view.

Is the available literature and scientific experience enough developed to establish "best practices" in bivalve molecular studies?

The methodologies involved in molecular approaches to bivalve studies follow analogous patterns. A molecular approach consists of sampling, storage for laboratory analysis and the adequate laboratory work (DNA isolation, amplification and sequencing), as it has been underlined in the present review. In detail, the methodology varies according to the specific aim of the study by choosing the right molecular marker to the proper future goal. Yet, despite this very often obtained results are different, leading to a result that is not in agreement with the expectations. This is bivalves phylogenetic studies were molecular methods leave still ambiguities due to the lack of general agreement upon the common agreed methodology to be used. A number of studies using morphological, molecular or both approaches have managed to infer more or less the taxonomy and phylogeny of Bivalvia (Giribet and Wheeler, 2002; Giribet and Distel, 2003; Harper et al. 2006; Mikkelsen et al; 2006; Olu-Le Roy et al, 2007; Plazzi and Passamonti 2010). On a synthesis mitochondrial markers are generally preferred in phylogenetics as they are single-copy gene; they unable to 'see' relationships in a very straight forward manner, being maternally inherited with the lonely exception of (DUI – double uniparentall inheritence). Nuclear markers are problematic in phylogenetic studies because their genes are found in more copies leading to wrong interpretations of results but they are very robust for population genetic structure investigations.

CONCLUSIVE REMARKS

The intensive use of molecular data for inferring all kind of relations in the living world had a simple approach in the early days of molecular studies, but as new tools were developed, also the progresses in this field increased. If for inferring phylogenies, phylogeographies, genetic structure, etc, most studies relied on single molecular markers in the past, today the great information provided by these molecules offers as many explanations as their number is. Giving this fact, a common methodology probably is not well defined due to numerous directions and approaches that researchers use to identify and classify the species, or populations. Bivalves are species that probably need common tools and methods in order to have a clear picture about their relationships. Different approaches in Bivalvia are using genetic markers to understand phylogenetics, population structure, taxonomy, ecology, etc. In phylogenetics, the COI (cytochrome oxidase subunit I) proved to be a good marker with a strong signal appropriate for this kind of relations. Although this marker was first employed in molecular studies, now its utility is being questioned (Galtier et al., 2009). In consequence, as it may be no longer used in molecular studies, there is the risk that all COI based research should be 'demolished' and new, maybe more powerful markers should be used in alternative. A common methodology shared by all researchers around the world should be employed for having a general view of the relations in class Bivalvia. COI should be the standard marker when inferring the phylogeography of bivalves, due to its strong phylogeographic signal which generally is enough to assess the distribution of the genes in space and time. If different researchers use different markers, the obtained results would be more or less different leading to misunderstandings. In the case of genetic structure of populations a good marker is ITS2. This marker has enough discrimination power to distinguish different population structures. Phylogenetic studies of Bivalvia could be better performed through a combination of genes like COI and 16S rDNA or 18S rDNA more than using a single target gene. As mitochondrial DNA is a circular molecule made up of genes that act together in the process of evolution, focusing on a single gene can lead to an incomplete assessment of the whole genetic variability.

After all, despite the multitude of the methods or markers employed, one major obstacle for obtaining common methodologies in Bivalve genetic studies is the unequal distribution of research funds amongst developed and developing countries (i.e Western vs Eastern Europe) as the latter cannot afford similar costs for the establishment of high-tech laboratories.

Nevertheless, it is obvious that a common methodology should be the best solution for bivalve population studies. This major objective is a fundamental prerequisite for developing 'best practices' in molecular studies.

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APPENDIX

TABLE 1

Bivalve sampling effort for molecular studies as suggested by literature

Endangered species	Nr. of samples (*whole ind; **only tissue)	Authors
Lampsilis sp.	18*	Roe et al., 2001
Margaritifera auricularia (Spengler, 1793 *Margaritifera margaritifera* (Linnaeus, 1758) *Potomida littoralis* (Cuvier, 1798)	36 ** 87** 10**	Machordom et al., 2003
Tridacna crocea (Lamarck, 1819)	414 **	DeBoer et al., 2008
Lasmigona subviridis (Conrad, 1835)	77**	King et al., 1999
Epioblasma triquetra (Rafinesque, 1820)	131**	Zanatta and Murphy, 2008
Epioblasma capsaiforms (I. Lea, 1834) *Epioblasma florentina walkeri* (Wilson and H.W. Clark, 1914)	32** 22**	Jones et al., 2006
Non endangered species		
Ostrea edulis (Linne, 1758)	575**	Diaz-Almela et al., 2004
Spisula spp.	56**	Hare and Winberg, 2005
Anodonta sp.	113**	Mock et al., 2004
Scapharca broughtonii (Schrenck, 1867)	100**	Cho et al., 2007
Mytilus californianus (Conrad, 1837)	150**	Ort and Pogson, 2007
Crassostrea gigas	120**	Dridi et al., 2008
Myitilus galloprovincialis	278**	Westfall and Gardner, 2010
Mactra chinensis (Philippi, 1846)	441**	Ni et al., 2011

TABLE 2

Species and tissue parts used in the protocol of Sokolov, 2000

Order	Specie	Tissue	Yeld of DNA tissue wet weight (ww)
Polyplacophora	*Tonicella marmoreal (Fabricius, 1780)*	whole body	0.48 μg mg $^{-1}$ ww
Gastropoda	*Cepaea* sp.	hepatopancreas and foot	3.5 and 3.2 μg mg $^{-1}$ ww
	Margarites helicinus (Phipps, 1774)	whole body	2.7 μg mg $^{-1}$ ww
	Littorina saxatilis (Olivi. 1792)	foot muscles	2.9 μg mg $^{-1}$ ww
	Littorina littorea (Linnaeus, 1758)	foot muscles	3.0 μg mg^{-1} ww
Bivalvia	*Mytilus edulis*	foot muscles	2.3 μg mg $^{-1}$ ww

Figure 1. Principal steps in molecular analysis

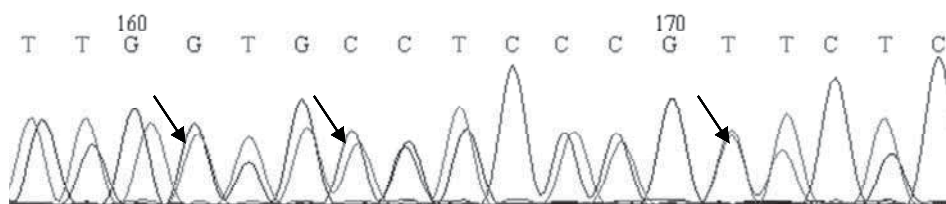

Figure 2. ITS2 sequence of *Cerastoderma glaucum* presenting double peaks (black arrows) – original data from David C. (in preparation)