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DNA BARCODING OF PLEURONECTIFORMES: *IN SILICO* ANALYSIS AND DEVELOPMENT OF MARKERS

Tesi di laurea in Struttura e Connettività delle Popolazioni Marine

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DNA barcoding of Pleuronectiformes: *in silico* analysis and development of markers.

Hokusai - Flat fish and pink.

Index.

Abstract.

DNA barcoding is a method used for the identification and discovery of animal species. It usually involved a 648 base pair fragment of the mitochondrial cytochrome *c* oxidase subunit I, known as COI. This work is focused on the study of the genetic identification in the families belonging to the order Pleuronectiformes, commonly known as flatfish, and the accuracy of the genetic marker most used for the study of their DNA barcodes. The results indicate possible existence of taxonomical mistakes because several families do not show a *gap* between maximum intraspecific distance - which is the maximum distance within a specie - and the minimum interspecific distance - which is the minimum distance between a species and its nearest neighbor (NN), meaning that the marker in use cannot reliably distinguish among those species. This study uses a bioinformatic approach to design new Pleuronectiformes barcodes and compares their coverage and resolving power with that of existing barcodes. The new primers, proposed by the program *ecoPrimer*, are based on two indices that estimate the resolution capacity of the barcodes and the taxonomic coverage of them, for the amplification. The performances of both barcoding regions already in use (COI and 16 rDNA genes), and the new primer pairs designed, were performed through a '*in silico* PCR'. The results show that the new primer pairs, located in a different regions of 16S rDNA gene compared to the universal barcode region used in fishes, present best resolution capacity and taxonomic coverage than the others already in use. This is an essential complement for future barcoding studies.

I. Introduction.

I.1. Pleuronectiformes.

I.1.1. Description and distribution.

Pleuronectiformes, (flatfish) is a ray-finned fish order (Actinopterygii) that comprises a large number of species distributed all over the world with a large economic interest. Flatfishes are instantly recognizable, with a unique asymmetric body form, developed as an adaptation to a bottom-living lifestyle. They all begin their life as pelagic, bilaterally symmetrical fishes, but during larval development undergo a spectacular metamorphosis where one eye migrates from one side of the head to the other (Brewester, 1987), right or left, depending upon the family and it could be genetically fixed. Basically, flatfishes are the only vertebrates that make so radical switch from a bilaterally symmetrical body plan. This extreme mutation of the head permits adults to rest on the seafloor on their blind side, blending with the sediment (Harrington *et al*., 2016). Afterwards they assume a benthic lifestyle, generally lying on the bottom on their blind side, on the top of the substratum or partially buried under a fine layer of sand with their eyes protruding outside. Some species appear to have preferences for particular substrata, while others can be found on many of that, like silt, mud, sand, rocky or pebbly bottoms (Gibson, 2005).

Pleuronectiformes represent a very specialized assemblage within ray-finned fish. Flatfish fossils date back to the Eocene (Verneau *et al*., 1994), but the origin of group remains still unknown (Chapleau, 1993). The oldest fossil found, known as *Eobothus minimus*, indicate the presence of flatfishes as far back as the early Tertiary, in the Eocene (approximately 53-57 million years ago; Schwarzhans, 1999).

It represents an advanced evolutionary line within the main suborder Pleuronectoidei. Others fossils are found from the Eocene with the structural features and diversity of the Order, indicating that the diversification occurred earlier than 45 million years ago, before Lutetian (Chanet 1997; Schwarzhans 1999). In addition, these fossils highlight the fact that all the anatomical specializations, such as cranial asymmetry and modifications of the caudal skeleton, occurred before. However 'when' is a question that recent molecular phylogenetic studies are trying to resolve. Harrington *et al*. (2016) claims that the flatfish asymmetry concerning the complete orbital migration is probably evolved over an interval of no more than 2.97 million years.

The life near the sea bottom allows flatfishes to live successfully and play the ecological roles as both benthic predator and prey (Gibson, 2005). Three-quarters of flatfishes show a tropical distribution, while one quarter is northerly and southerly distributed in temperate waters (Pardo *et al.,* 2005). In tropical areas, flatfishes can be found in a variety of habitats including mangrove estuaries, nearby mud flats, seagrass bed and on mud bottoms. They can be generally found where substrata mostly consisted of sand with algae, around coral, and in lagoons associated with reefs. The majority of flatfishes (such as Bothidae, Samaridae, Poecilopsettidae), inhabiting the Indo-Pacific region, are small fishes without any commercial importance. The larger species (like Psettodidae and some Paralichthyidae and Soleidae) are captured, in the majority, in tropical fisheries, but also in temperate and subartic zones (Gibson, 2005).

I.1.2. Importance in the Europe economy.

Flatfish are sold in European markets in different ways, mostly as frozen fillets (Sotelo *et al*., 2001). The high-value species are sole (*Solea solea)*, turbot (*Scophthalmus maximus*), European plaice (*Pleuronectes platessa*) and flounder (*Platichtys flesus*) (Cerdà *et al.,* 2013). In the Northeast Atlantic, there are a total

of 11 relevant species for fisheries including representatives of Pleuronectidae (such as North Sea plaice *Pleuronectes platessa*, and the Atlantic halibut *Hippoglossus hippoglossus*), Soleidae (such as the common sole *Solea solea*, and Senegalese sole *S. senegalensis*), and Scophthalmidae (with the turbot *Scophthalmus maximus*, the brill *S. rhombus*, and the megrim *Lepidorhombus whiffiagonis*). In Europe, the main flatfishes used in aquaculture are the common sole, the Senegalese sole, that is especially commercialized in Southern Europe (Garcia-Cegarra *et al*., 2013), the turbot and the Atlantic halibut. Currently, the aquaculture of *S. senegalensis* in Spain and other European countries is seriously impaired because of difficulties in controlling reproduction in captivity and suboptimal larval nutrition (Cerdà *et al*., 2013).

I.1.3. Taxonomy.

According to Chapleau (1993), Pleuronectiformes can be divided in the suborders Psettodoidei and Pleuronectoidei and thirteen families (Fig. 1). The NCBI (National Center for Biotechnology Information) places the Order of Pleuronectiformes using this taxonomy:

Kingdom: Animalia → Phylum: Chordata → Subphylum: Vertebrata → Class: Actinopterygii → Infraclass: Teleostei → Order: Pleuronectiformes → Suborders:

1. Pleuronectoidei → Family: Achiridae, Bothidae, Citharidae, Cynoglossidae, Paralichthodidae, Paralichthyidae, Pleuronectidae, Poecilopsettidae, Rhombosoleidae, Samaridae, Scophthalmidae, Soleidae
2. Psettodoidei → Family: Psettodidae

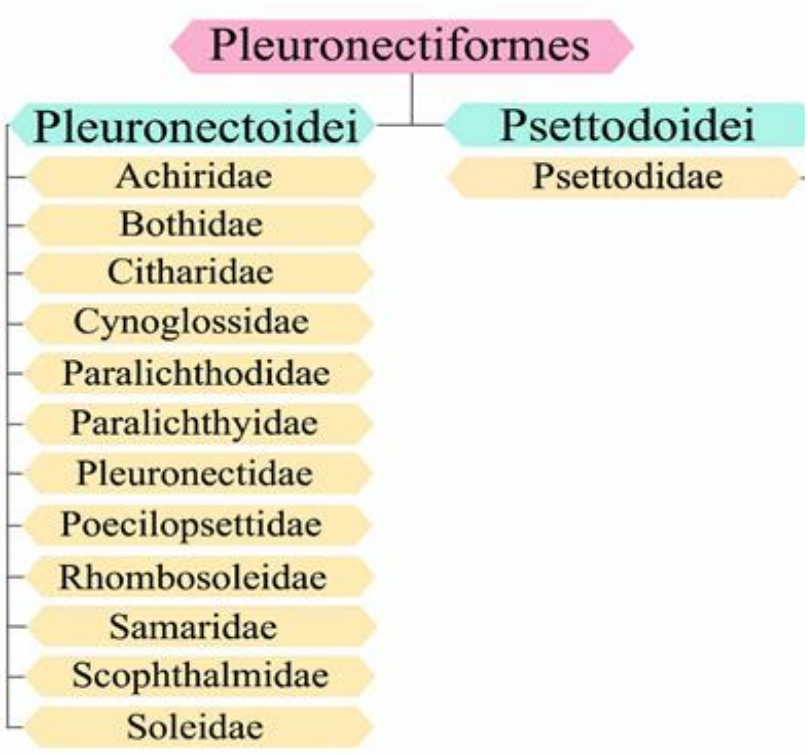

Fig.1: Taxonomy of Pleuronectiformes as proposed by NCBI.

Taxonomically, the best known flatfishes are those living in areas with large commercial fisheries of the northern hemisphere, such as both Atlantic and Pacific Oceans (Pleuronectidae, Scophthalmidae and some representatives of Soleidae and Paralichtyidae). In the Southern temperate regions like Australia-New Zealand and South America, commercial fisheries were also supported by Rhombosoleidae and Paralichthydae. In spite of the greatest species diversity of flatfish in the Indo-Pacific tropical areas, the taxonomy is still unclear because of taxonomy difficulties to the species identifications and the small size of species (Gibson, 2005). However, one of the major questions concerning flatfish phylogeny is the presumptive monophyly of the order, due to three synapomorphic characters:

1. Migration of one eye during ontogeny
2. Anterior position of the origin of the dorsal fin
3. Presence of a *recessus orbitalis* (accessory organ associated with eyes)

A recent phylogenetic study based on UCE (ultraconserved DNA element), with over 1,000 loci sampled from 45 carangimorph species, supports flatfish monophyly, assuming that *Psettodes* is a sister lineage to Pleuronectoidei, which contains all other flatfish species, and that they all belong to an unique branch within Carangimorpha (Harrington *et al.*, 2016). On the contrary, previous studies claimed a polyphyletic origin, suggesting a multiple origin from different groups of symmetrical fishes (Pardo *et al*., 2005).

I.1.4. Flatfish Genomics and Genetics.

Fish aquaculture is one of the most sustainable source of food for humans, mostly because of its high content in proteins and lipids (Hibblen *et al*., 2006). In order to protect the consumer, the EU has strict regulations for seafood labelling, which most include the species name (EU Council Regulation No 104/2000, EU Commission Regulation No 2065/2001). It is extremely important to trace the products in order to avoid and to detect commercial fraud, but this is not so easy, because processed aquatic food is the most widely traded type of food since it lost all the morphological characters suitable for species identification during the conservation procedures (Benard-Capelle *et al.*, 2015). The genetic identification of species can help to solve this problem (Kochzius *et al*., 2010).

The high demands in flatfishes, due their economic importance, increases fishing pressure with the serious consequence that wild stocks has reduced genetic diversity in plaice, with a shift towards earlier sexual maturation at smaller size in sole and plaice as well (Hoarau *et al*., 2005; Mollet *et al*., 2007; van Walraven *et al*., 2010). As a consequence, the development of aquaculture has been proposed for some of these species to supplement the demands for human consumption and reduce the pressure on natural populations. Identification and characterization of the genes, as well as genetic networks controlling traits like growth rates, reproduction, larval development and disease resistance, would

allow for a better optimization of production and management procedures in the industry. The lack of knowledge concerning pathologies and their prevention, the procedures associated with the control of reproduction in captivity, the proper amount of diet to reduce malformations and pigmentation anomalies, and, instead, to improve growth and disease resistance with all the physiological mechanism involved, are some of the major problems in flatfish aquaculture (Millàn *et al*., 2011; Agulleiro *et al*., 2006). In the last few years an important effort has been directed towards the use of functional genomics, metabolomics and proteomics to better characterize, as has already been mentioned, reproduction, development, nutrition, immunity and toxicology of flatfishes. The aim is to identify the critical genes and molecules that control physiological traits in order to improve current flatfish aquaculture structure, and several studies have proceeded on this way (Garcia-Cegarra *et al*., 2013; Portela-Bens *et al*., 2016). However, since flatfishes are non-model organisms, the genomic information for this Order has remained very limited. The usual techniques used for genome mapping studies, such as EST database or micro-arrays, are very limited because of high cost and time-consuming with a low analysis number, so the use of new sequences technologies that allow massive-scale DNA sequencing (Next Generation Sequencing) with a feasible and cost-effective way, is the newest approach (Cerdà *et al*., 2013).

I.2. Importance of DNA genetic markers for species identification.

The genetic marker analysis is an important tool for identify populations with genetic or taxonomic uncertainty by comparing the genotypes at a number of polymorphic loci, in order to establish management units within species. It also provides useful tools for preventing illegal hunting and protecting endangered species (Arif *et al*., 2011). In the animal realm, huge number of studies, for

conservation genetics, are based on the sequence variation of the mitochondrial DNA (mtDNA) which consists of a haploid, maternally-inherited circular chromosome and, ordinarily contains 36 or 37 genes: 2 for rRNAs, 22 for tRNAs and 12 or 13 coding genes that translate subunits of multimeric proteins of the inner mitochondrial membrane (Fig. 2). This type of genetic marker has its own particularities such as histone-free, limited repair ability with a relatively high mutation fixation rate (5-10 times than nuclear DNA), and it's a better target for analysis because of its lack of introns, its limited exposure to recombination and its haploid mode of inheritance (Saccone *et al*., 1999). Mitochondrial DNA has been evolved faster than nuclear genome, the rate of evolution is different across mtDNA genes, and it has been used for examine various phylogenetic relationship. Furthermore, most cells contain multiple copies of the mtDNA molecule so it can be obtained from very small amounts of cell tissue that contained degraded DNA. The mtDNA sequences were used in conservation genetics for resolving taxonomies, establishing interspecific hybridization, population structuring and the detection of illegal hunting and conservation of endangered animals (Arif *et al*., 2011). It is a tool for structural, evolutionary and population studies in several eukaryotic organisms because of the easy way in how sequence information can be obtained, by selective gene amplification with universally conserved primers (Tinti *et al*., 1999). However, mtDNA phylogeny represents only the genealogy of a gene that is almost only maternally transmitted, so for a more accurate interpretation of population biodiversity, genetics or phylogeny, additional markers targeting nuclear DNA need to be incorporated.

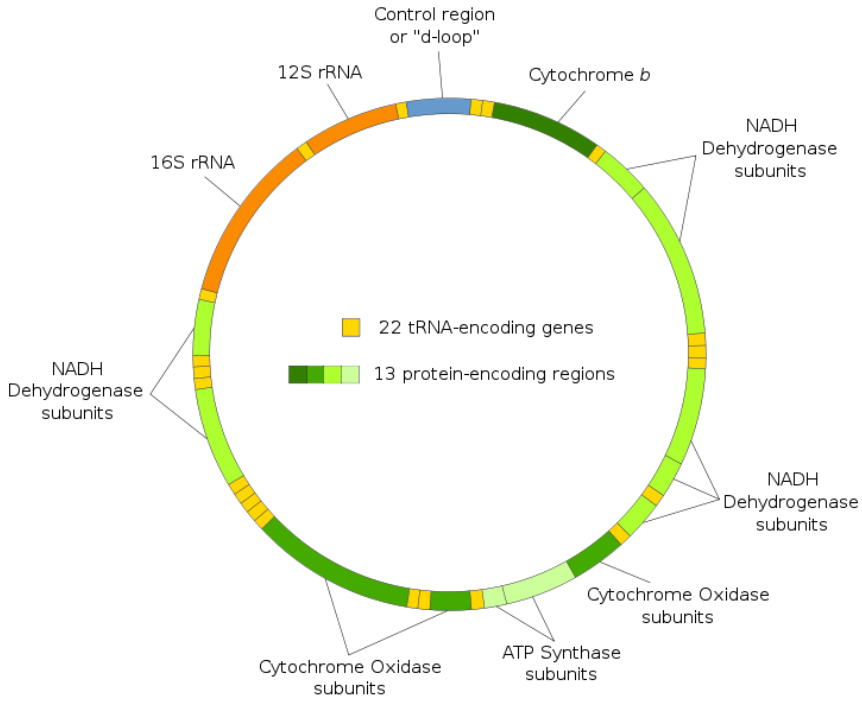

Fig. 2: mtDNA genome, modelled as a circle.

I.2.1. Use of Cytochrome Oxidase I and 16S rDNA for the flatfish DNA barcoding.

At the beginning, the resolution of the phylogenetic relationships within Pleuronectiformes was provided by using proteins as biochemical taxonomical markers, just in those cases where the product to be identified has not subjected to thermal treatment, because otherwise proteins become denatured and difficult to be analyzed. This treatment did not provide a solid species identification, because it also requires the use of authentic species protein extracts to be analyzed together with the unknown samples (Sotelo *et al*., 2001). DNA, instead, is now used as a biochemical, taxonomical marker in a high variety of process treatments, even when the product is heated, because is still possible the amplification of a short fragment containing species diagnostic value (Quintero *et al*., 1998). The partial DNA sequences of the cytochrome *c* oxidase subunit I (COI) and of the large RNA ribosomal subunit gene (16S rDNA), are the most used genetic markers for fish species identification, seafood control, fisheries control and

species delineation (Kochzius *et al*., 2010). Several molecular studies addressing in the species identification of flatfishes with these mtDNA fragments (Tinti *et al*., 2000; Tinti & Piccinetti 2000; Sotelo *et al*., 2001; Pardo *et al*., 2005).

I.3. Barcoding and Metabarcoding concepts.

DNA barcoding is a method specialized in species identification and a key tool for assessing biodiversity in both taxonomic and environmental studies. It is proposed to assign an unambiguous tag to each species in order to discriminate between taxa (Ficetola *et al*., 2010). It identifies biological diversity using standardized DNA regions, called marker, that must be as universal as possible and must contain enough information to discriminate between closely related species, and may also to discover new ones (Riaz *et al*., 2011). Therefore, DNA barcode is a small piece of the genome found in a large range of species and is usually located on the mitochondrial genome for animals or on the chloroplast genome for plants (Coissac *et al*., 2012). Barcoding has shown that DNA barcode can discriminate species across the whole animal kingdom (Tyagi *et al*., 2010) and in all kinds of animal groups such as mammals, fishes, birds, insects and others (Hebert *et al*., 2004; Hubert *et al*., 2008; Clare *et al*., 2011). Certain genes can be used because some regions of these are well conserved, so they show a very slow rate of evolution and very little change in their DNA sequence. One of the most used barcode in animals is the cytochrome *c* oxidase subunit I (COI), with 648 base pair fragment near the 5' end, as a standard barcode for animal identification (Hebert *et al*., 2003).

In this context, it is established that the *gap* between maximum intraspecific distance - which is the maximum distance within a species - and the minimum interspecific distance - which is the minimum distance between a species and its nearest neighbor (NN) – can be used for species delimitation (Fig. 3-A) (Ashfaq

et al., 2013). The extent and the separation between intraspecific variation and interspecific divergence for the selected marker is extremely important in order to obtain the best accuracy in the taxonomic work. The much bigger is the overlap between genetic variation between species and the divergence that separate sister species (Fig. 3-B), the less effective barcoding will be (Meyer and Paulay, 2005).

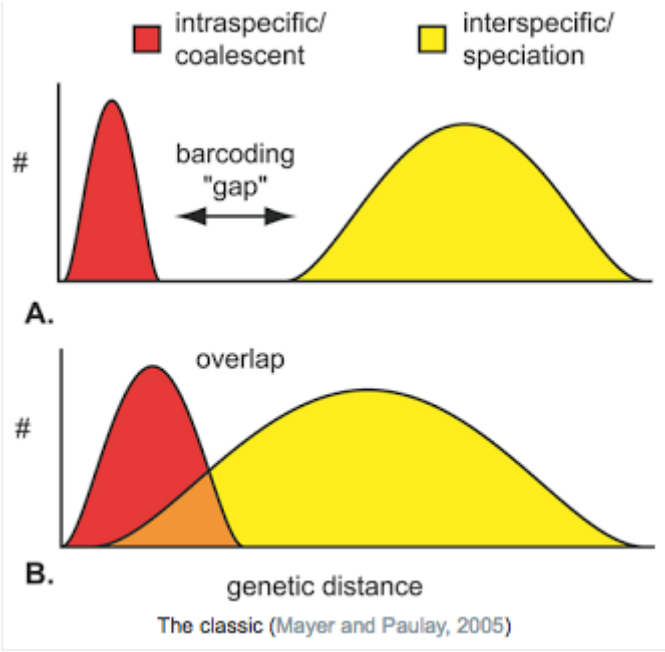

Fig. 3: Schematic of the Inferred Barcoding Gap for two species. A good Barcoding need to have no overlap to discriminate between species (A). Overlap means "no gap" (B).

When such overlap is real, it means that the marker used for the study cannot reliably distinguish among those species. Subsequently other portions of DNA have been proposed as barcodes, because the performances of different DNA regions may change in different taxa (Ficetola *et al*., 2010).

Many DNA barcoding resources have been developed since 2003 and the researchers from all over the globe have joined two major international initiatives in order to develop an efficient DNA barcoding based species identification system, universally applicable:

1. The iBOL (International Barcode of Life), activated in 2010 by the Biodiversity Institute of Ontario at the University of Guelph, Canada. They

created the barcode reference library BOLD (Barcode of Life Data System) (http://boldsystems.org) which is a barcode database assembling a global network of taxonomist, biologists and geneticists, available to the public.

2. The CBoL (Consortium for the Barcoding of Life), activated in 2004 by the grants from Alfred P. Sloan foundation (Bhargava and Sharma, 2013).

Reflecting the rapid growth in barcode coverage (Jinbo *et al*., 2011), the Barcoding of Life Data Systems (BOLD; Ratnasingham & Hebert 2007), now includes records for more than 174K animal species with more than 5 million barcode sequences (Access in Nov 2016). The order Pleuronectiformes contains 4,548 specimens with barcodes collected from 46 countries (Access in Nov 2016).

DNA barcoding can be divided into two main types depending on its application in different fields: DNA barcoding *sensu stricto* and DNA barcoding *sensu lato* (Valentini *et al*., 2009). The *sensu stricto* barcoding is the standard barcoding defined by CBoL and described above. On the other hand, the *sensu lato* corresponds to a DNA-based taxon identification using diverse techniques that lie outside the CBoL approach and it is known as DNA metabarcoding or environmental barcoding, which could be defined as the simultaneous identification of several species, even from environmental samples, using high throughput sequencing techniques (Clarke *et al*., 2014). Manipulation of such large datasets requires very specifically program, such as OBITOOLS package (http://metabarcoding.org/obitools) (Boyer *et al*., 2016).

I.3.1. Environmental DNA.

Advances in DNA sequencing technology and bioinformatics have significant potential to strengthen biological monitoring in the ocean. All living things contain DNA and through metabolic waste or sloughed cells, they generate waste that will persist in the environment for some period of time (Kelly *et al*.,

2014). This DNA obtained directly from environmental samples (sediments, soil, water, air) represents the Environmental DNA (eDNA), that could be used to detect individual animal species of interest even if they are present at very low abundances (rare or endangered species). In the beginning, it was a method to assess the diversity of macro-organism communities applied to ancient sediments, revealing the past of extinct and extant mammals, plants and birds (Willerslev *et al*., 2003). Only recently the approach has successfully used on several samples, including marine and terrestrial environments, to increase the accuracy of the distribution of vertebrate species, and decrease the cost of survey (Maruyama *et al*., 2014). Obviously, eDNA monitoring cannot replace field observation by experienced ecologists and taxonomists, but its integration it will be helpful to obtain basic data on distribution and abundance of species (Thomsen *et al.*, 2012). As mentioned before, the amplification of barcode markers from eDNA with PCR, leads to a sequence that can became a proxy for the biodiversity present in the collected samples. Thus, one of the major challenges of metabarcoding is to find new primers pair, with their associated barcode regions, which are appropriate for particular environmental applications (Coissac *et al.*, 2012).

I.4. Importance of good primers to amplify mtDNA sequences.

Other than all the properties of an ideal DNA barcode, high taxonomic coverage and high resolution are essential for the application of barcodes to a number of taxa as large as possible, and it is necessary that the DNA barcode region should have sufficiently conserved flanking regions to design universal primers (Ficetola *et al*., 2010). These primers should be developed in order to amplify a specific number of regions of the mtDNA genome in a wide range of taxa (Arif *et al.*, 2011). Robust primers enable the routine recovery of specific

segments of the mitochondrial genomes (Hebert *et al.*, 2003). In literature there are various sequences utilized for flatfish DNA barcoding (Kochzius *et al.*, 2010). However, the most used primer pair to resolve Pleuronectiformes genetic structure is that designed by Palumbi (1994), which amplify a 500-600 bp fragment of the 16S rDNA. In the BOLD (http://www.boldsystems.org/index.php/databases), it is also possible to find a comprehensive registry of primers created from the users, which can be used for identify closely related species, as well as higher taxa, in many animal phyla. All this indicates that exist a high number of available primers that will be helpful to achieve the same objective, but just recently, thanks to the new generation software tools that can handle large dimension data, in association with the enough information of the complete mitochondrial DNA available, it is possible to find and create the perfect primers pair for species identification.

This study propose an approach for comparing the performance of potential barcoding regions, through a '*in silico* PCR' performed on the mitochondrial Pleuronectiformes dataset, and based on two indices that estimate the resolution capacity of the barcodes and the taxonomic coverage of the primers used for the amplification. The program used for this aim, was the *ecoPrimers* software that selects highly conserved primer pairs and evaluates the quality of these primer pairs using two evaluation indices (Fig. 4) as proposed by Ficetola (2013):

- Bc → Barcode coverage → represents the proportion of amplified taxa for a specified taxonomic rank, among the total number of taxa of the same level, and estimates the amplification range of a primer pair.
- Bs → Barcode specificity → represents the proportion of specifically identified taxa among amplified taxa and evaluates the discrimination capacity of the amplified marker.

After that, it is necessary to compare the resolution and specificity of different primer set on the same set of mitochondrial sequence data by performing an *in silico* PCR with the program ecoPCR.

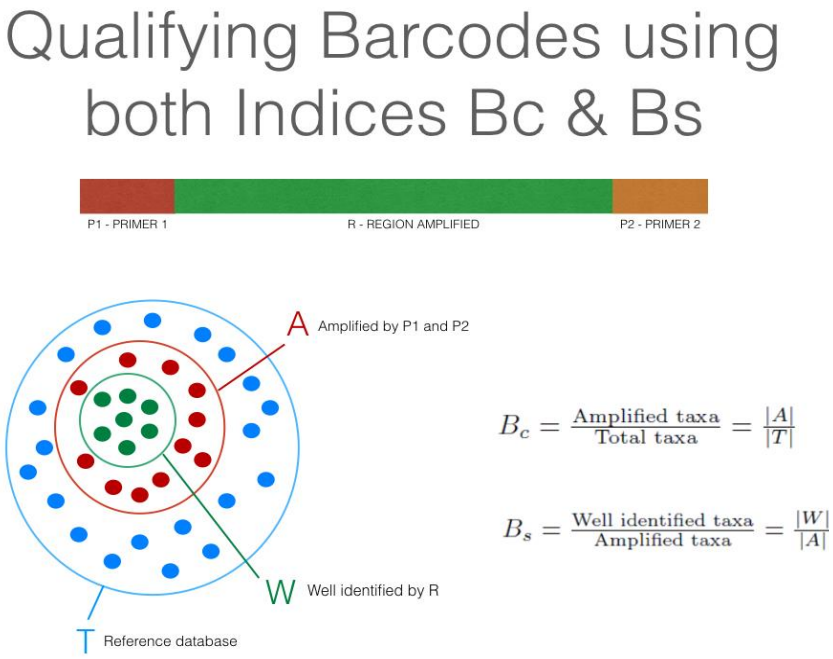

Fig. 4: The concept of Primer Evaluation Indices Bc and Bs.

II. Objectives of the study.

1. Develop a report of the characteristics and current genetic knowledge of species and families belonging to the order Pleuronectiformes.
2. Describe the current state of both COI and 16S rDNA sequences of the order Pleuronectiformes included in the public database used for the achievement of the DNA barcoding studies.
3. Analyze the *gaps* between both maximum and avarage intraspecific distance with minimum interspecific distance of the target species of this study.
4. Design new primers using the bioinformatic software *ecoPrimers*, to improve the DNA barcoding of Pleuronectiformes, electing the most useful pair of primers in order to amplify and sequence mtDNA markers.
5. Compare and analyze, via PCR *in silico*, different DNA barcodes already used in previous studies of flatfish, along with the new primers obtained in the previous objective 4.

III. Materials and Methods.

III.1. DNA barcoding.

III.1.1. DNA sequences database.

For the purpose of this work, as well as for surveying the variation of the mitochondrial genes COI and 16S rDNA, all the 12 families of Pleuronectoidei, plus the Suborder Psettoidei itself, were targeted. Orthologous sequences of flatfish species were retrieved from the *National Center for Biotechnology Information* (NCBI), which contains information provided from different sources, such as GenBank, RefSeq, TPA y PDB. For each family, both COI and 16S rDNA species-specific sequences, were downloaded in a *fasta* format and, using the software *Obigrep* of the Obitools package for Linux (Boyer *et al*., 2016), they were cleaned from all the possible errors. The software *Obigrep* is specifically designed for filtering the sequences files, taking account of theirs taxonomic information and to simplify the manipulation of sequence files (https://git.metabarcoding.org/obitools/obitools/wikis/home). After cleaning, the sequence data are uploaded in the *SpeciesIdentifier* program (Meier *et al*., 2006) for performing the statistical description, using the information provided, like the species of each sequences, the accession number and the complete sequence.

III.1.2. Nucleotide sequences alignment.

Family-specific alignments were created from cleaned sequences using the program Multiple Alignment Using Fast Fourier Transform (MAFFT) (Katoh *et al*., 2002) with the default parameters (Strategy: Auto). The sequences that showed reverse strands marked by the blue line (instead the red line) were reported and appropriately converted into theirs reverse-complement sequences, with the web program Reverse complement (http://www.bioinformatics.org/sms/rev_comp.html).

III.1.3. Gap analysis: comparing the intraspecific and interspecific genetic distances.

With the aligned sequences obtained in MAFFT, the pairwise genetic distances among all the sequences from each family were estimated. For the study of *gap* between the maximum intraspecific and the minim interspecific distance, it is necessary to have all these genetic distance values between each pair of sequences, for each flatfish family. This will generate an enormous number of comparisons that it must be treated later to perform the barcode gap analysis, with the use of dedicated programs. To do that, for each family and from the alignments obtained, the genetic distance between each pair of sequences was calculated with the program Molecular Evolutionary Genetics Analysis (MEGA) using the evolutionary model Kimura-2-parameter (Kimura, 1980) with the pairwise-deletion option, to eliminate nucleotide gaps (Ashfaq *et al*., 2013). Thus, the maximum and the average intraspecific distance (among sequences from each species) and the minimum interspecific distance (the smallest value among all the comparisons made between sequences from all species) were obtained by ranking pairwise distance values according to taxonomy.

The data obtained in MEGA were sequentially reorganized and processed in Linux with the use of scripts realized for this aim, in order to get the values of maximum and the average distance between sequences inside a species, and the minimum distance with the other species, within every family analyzed.

All the files generated with this script were processed in Microsoft Excel for visualizing gaps i) between the maximum interspecific distance and the minimum interspecific distance and ii) and between the average intraspecific distance and minimum interspecific distance. In addition, the comparison of the maximum intraspecific distance with the number of the sequences for each species for each family of Pleuronectiformes was also represented. For the interpretation of the information that were obtained with the elaboration of these comparative graphs,

it is necessary to keep in mind the fact that a species is different from its Nearest Neighbor (NN) if its maximum intraspecific distance is less than the minimum genetic distance between them (Ashfaq *et al*., 2013). To better understand that, a red line was diagonally designed in the graphics, which represents the points where the maximum or average intraspecific distance value is equal to the minimum interspecific distance value and theirs ratio is 1.

III.2. Primer design.

The design of new barcoding primers for Pleuronectiformes is one of the most interesting goals of this work. This aim can be addressed using the mitochondrial genomes of the Pleuronectiformes, available in the NCBI database, and the *ecoPrimers* program (Riaz *et al*., 2011) from the Obitools package (Boyer *et al*., 2016). The primer design was carried out firstly at the Order level, to obtain a perfect primer pair for this taxonomic group. The *ecoPrimer* program was then used also for obtain primer pairs at the Family, as well as for any different taxonomic level. To use *ecoPrimer* program, the following steps must be done:

1. Available complete mitochondrial flatfish genomes were downloaded from GenBank (accessed in July 2016), representing 120 sequences of 11 Family of the Pleuronectiformes.
2. The *ecoPrimer* program designs the most efficient barcode primers and markers, based on the set of reference sequence records downloaded, and identifies highly conserved and useful sequences to use them as sites of primer annealing. It is an useful tool because it maximize the potential of the intervening sequence among these primers to discriminate between taxa and to amplify a variable DNA region. The ranking of the primer pairs is based on the two indexes Bc and Bs that assess the taxonomic range potentially amplified by a primer pair (Bc) and the discrimination capacity

of the amplified region (Riaz *et al*., 2011). The parameters used in the program were:

- O = 20, as the primer length;
- e = 2, as the maximum numbers of errors allowed per primer;
- 3 nucleotides on the ‘3 end of the primers that must have a strict match with their target sequences;
- q = 0.7, as the proportion of the sequence records in which a strict match between the primers and their targets occurs;
- s = 0.7, as the proportion of the example sequence records that must fulfill the specified parameters for designing the barcodes and the primers;
- l = 10, as the minimum length of the barcode, excluding primers;
- L = 300 – 800, as two different maximum length of the barcode;
- c, because the sequences of the mtDNA are circular

III.3. PCR *in silico*

Using the results obtained with the *ecoPrimers* program, along with the combinations of primers already in use widely in the literature, it was realized the *in silico* amplification of the different sequences of Pleuronectiformes for each family. It was performed with the program *ecoPCR* (Clarke *et al*., 2014), included in the Obitools package, that uses a pattern-matching algorithm to identify sequences within a database that can be amplified with a given primer pair. It compares the degree of taxonomic coverage and the amplification of the sequences belonging to the different family of the order flatfish.

For the accomplishment of this bioinformatics study, a local dataset of complete Pleuronectiformes was used in order to test whether metabarcodes provided better taxonomic coverage. To verify the best coverage of every pair of primers, that join the end-regions of the DNA sequences, the PCR *in silico* was realized for

each family excluding Cytharidae and Paralichthididae because no data was available for the entire mtDNA. To carry out this goal, all the sequences, along with their Taxonomic information, were downloaded from NCBI nucleotide and taxonomy, respectively. Then they were converted into the local database with *ecoPCR* by using the *ecoPCRFormat.py* script (script in Phyton of the Obitools program). After that, the different primer pairs (Table 1) were tested separately on each family of the database using the same parameters:

- e = 4, as the maximum number of errors allowed per primer;
- the taxonomic group identified by its "taxid" specific for each family (R=accession number of the family);
- c, because the sequences of the mtDNA are circular

The results obtained for each family and for each pairs of primer include: the length of the original sequences, the scientific name of the species, the length of the amplified fragment (excluding primers), the number of allowed mistakes, the temperature of denaturation (Tm), etc. From these data, a statistic summary is realized with the Bc value of each pair of primers using the *ecotaxstat* program of the Obitools package.

Table 1: Pairs of primers used for the PCR *in silico.*

Locus	Primer sequences	Reference
COI	FishF1- TCAACCAACCACAAAGACATTGGCAC FishF2- TCGACTAATCATAAAGATATCGGCAC FishR1- TAGACTTCTGGGTGGCCAAAGAATCA FishR2- ACTTCAGGGTGACCGAAGAATCAGAA	Ward *et al.* (2005)
16S rDNA	16Sbr- CCGGTCTGAACTCAGATCACGT 16Sar- CGCCTGTTTAACAAAAACAT	Palumbi *et al.* (1991)
16S rDNA	16S_300F- GGAGACAGTTAAGCCCTCGT 16S_300R- TACCAAAAACATCGCCTCTT	*ecoPrimer* 300bp (in this study)
16S rDNA	16S_800F- CTCGTACCTTTTGCATCATG 16S_800R- GCGATGTTTTTGGTAAACAG	*ecoPrimer* 800bp (in this study)

IV. Results and Discussion.

IV.1. Results.

IV.1.1. Current flatfish resources of the COI and 16S rDNA genes.

The taxonomical information of the NCBI Taxonomy and the bibliographical reference of Chapleau (1993) were used to describe the total number of species for each Family of Pleuronectiformes. The Paralichthodidae family contains one species, so it was excluded from the barcode gap analysis because it is not possible to perform pairwise intraspecific comparisons with only one species.

The genomic flatfish information available from NCBI (access in Nov 2016) indicates that there are 221,090 DNA and RNA sequences, more than 50,000 described proteins, 2 completely sequenced genomes and almost 27,000 gene loci collected (Table 2). In the BOLD database 474 species are reported, from which 446 present barcodes, and 1,470 primers are published in the Primer database section from BOLD (access Nov 2016).

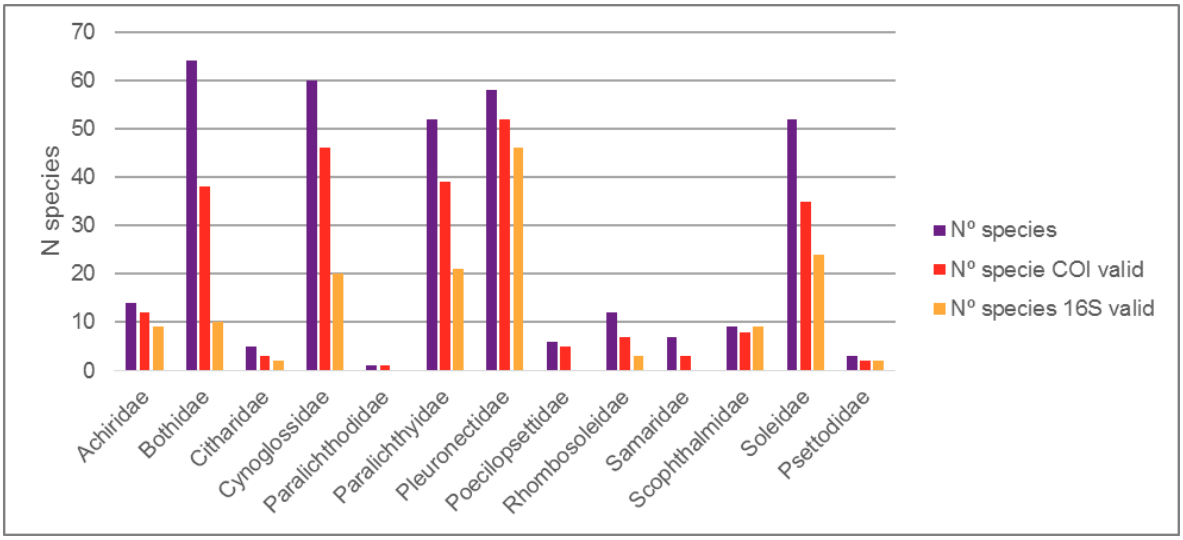

Fig. 5: Graphic representation of the total number of species of the Order Pleuronectiformes divided by Family and the total number of species that have COI and 16S valid sequences.

Table 2: Actual information available in NCBI database about Genes of the Pleuronectiformes (a) and Genomes (b).

Genomes		
Assemby	2	genome assembly information
BioProject	96	biological projects providing data to NCBI
BioSample	988	descriptions of biological source materials
Clone	0	genomic and cDNA clones
dbVar	0	genome structural variation studies
Genome	2	genome sequencing projects by organism
GSS	24	genome survey sequences
Nucleotide	221090	DNA and RNA sequences
Probe	9775	sequence-based probes and primers
SNP	90	short genetic variations
SRP	560	high-throughput DNA and RNA sequence read archive
Taxonomy	1	taxonomic classification and nomenclature catalog

(a)

Genes		
EST	84625	expressed sequence tag sequences
Gene	26951	collected information about gene loci
GEO DataSets	640	functional genomics studies
Geo Profiles	0	gene expression and molecular abundance profiles
HomoloGene	0	homologous gene sets for selected organisms
PopSet	337	sequence sets from phylogenetic and population studies
UniGene	0	clusters of expressed transcripts

(b)

The number of barcoded species with the Cytochrome Oxidase I sequences are less than the total number for 11 families out of 12 (Table 3; Fig. 5), especially after the sequence cleaning of the all possible errors with the *Obigrep* software. The family with highest number of valid COI sequences is Pleuronectidae with 926, on a total of 2,100 COI valid sequences (Table 3). The number of species barcoded with the 16S rDNA sequences are definitely much less than those barcoded with the COI (Fig. 5), and there are five more families with no adequate

data to be used for this work: Citharidae, Poecilopsettidae, Rhombosoleidae, Samaridae and Psettodoidei.

Table 3: Data of the total number of the species according to NCBI, the total number of species with COI sequences and 16S sequences, and the total number of the COI and 16S sequences used for this work.

	Nº species	Nº specie COI valid	Nº species 16S valid	Nº sequences COI valid	Nº sequences 16S valid
Achiridae	14	12	9	60	19
Bothidae	64	38	10	181	13
Citharidae	5	3	2	61	2
Cynoglossidae	60	46	20	243	108
Paralichthodidae	1	1	0	2	0
Paralichthyidae	52	39	21	234	40
Pleuronectidae	58	52	46	926	163
Poecilopsettidae	6	5	0	10	0
Rhombosoleidae	12	7	3	30	3
Samaridae	7	3	0	6	0
Scophthalmidae	9	8	9	109	20
Soleidae	52	35	24	224	47
Psettodidae	3	2	2	14	3
TOTAL	343	251	146	2100	418

IV.1.2. Comparison of intra- and interspecific genetic distances (barcode gap analysis).

A comparative analysis of the genetic interspecific distances (differences between sequences of different species) with both maximum and average genetic distances at the intraspecific level (differences between individual sequences attributed to the same species) was performed and illustrated for each Family of the Pleuronectiformes in order to analyze the barcode *gap*. For a better understanding it was used a red line representing the point where the ratio between maximum/average intraspecific distance and minimum interspecific distance is 1, i.e. the values are equal. The available data were not adequate for the barcoding

gap analysis of Citharidae, Poecilopsettidae, Rhombosoleidae, Samaridae and Psettodoidei at 16S rDNA marker, and of Paralichthodidae both at the COI and 16S rDNA markers.

Two alternative scenarios were obtained:

- All the genetic distance values show a gap between intraspecific and interspecific distance because they are above the red line. As well, both the maximum and average distance to minimum, are higher than the respective intraspecific distances for all the species (Fig 6.a).
  The families showing this pattern are: Achiridae, Citharidae, Poecilopsettidae, Rhombosoleidae and Samaridae at COI, Bothidae and Paralichthyidae at 16S rDNA (Appendix, Fig. 1.1; 2.2; 3.1; 5.2; 7.1; 8.1; 9.1).
- One or several genetic distance values are below the red line, indicating that the maximum and/or the average intraspecific values are higher than the minimum distance within a species (Fig 6.b).
  The families showing this pattern are: Achiridae at 16S rDNA, Bothidae and Paralichthyidae at COI, Cynoglossidae, Pleuronectidae, Scophthalmidae and Soleidae at both COI and 16 rDNA. More specific:
  - Achiridae 16S rDNA → all species show the barcode gap between intraspecific and interspecific distance except 2: *Trinecte paulistanus* and *Achirus lineautus* (Appendix, Fig. 1.2);
  - Bothidae COI → all species show the barcode gap except *Bothus ocellatus*, that shows the value under the red line in the comparison between the maximum intraspecific and average intraspecific, and *Laeops nigromaculatus*, which shows the average intraspecific/minimum interspecific plotted value under the red line (Appendix, Fig. 2.1);

- Cynoglossidae COI → eleven species, such as *Cynoglossus acaudatus, Cynoglossus capensis, Cynoglossus macrostomus, Cynoglossus itinus, Paraplagusia japonica, Symphurus civitatium,* do not exhibited the barcode gap because they show the maximum and the average intraspecific values higher than the minimum interspecific distance (Appendix, Fig. 4.1);
- Cynoglossidae 16S rDNA → all species show the barcode gap except *Symphurus plagusia, Symphurus plagiusa* and *Cynoglossus semilaevis*, which show the value under the red line indicating the absence of a gap (Appendix, Fig. 4.2);
- Paralichthyidae COI → five species, such as for *Pseudorhombus natalensis, Pseudorhombus arsius, Syacium papillosum, Paralichthys isosceles*, do not show the barcode gap because the maximum and the average intraspecific values are higher than the minimum distance (Appendix, Fig. 5.1);
- Pleuronectidae COI → twelve species, such as for *Lepidopsetta bilineata, Hippoglossoides dubius, Platichthys flesus, Kareius bicoloratus*, do not show the barcode gap because the maximum and the average intraspecific values are higher than the minimum distance (Appendix, Fig. 6.1);
- Pleuronectidae 16S rDNA → five species do not show the barcode gap, such as for *Glyptocephalus cynoglossus*, *Pleuronectes platessa* because the maximum and the average intraspecific values are higher than the minimum distance (Appendix, Fig. 6.2);
- Scophthalmidae COI → all species show the barcode gap between intraspecific and interspecific distances except for *Psetta maxima*, (Appendix, Fig. 10.1);

- Scophthalmidae 16S rDNA → all species show the barcode gap except for *Lepidorhombus whiffiagonis*, which shows the value under the red line indicating the absence of a gap (Appendix, Fig. 10.2);
- Soleidae COI → five species were plotted below the red line, such as for, *Austroglossus pectoralis*, *Pegusa impar*, *Pardachirus pavoninus*, *Pardachirus pavoninus*, showing that the maximum and the average intraspecific values are higher than the minimum distance (Appendix, Fig 11.1);
- Soleidae 16S rDNA → all species show the barcode gap except for *Dicologlossa cuneata*, which shows the value under the red line indicating the absence of a gap (Appendix, Fig. 11.2);
- Psettodoidei COI → *Psettodes bennettii* and *Psettodes erumei* are below the red line in the maximum intraspecific distance vs the minimum interspecific distance (Appendix, Fig. 12.1).

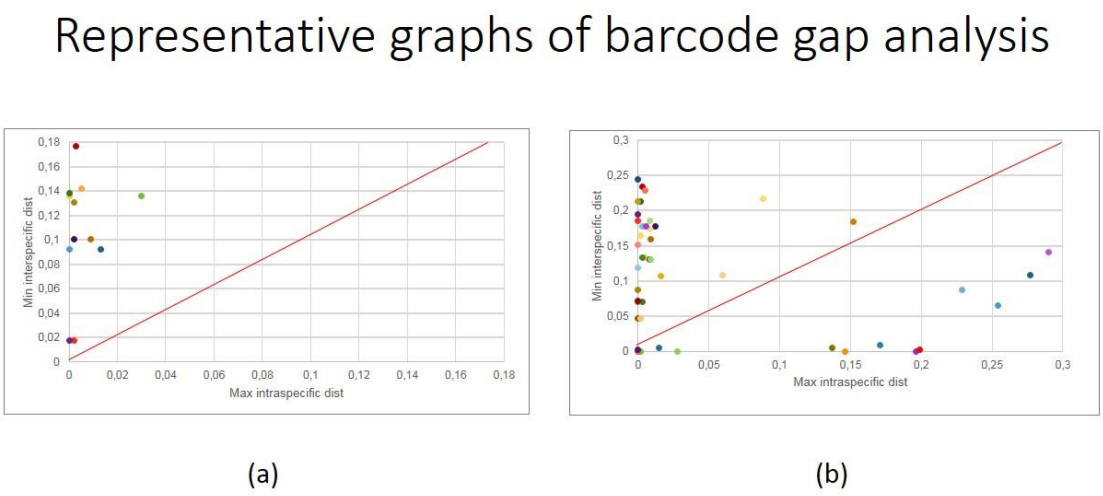

Fig. 6: Chart showing two representative results of barcode gap analysis for flatfish obtained in this study. (a) Family with all species above diagonal (max. intraspecific distance lower than distance to Nearest Neighbor); (b) family with some species below diagonal (max. intraspecific distance higher than distance to Nearest Neighbor).

IV.1.3. Primer design.

In order to realize an adequate worldwide DNA barcode study, new primers pair have been developed with the *ecoPrimers* program using two different maximum sequence length parameters for the barcode with theirs own characteristic (Table 4). The partial region of genes amplified by these two specific primer pairs turns out to be in the 16S rDNA gene (Fig. 7):

- 300 → 16S_300F- GGAGACAGTTAAGCCCTCGT
  16S_300R- TACCAAAAACATCGCCTCTT
- 800 → 16S_800F- CTCGTACCTTTTGCATCATG
  16S_800R- GCGATGTTTTTGGTAAACAG

Table 4: The characteristics of each barcode and its associated primers designed, according to *ecoPrimer:*

1. Tm (melting temperature) of primer Forward, without mismatch
2. Tm of primer Reverse, without mismatch
3. Bc index
4. Bs index
5. Number of taxa of the example dataset properly amplified according to the specific parameters
6. Number of taxa of the example dataset that are properly identified
7. Number of sequence records of the example dataset that are properly amplified according to the specific parameters
8. Minimum length of the barcode in base pairs for the example sequence records (excluding primer)
9. Maximum length of the barcode in base pairs for the example sequence records (excluding primers)

	1	2	3	4	5	6	7	8	9
16S-Ecoprimer 300pb max	59,2	55,3	0,983	0,831	59	49	118	121	133
16S-Ecoprimer 800pb max	55,1	54	1000	1000	60	60	120	687	717

In Fig. 7, it can be noted that these primer pairs amplified regions that are different respect to those amplified by the 16S primer pair developed by Palumbi *et al.* (1991).

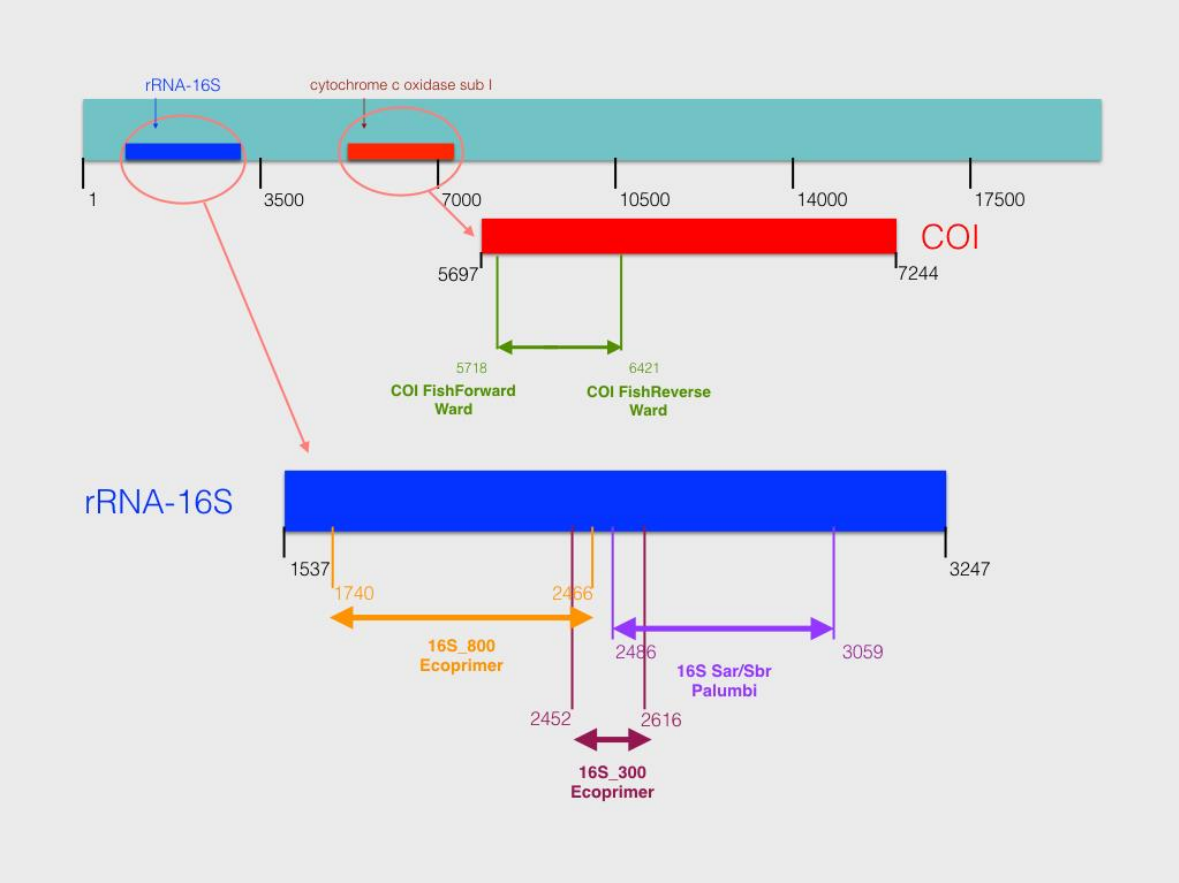

Fig. 7: Location on mtDNA of primers used in fish barcode analysis. A sequence (Acc. Number: NC_030367 sequence) from *Lophonectes gallus* species of Bothidae, was used to show both the universal primers described by Ward *et al.* (2005) and Palumbi (1991) as well as the new primers designed in this study, using Nucleotide BLAST

IV.1.4. PCR *in silico*.

With the newly designed primer pairs, along with the primers already in use for fish barcoding, a PCR *in silico* has been realized with the program *ecoPCR* in order to obtain different amplification percentages for each family of the flatfish Order (Table 5).

- COI - Ward *et al.* (2005) → there are four possible combinations for the four primers proposed by these authors, which give different percentage depending on the forward or reverse primers in use (Fig. 8). In Table 5 it is possible to see the various percentage for each family, where most of them

show a complete success of the amplification (Paralichthydae, Pleuronectidae, Poecilopsettidae, Psettodoidei) independently from the primer combination used. Other primer pairs showed a success of amplification similar to that obtained on the average with the F1R1 and F1R2 combinations, but show lower percentage on the other two F2R1 and F2R2 (Achiridae, Cynoglossidae, Soleidae); Samaridae and Scophthalmidae have 100% in F1R1 and F1R2 but 0% in the other two F2R1 and F2R2; Rhombosoleidae has lower percentage in F1R1 and F1R2 than in the other two F2R1 and F2R2; and Bothidae shows lower percentage in F1R1 and F2R2 than in F1R2 and 0% in F2R1.

- 16S - Palumbi *et al.* (1991) → all the families show a full 100% percentage for this pair of primer.
- 16S – 300L *ecoPrimer* → all the families show a full 100% percentage for this pair of primer.
- 16S – 800L *ecoPrimer* → all the families show a full 100% percentage for this pair of primer.

Table 5: Percentage amplification of the flatfish family for each primer pair proposed.

			COI				16S rDNA		
Family	Sequen mtDNA	Species mtDNA	Ward F1R1	Ward F1R2	Ward F2R1	Ward F2R2	Palumbi 16S	16S-ecoprimer 300pb max	16S-ecoprimer 800bp max
Achiridae	4	2	50	50	0	0	100	100	100
Bothidae	8	4	25	75	0	25	100	100	100
Cytharidae									
Cynoglossidae	32	16	81,25	81,25	18,75	12,5	100	100	100
Paralichthididae									
Paralichthydae	9	5	100	100	100	100	100	100	100
Pleuronectidae	20	11	100	100	100	100	100	100	100
Poecilopsettidae	1	1	100	100	100	100	100	100	100
Psettodoidei	2	1	100	100	100	100	100	100	100
Rhombosoleidae	7	4	25	25	50	50	100	100	100
Samaridae	4	2	100	100	0	0	100	100	100
Scophthalmidae	2	1	100	100	0	0	100	100	100
Soleidae	31	13	76,92	69,23	46,15	46,15	100	100	100

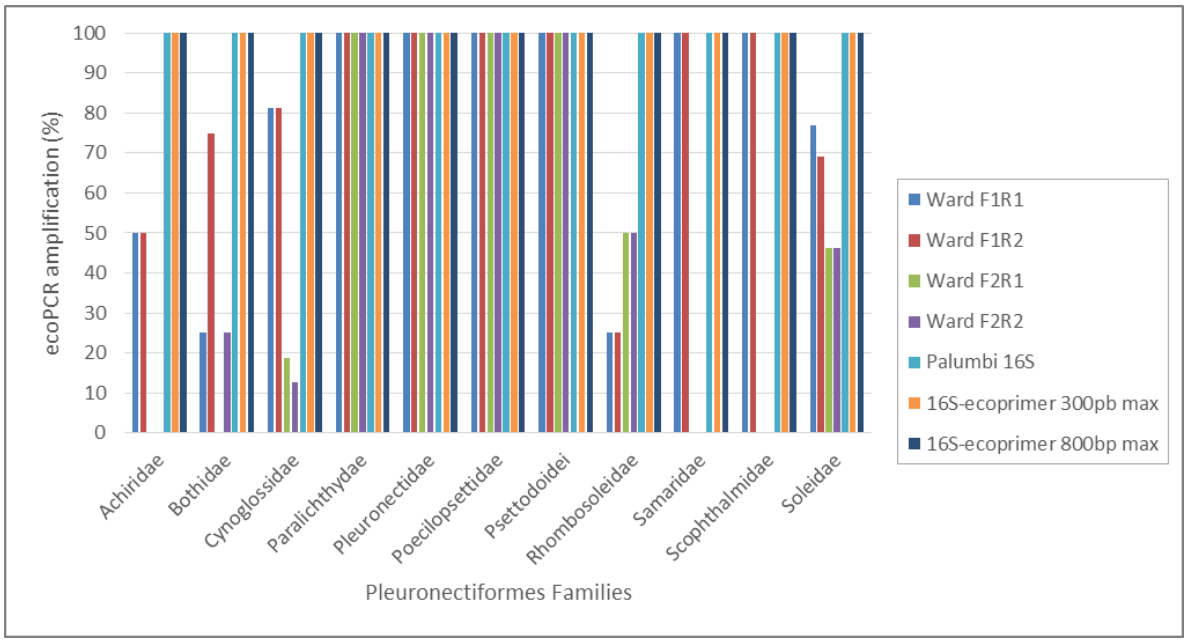

Fig. 8: Histogram representing the percentage amplification of the flatfish family for each primer pair proposed.

IV.2. Discussion.

This Thesis has laid the groundwork for an integrative study that consolidate the construction of a DNA barcode reference library, and the design of the most appropriate barcode primer that well discriminate between species. The flatfish taxonomic information described with the COI and 16S rDNA sequences, available in, and downloaded from NCBI, showed that there is a lack in the Pleuronectiformes resource data (Table 3). Presently, the existing marker sequences do not cover the totality of the species for each family, most for the 16S rDNA, lesser for the COI marker. Bothidae, which is the most numerous family including 64 species, has available COI sequences just for 38 species, and for the 16S rDNA they are merely 10. Other families, with high-commercial species, such as Soleidae (Tinti *et al*., 2000; Cerdà *at al*., 2008; Boukouvala *et al*., 2012; Garcia-Cegarra *et al*., 2013), and Scophthalmidae (Figueras *et al*., 2016), have, proportionally, more COI and 16S rDNA sequences than the others, indicating their particular economic interest. Generally, this paucity in the genetic information concerning this Order could be attributed to the fact that flatfish do not represent biological model for basic or biomedical research (Cerdà *et al*., 2013), which is in net contrast to their increasing importance of aquaculture, in order to strongly reduce the trawl demersal fishery (Cerdà *et al*., 2008).

The results from the barcode *gap* analysis with the comparisons between the genetic interspecific and intraspecific distances, in order to assume if that particular specie is well characterized (Meyer and Paulay, 2005), showed different scenarios for the various families (Appendix, Fig. 1.1-12.1). In the case where there were no data under the red line in both maximum and average intraspecific vs the minimum interspecific distance, like in Achiridae, Citharidae, Poecilopsettidae, Rhombosoleidae and Samaridae at the COI marker, and Bothidae and Paralichthyidae at the 16S rDNA marker, it does mean that the

marker (barcode) is well representative for those species of the family. As well, the minimum interspecific distance value, i.e. the minimum distance value between all the species belonging to that family, are higher than both average and maximum intraspecific distance value, i.e. distance value of the all sequences for that particular specie, which means that they are well ranked according to other similar studies (Tyagi *et al*., 2010; Ashfaq *et al*., 2013; Blagoev *et al*., 2013).

Several families showed inadequate quantity of resource sequence data, like as the case of Achiridae, Cynoglossidae and Pleuronectidae at the 16S rDNA marker, and Bothidae and Paralichthyidae at the COI marker. Scophthalmidae and Soleidae showed this scarcity at both COI and 16 markers, which are under the red line, indicating that the maximum and the average intraspecific, are higher than the minimum interspecific, with the absence of a gap between that specific species from the others (Meyer and Paulay, 2005). The COI marker of Cynoglossidae and Pleuronectidae, which are respectively the second and the first in high number of valid COI sequences, indicate the most numerous data with the overlap between genetic variation in species and the divergence that separate sister species. This means that all these cases seems to be candidate for cryptic species (Blagoev *et al*., 2013), but this should not be the case, it could be that those specific barcode marker cannot reliably distinguish among those species (Meyer and Paulay, 2005).

The challenge for defining a barcode of good quality consists in finding a quite short enough variable DNA sequence with highly conserved regions (Ficetola *et al*., 2010). The new primer pairs created by the *ecoPrimers* program, which in the whole mtDNA sequences from Pleuronectiformes, finds the best region for barcode analysis according to Bs and Bc indices, amplify two different partial regions of the 16S rDNA genes. Certainly, they are not the same regions amplified by the primer pairs described by Palumbi (1991), but they show better values in both Bs and Bc indices than the generally used. The primer pair designed with the

300 length parameters have a proportion of taxa that are properly identified (Bs) at 0.831, and a proportion of taxa that are properly amplified according to the specified parameters (Bc) at 0.983, resulting very promising for the taxonomic resolution in a future DNA barcode study. The primer pair designed with the 800 length parameters is much longer and have both Bs and Bc at 1.000, which means 100% of taxa properly amplified (Boyer *et al*., 2016). The resolution of the barcode markers associated with their PCR primer pairs, tested through a PCR *in silico*, in conjunction with the pair of primers already in use, show different taxonomic coverage. It is demonstrated by the differences observed in the amplification results of the flatfish families (Table 5) between those carried out with the universal marker for animal, the COI (Hebert *et al*., 2003) and those obtained by each of the 16S rDNA primer pairs. The 16S rDNA primer pairs developed by Palumbi *et al*. (1991), and the two new primer combinations designed in this work, exhibited a full 100% PCR amplification *in silico*, proving that for animals mitochondrial rDNA genes provide taxonomic resolution power similar to that of COI, but will allow the design of more conserved primers (Deagle *et al*., 2014). Previous studies demonstrated that COI metabarcodes provided lower taxonomic coverage than the 16S rDNA metabarcodes of similar length (Tang *et al*., 2012; Clarke *et al*., 2014) or than other different mitochondrial regions (van Steenkiste *et al*., 2014). *In silico* PCRs consist in selecting within a database the sequences that exhibit similarity with two PCR primers, and the regions that have this match should be localized on the selected sequence in order to allow PCR amplification to force the relative orientation of the matches and the distance between them (Ficetola *et al*., 2010). Results of the *in silico* and *in vitro* PCRs can differ somewhat: *ecoPCR* is a useful tool for predicting taxonomic amplification and improving the performance of a study, and it could successfully predicted many taxa that would not be amplified by the COI marker (Clarke *et al*., 2014). An *in vitro* analysis could be integrated in the future to validate the correspondence between *in silico* and real world PCR.

V. Conclusions.

1. The analysis of the mtDNA sequences available for the Pleuronectiformes revealed an overall low coverage of information for the Families of low economic interest, more for the 16S rDNA marker, than for the COI, emphasizing that they do not characterize totally the current state of this Order of ray-finned fish.
2. The analysis of the barcode gaps existing between the intraspecific and interspecific distance in flatfishes in both COI and 16S rDNA regions, showed the existence of several problems in the taxonomical identification of some families, such as Bothidae, Cynoglossidae, Pleuronectidae, Scophtalmidae and Soleidae, in which the intraspecific distance values are higher than the genetic distances between species (interspecific). This pattern denotes possible occurrence of taxonomic uncertainties or errors, which can derive from faults at the moment of a taxonomical classification, based just on morphological analysis of the species. It is important to emphasize the need of tools that will complement their taxonomical classification.
3. The new primer pairs designed, through the *ecoPrimer* bioinformatic software, elected on the basis of the two indices Bs and Bc, showed greater taxonomic coverage than the COI universal primers, but similar to that of the 16S universal primers decribed by Palumbi (1991). This is an essential complement for future metabarcoding studies.

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"Happiness can be found in the darkest of times, if one only remembers to turn on the light." – J.K.Rowling

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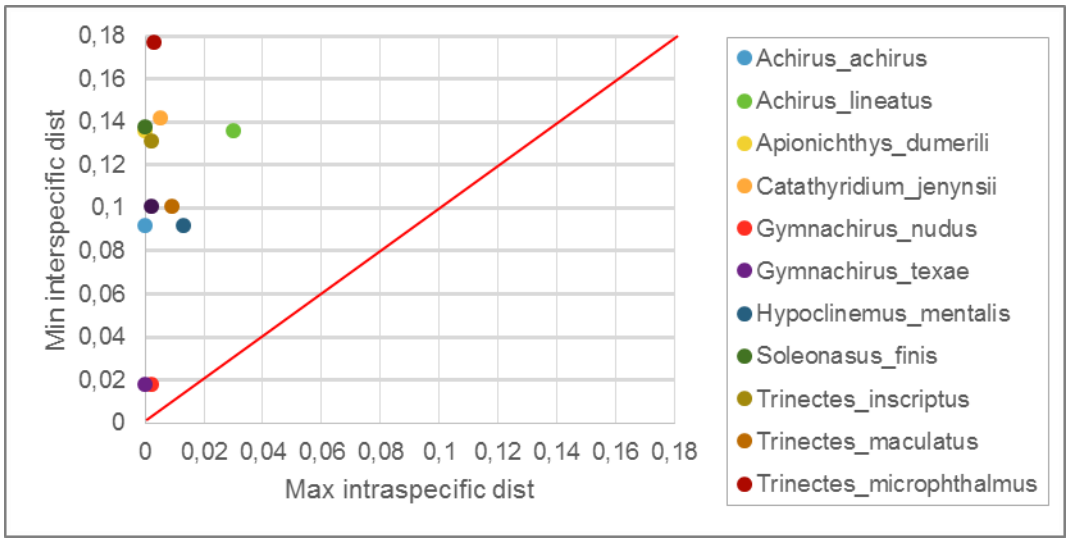
Appendix

Barcode gap analysis.

- **Achiridae** → Fig. 1.1 (COI) and Fig. 1.2 (16S rDNA)
- **Bothidae** → Fig. 2.1 (COI) and Fig. 2.2 (16S rDNA)
- **Citharidae** → Fig. 3.1 (COI)
- **Cynoglossidae** → Fig. 4.1 (COI) and Fig. 4.2 (16S rDNA)
- **Paralichthyidae** → Fig. 5.1 (COI) and Fig. 5.2 (16S rDNA)
- **Pleuronectidae** → Fig 6.1 (COI) and Fig 6.2 (16S rDNA)
- **Poecilopsettidae** → Fig. 7.1 (COI)
- **Rhombosoleidae** → Fig. 8.1 (COI)
- **Samaridae** → Fig. 9.1 (COI)
- **Scophthalmidae** → Fig. 10.1 (COI) and Fig 10.2 (16S rDNA)
- **Soleidae** → Fig. 11.1 (COI) and Fig. 11.2 (16S rDNA)
- **Psettodoidei** → Fig. 12.1 (COI)



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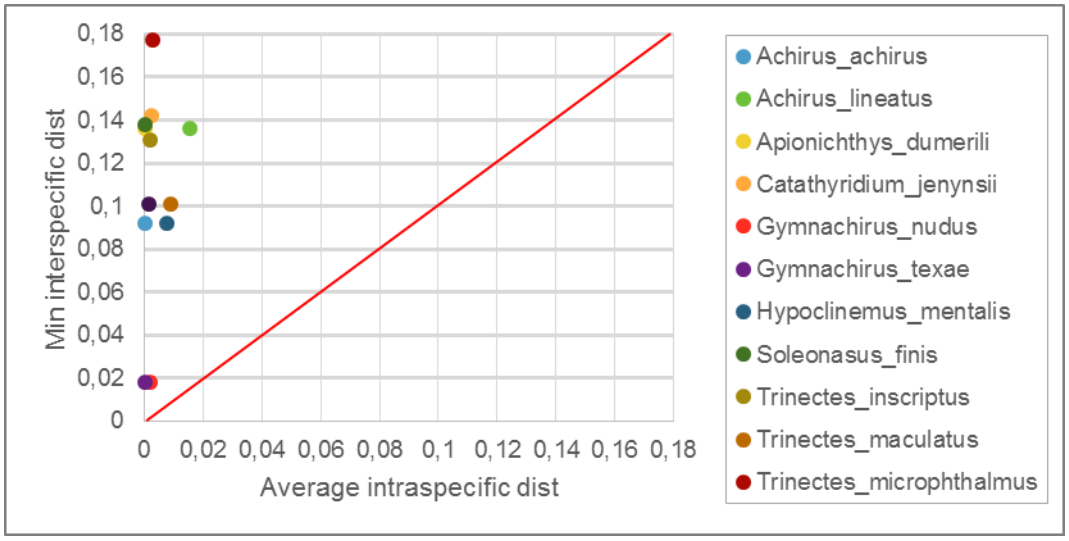
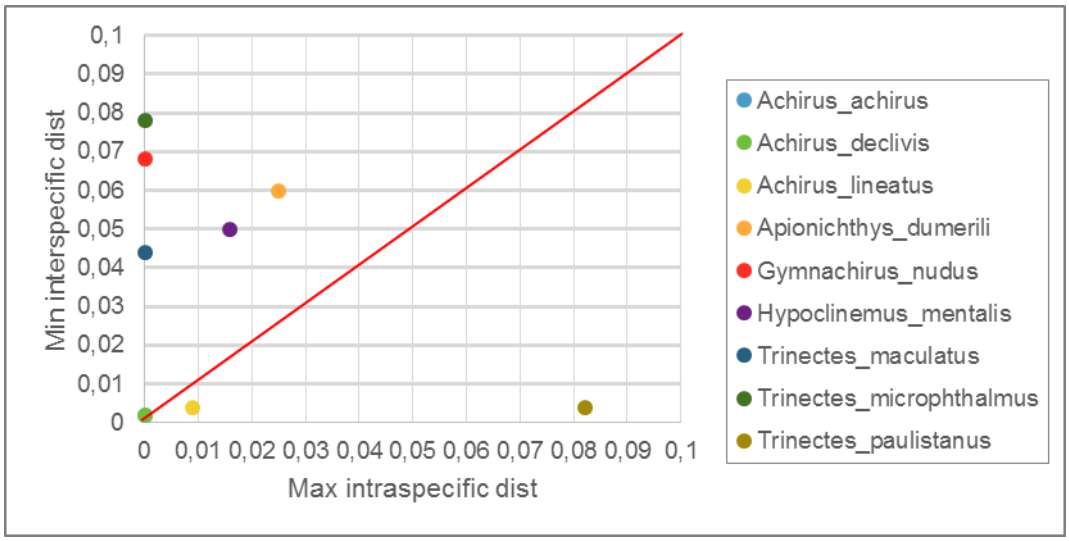


(b)

Fig. 1.1: Barcode gap analysis for **Achiridae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker COI



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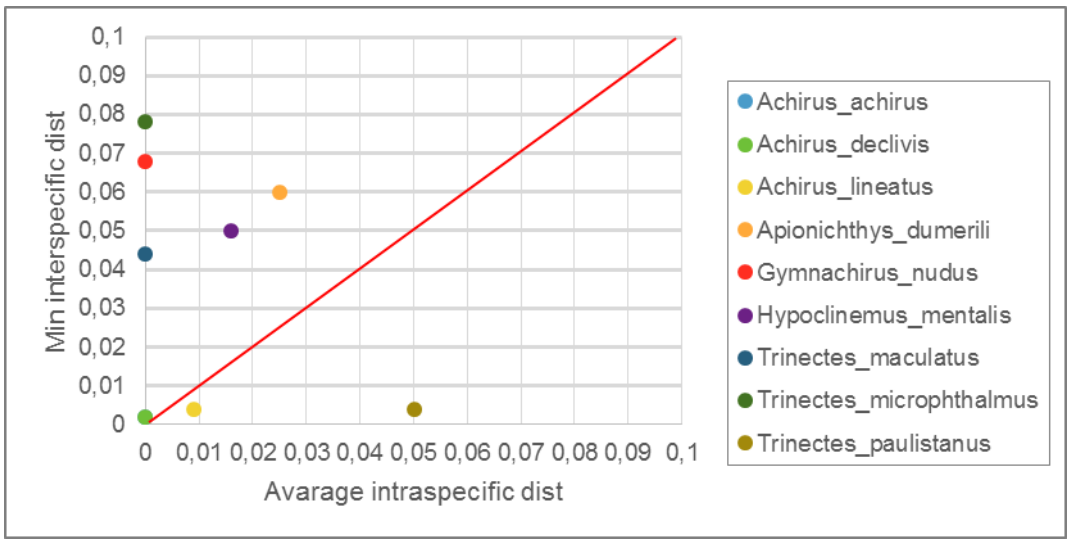
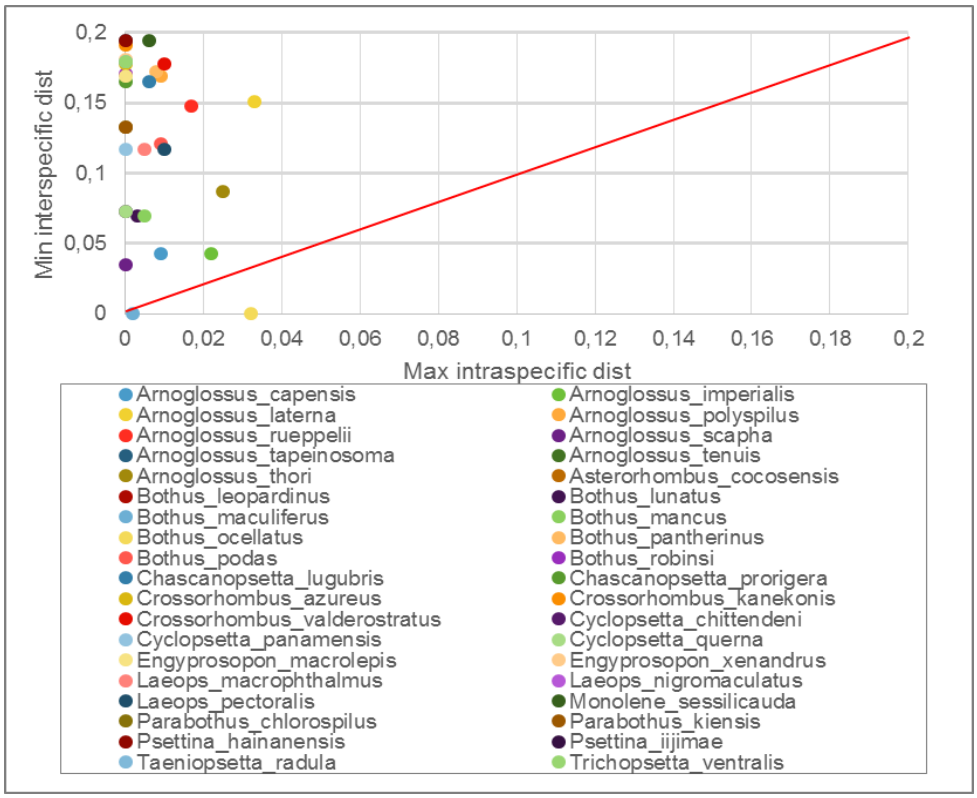


(b)

Fig. 1.2: Barcode gap analysis for **Achiridae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker 16S rDNA



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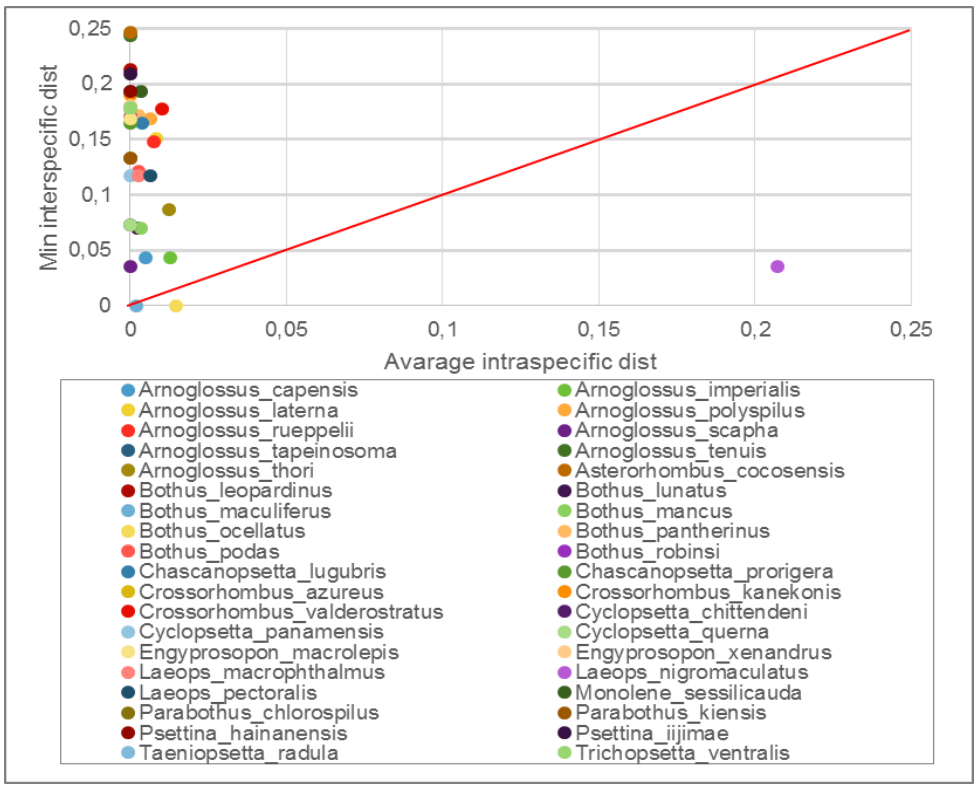
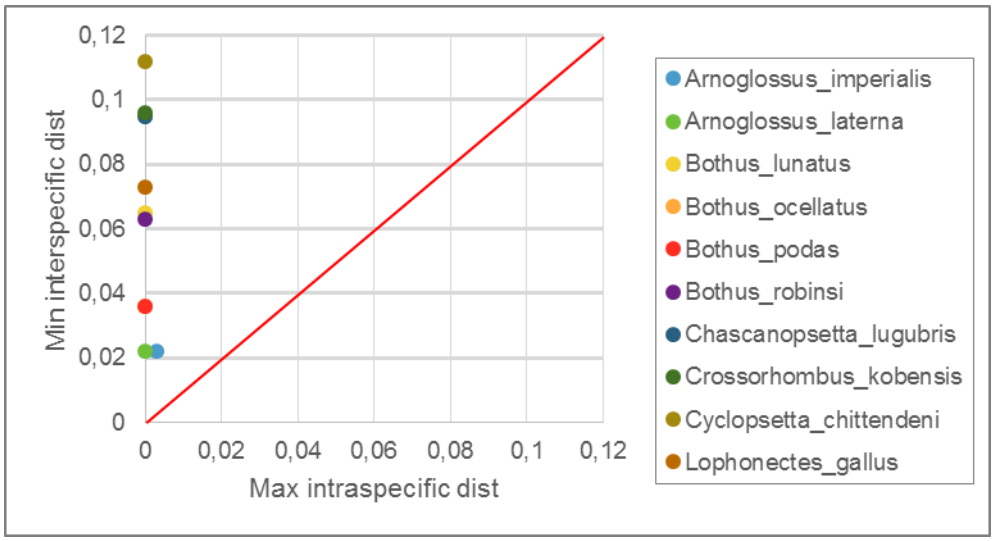


(b)

Fig. 2.1: Barcode gap analysis for **Bothidae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker COI



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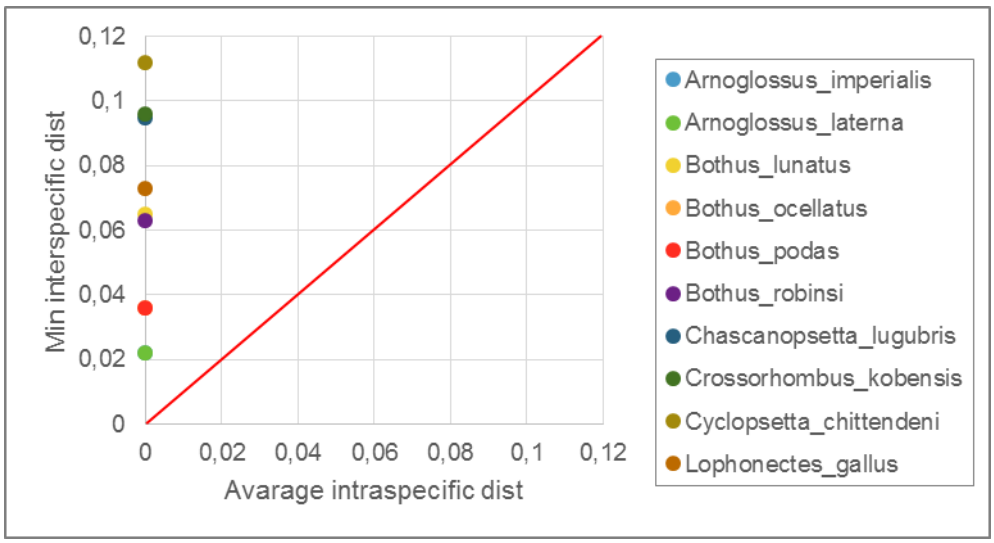

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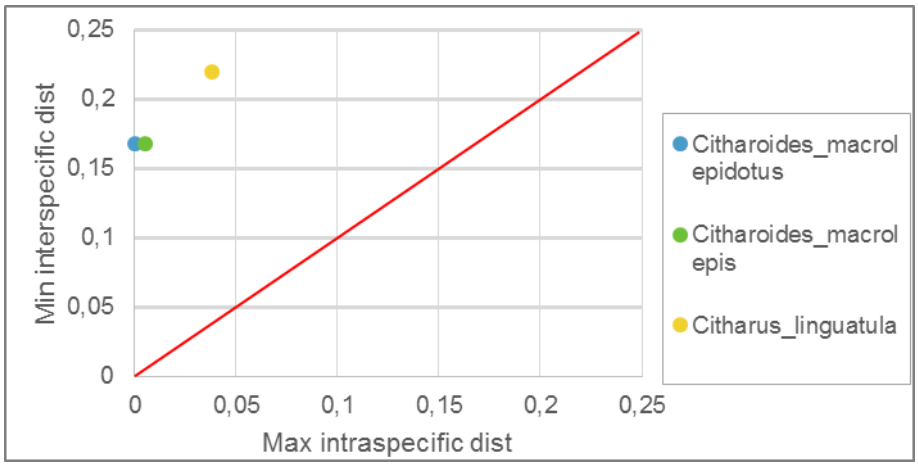
Fig. 2.2: Barcode gap analisys for **Bothidae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker 16S rDNA



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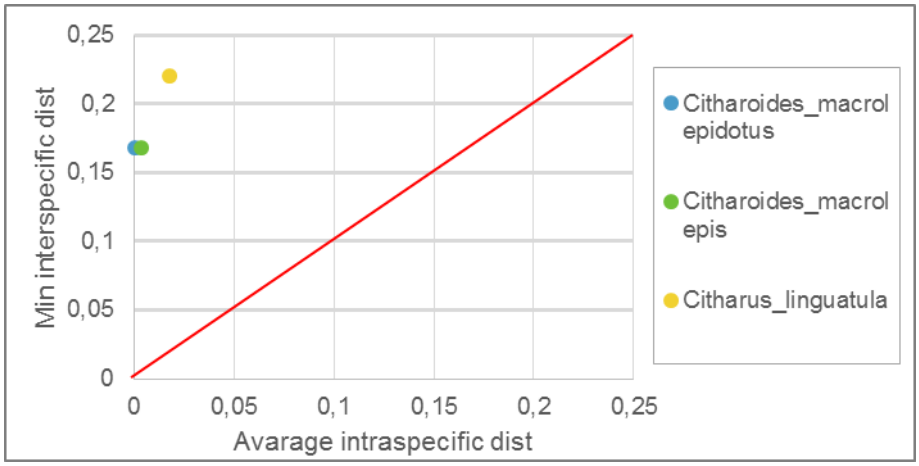
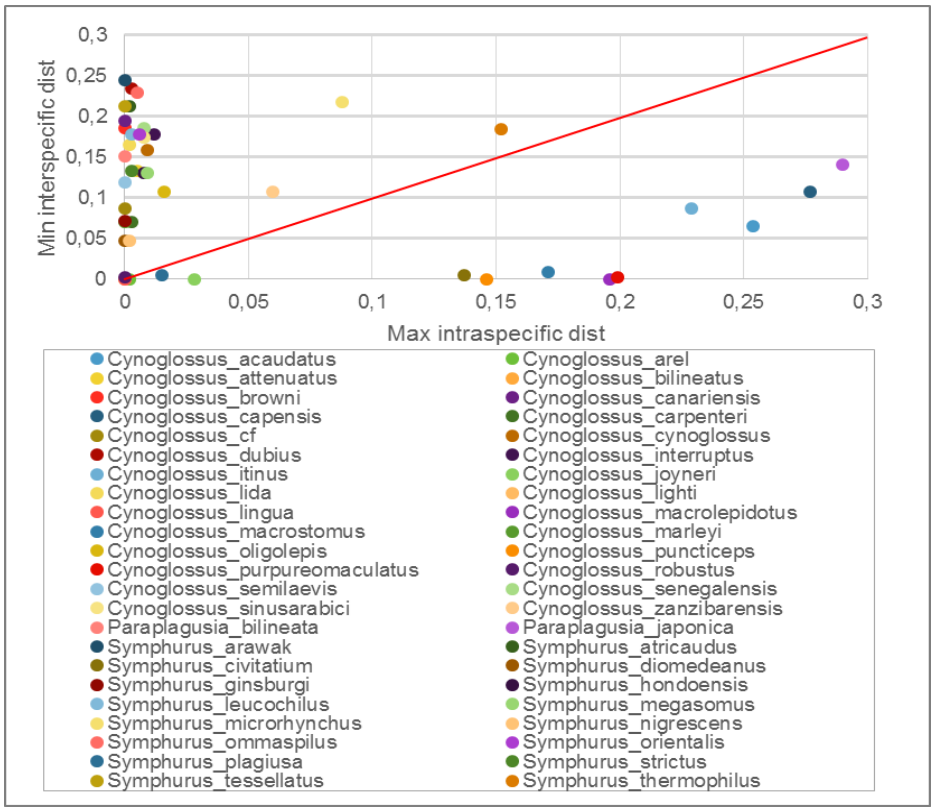


(b)

Fig. 3.1: Barcode gap analysis for **Citharidae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker COI



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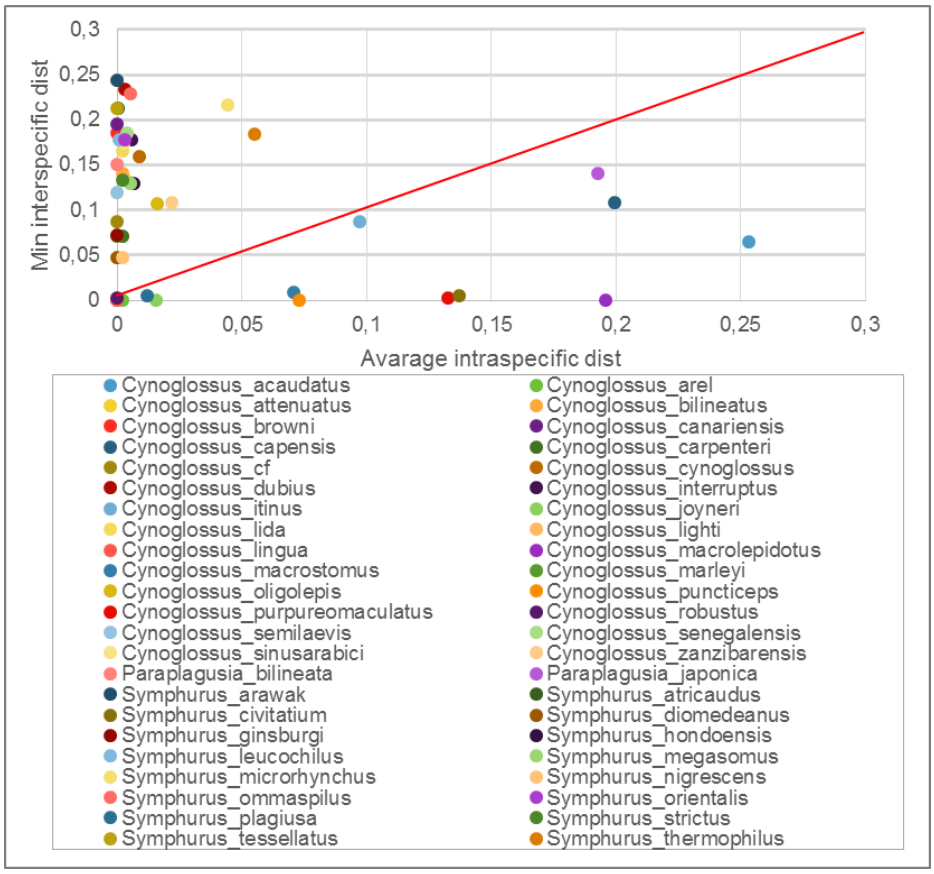
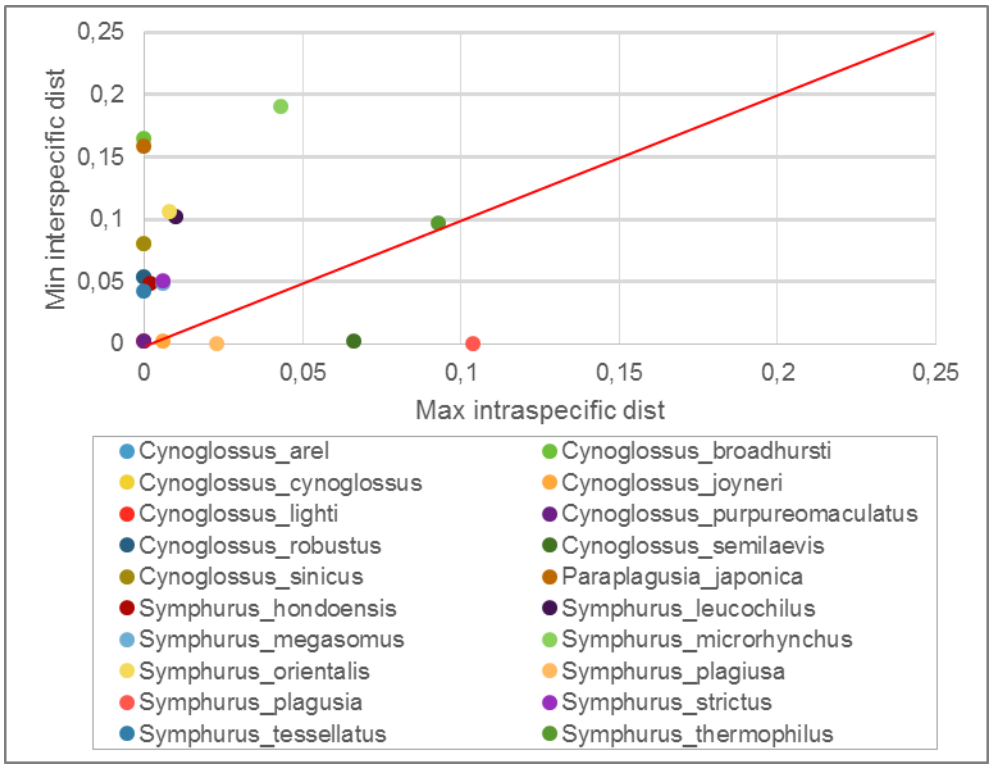


(b)

Fig. 4.1: Barcode gap analysis for **Cynoglossidae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker COI



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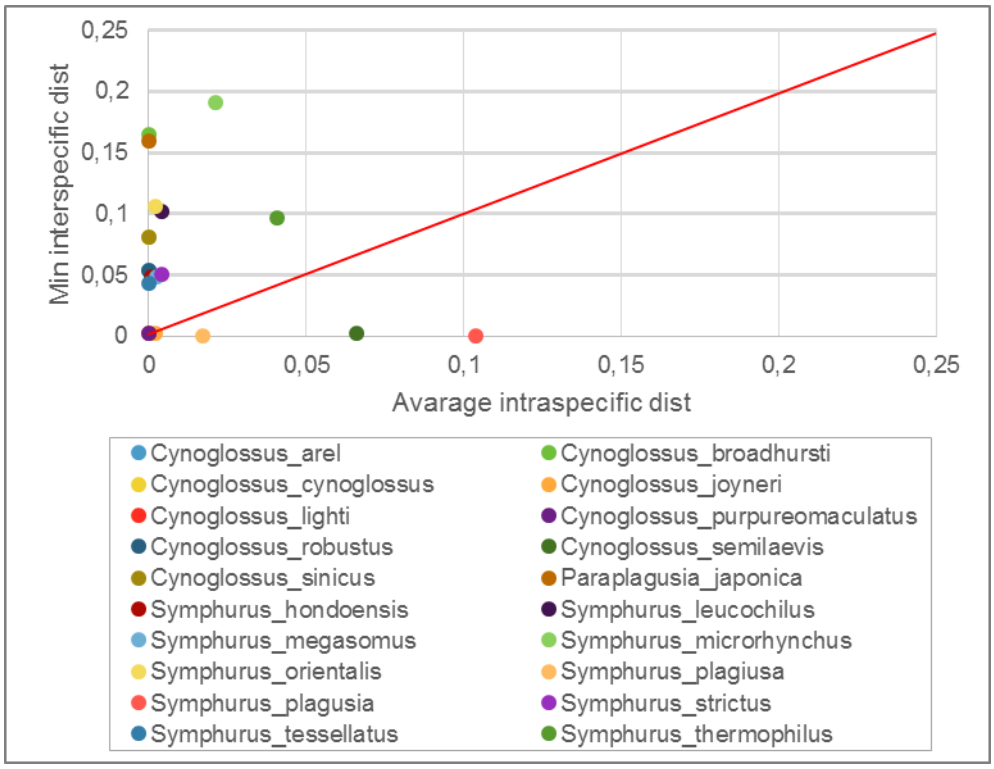
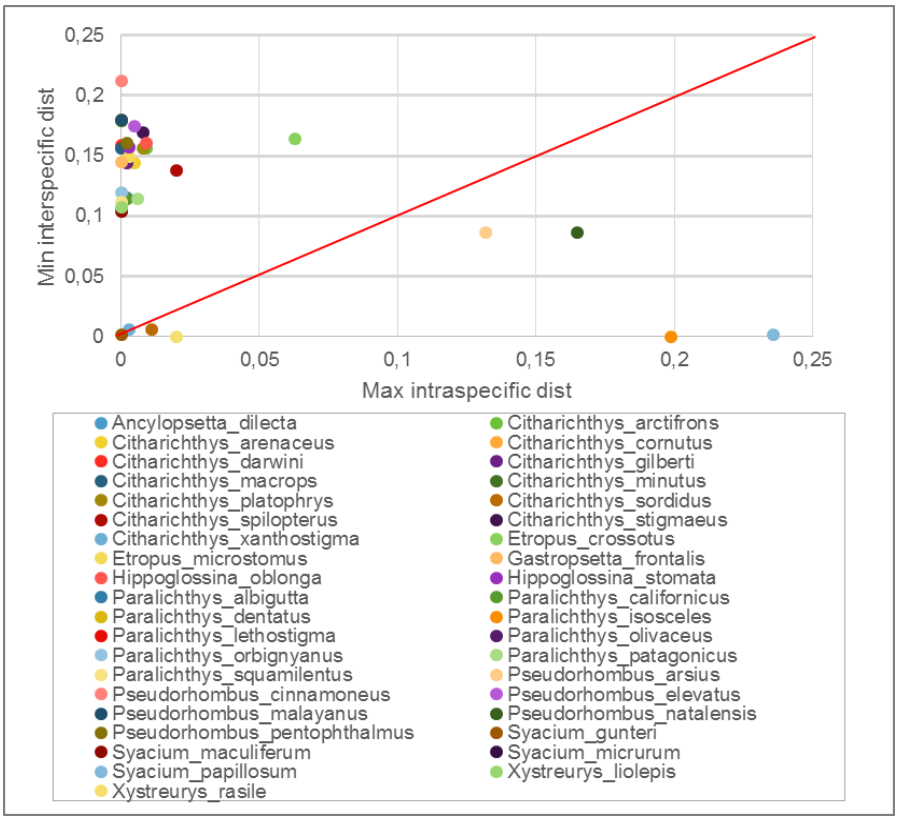


(b)

Fig. 4.2: Barcode gap analysis for **Cynoglossidae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker 16S rDNA



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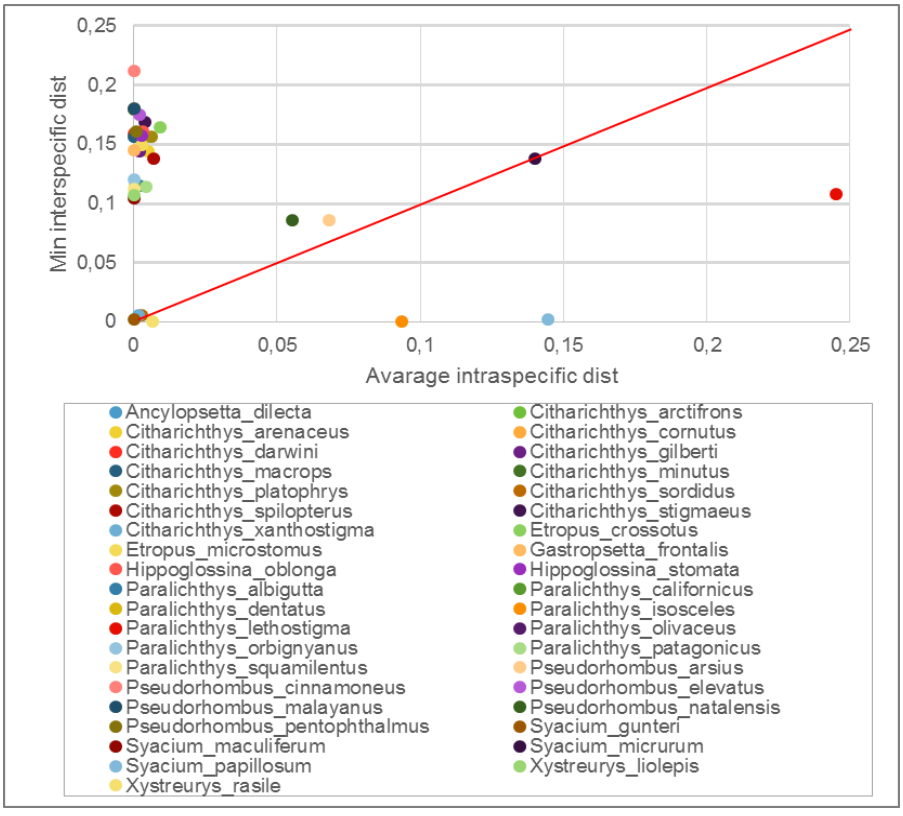
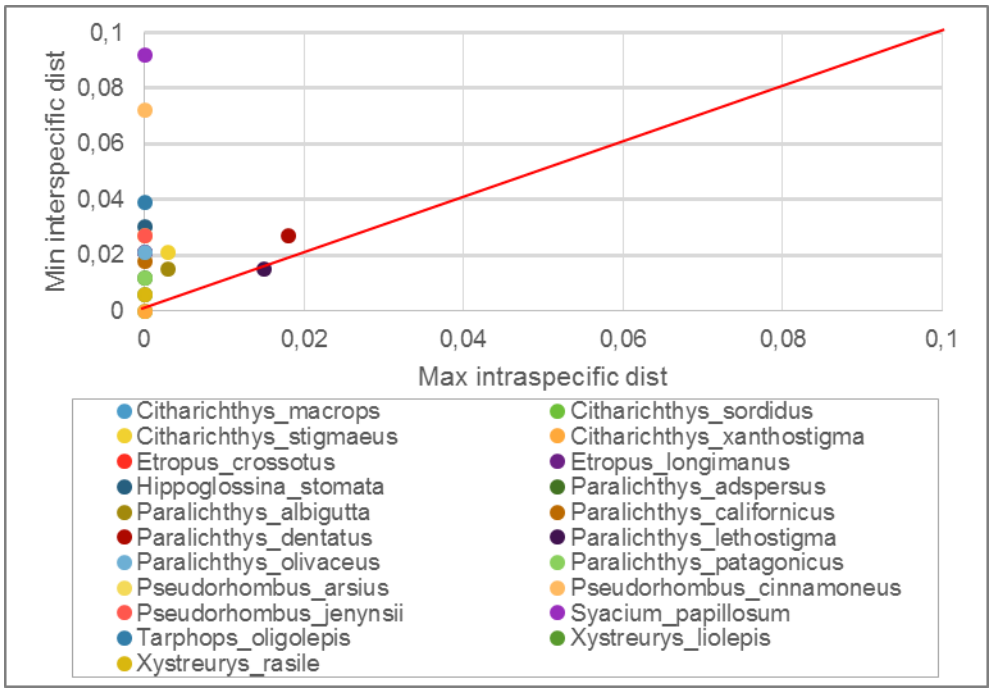


(b)

Fig. 5.1: Barcode gap analysis for **Paralichthyidae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker COI



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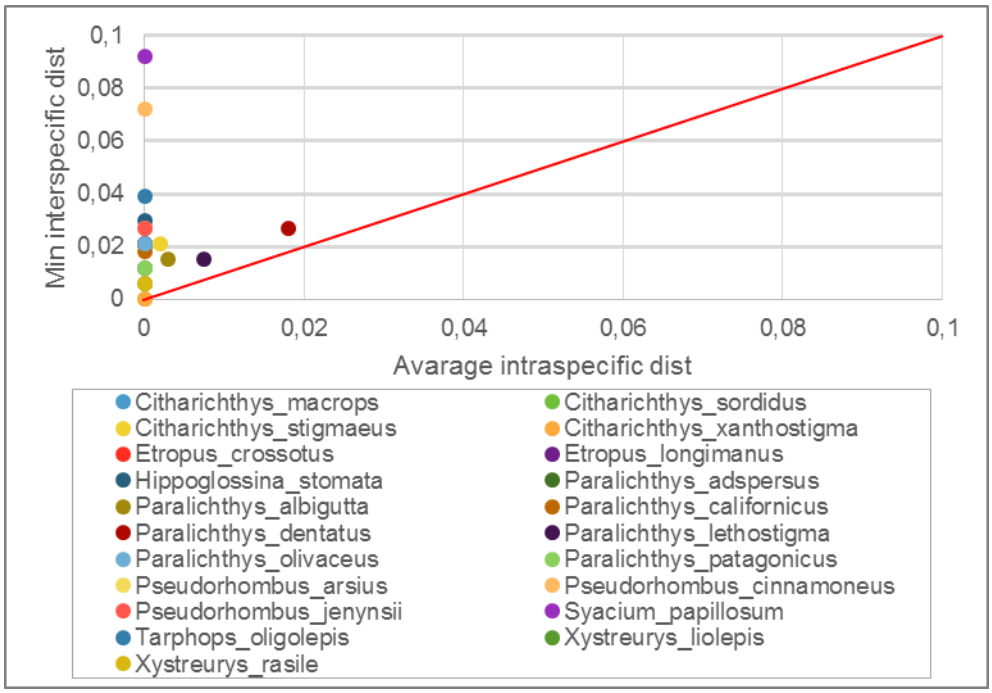

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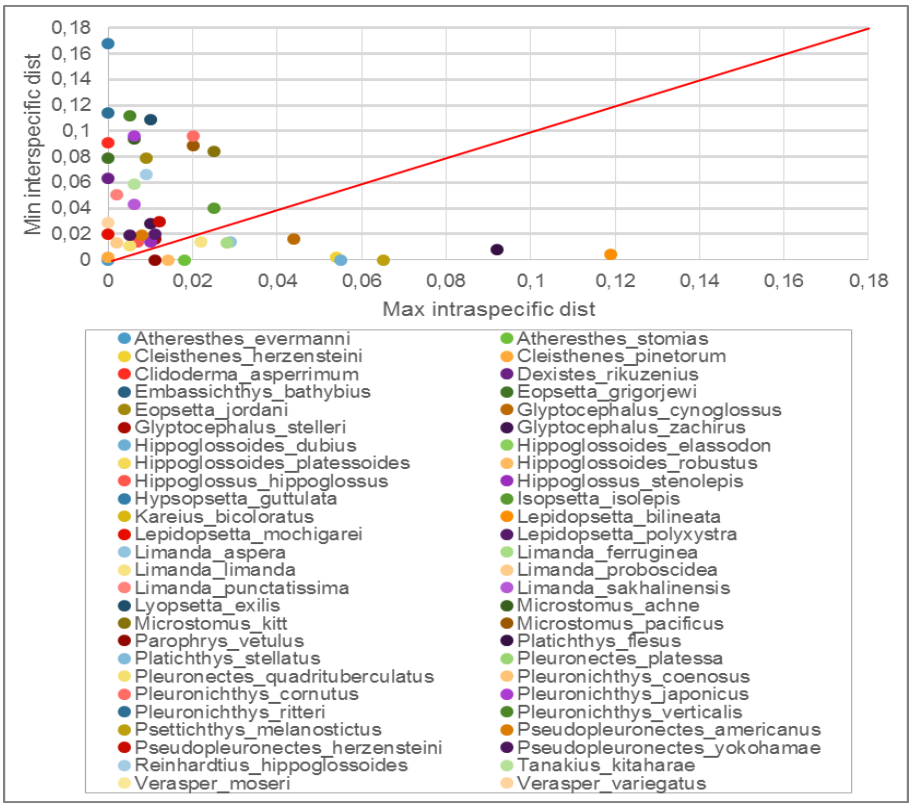
Fig. 5.2: Barcode gap analysis for **Paralichthyidae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker 16S rDNA



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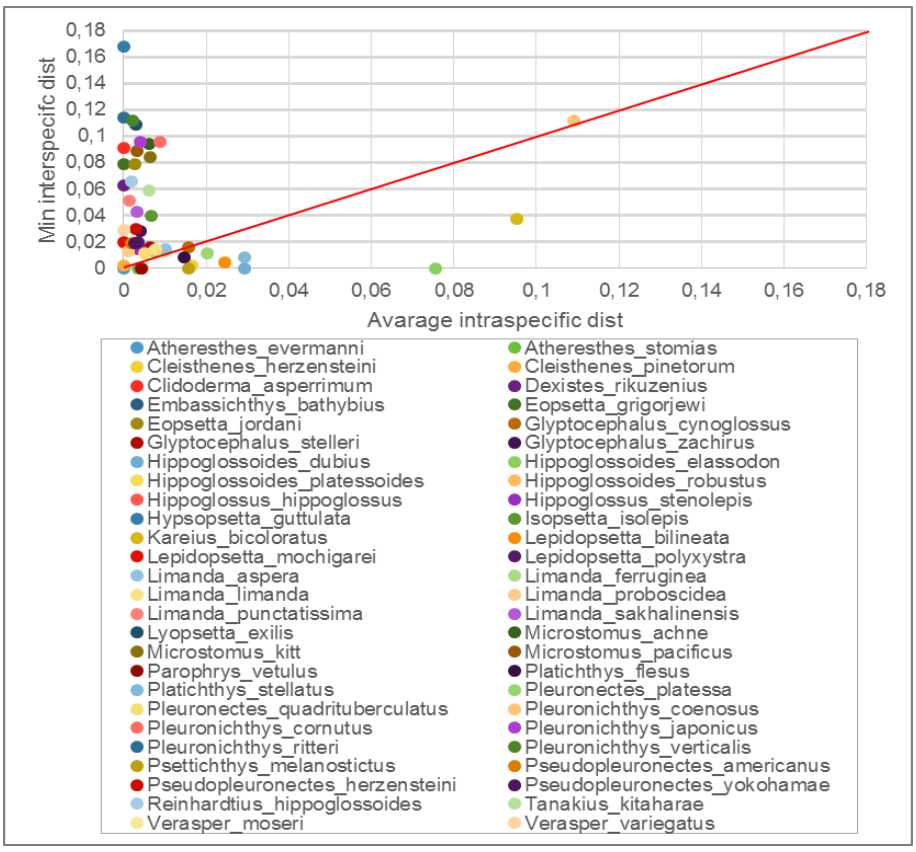

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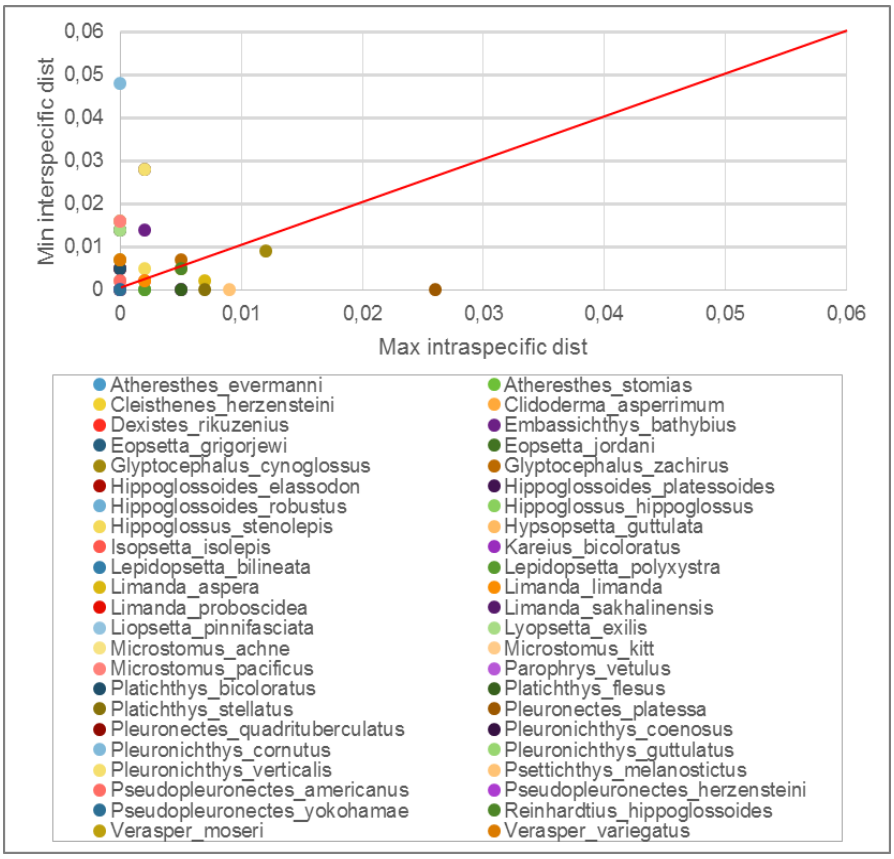
Fig. 6.1: Barcode gap analysis for **Pleuronectidae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker COI



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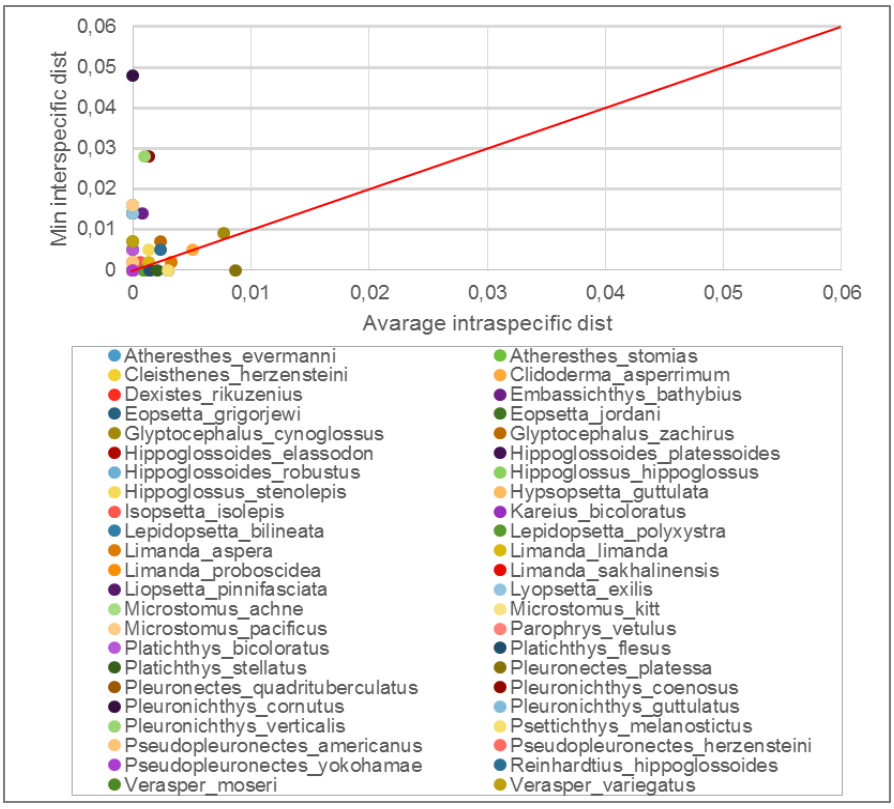

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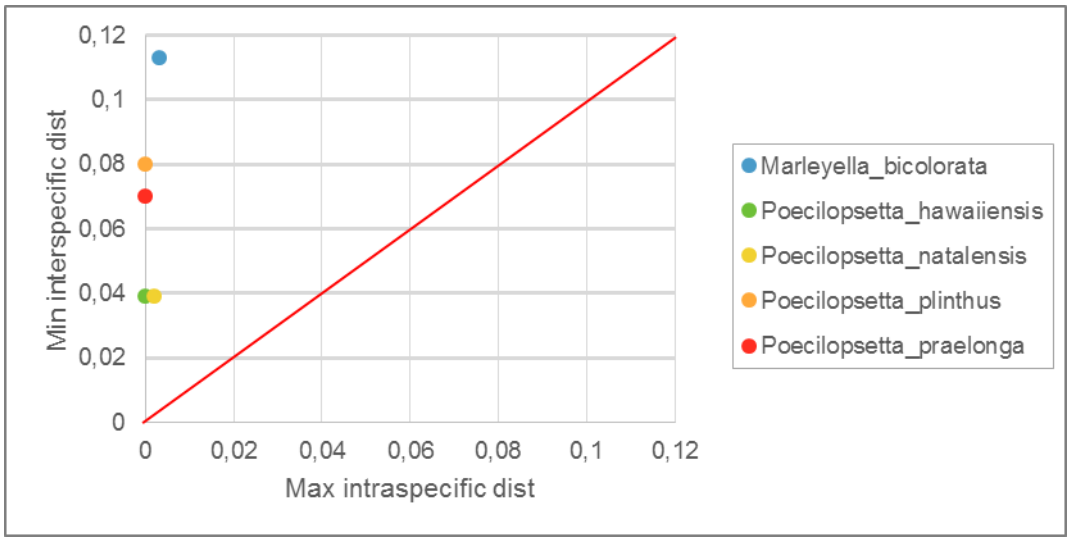

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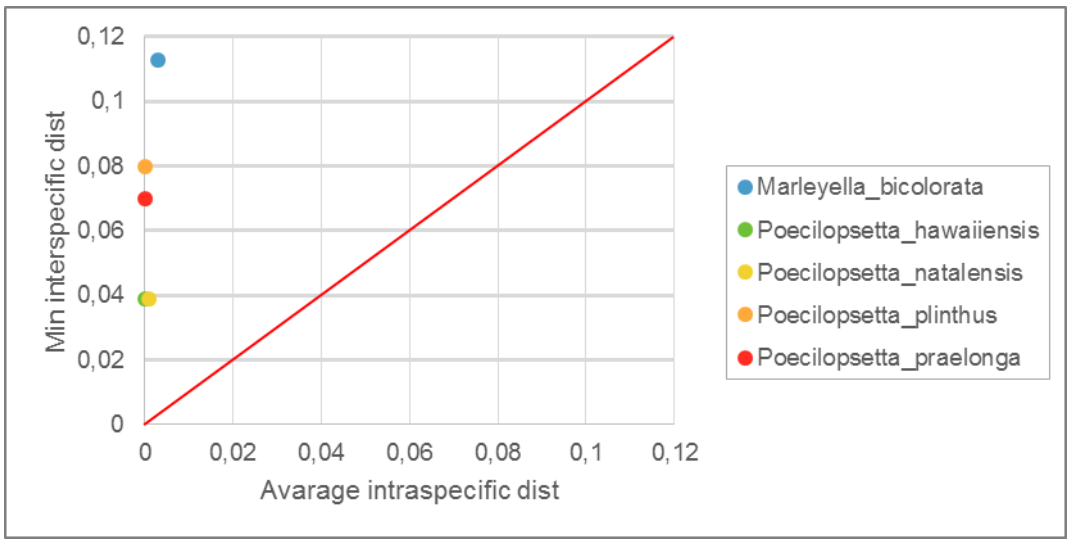

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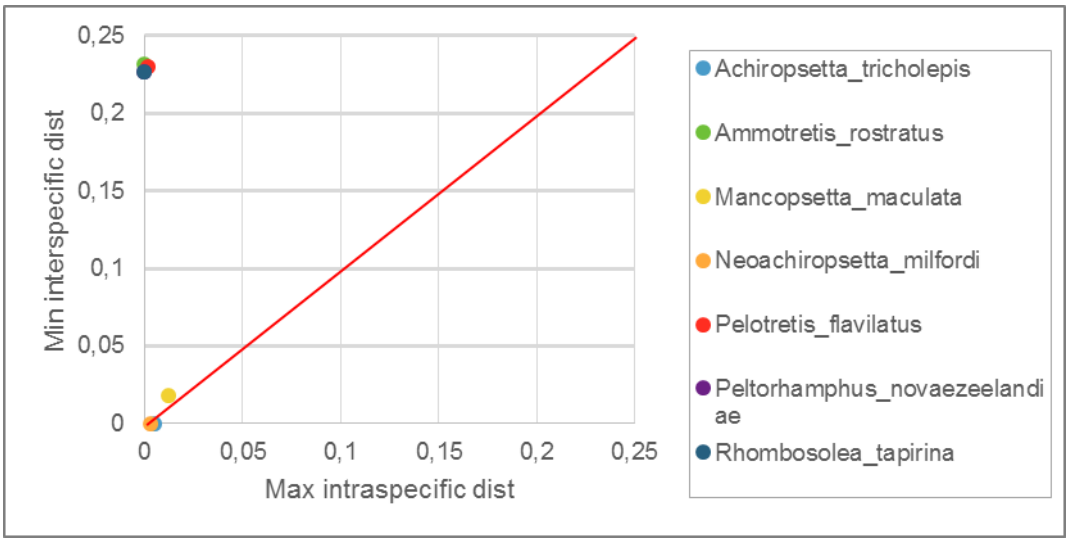
Fig. 7.1: Barcode gap analysis for **Poecilopsettidae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker COI



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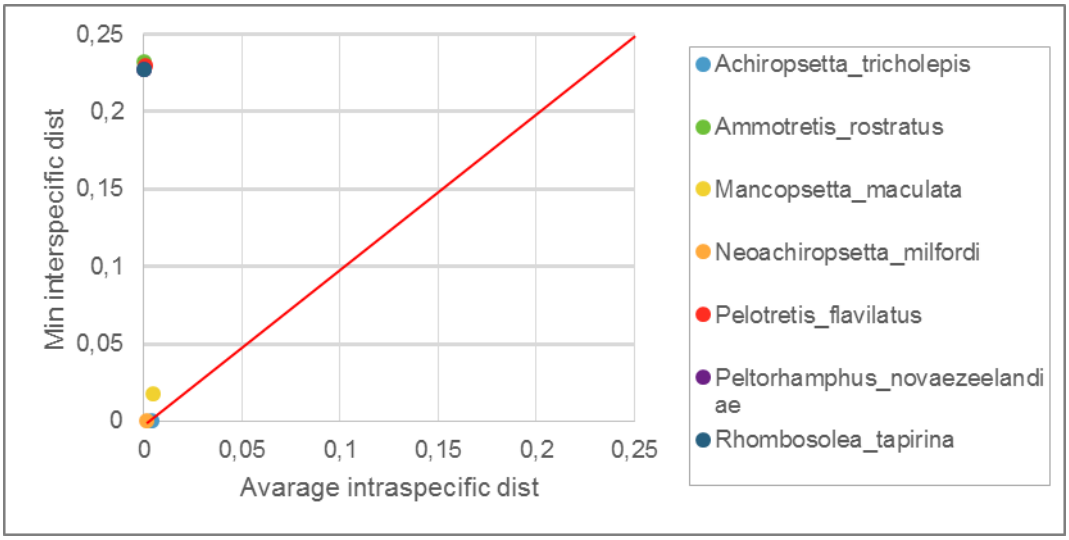

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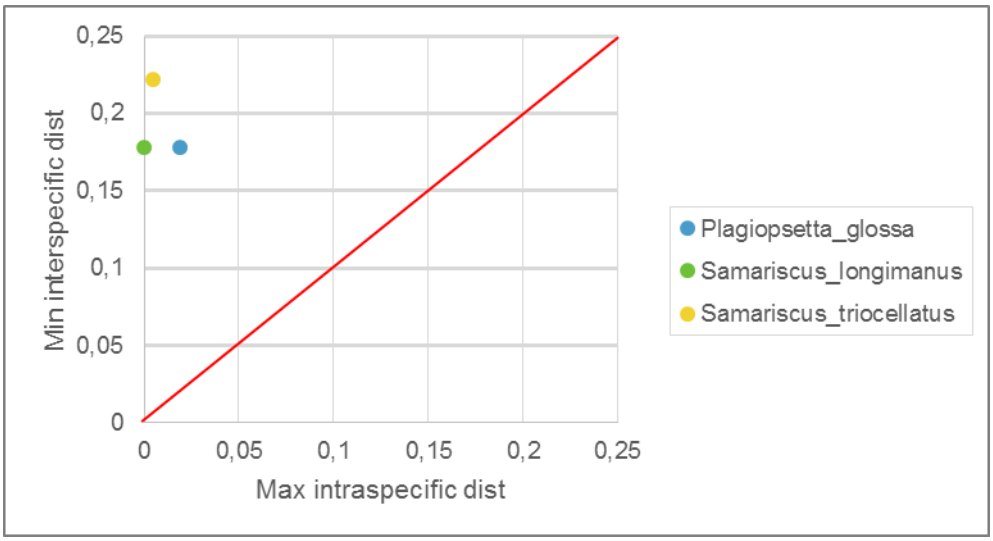
Fig. 8.1: Barcode gap analysis for **Rhombosoleidae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker COI



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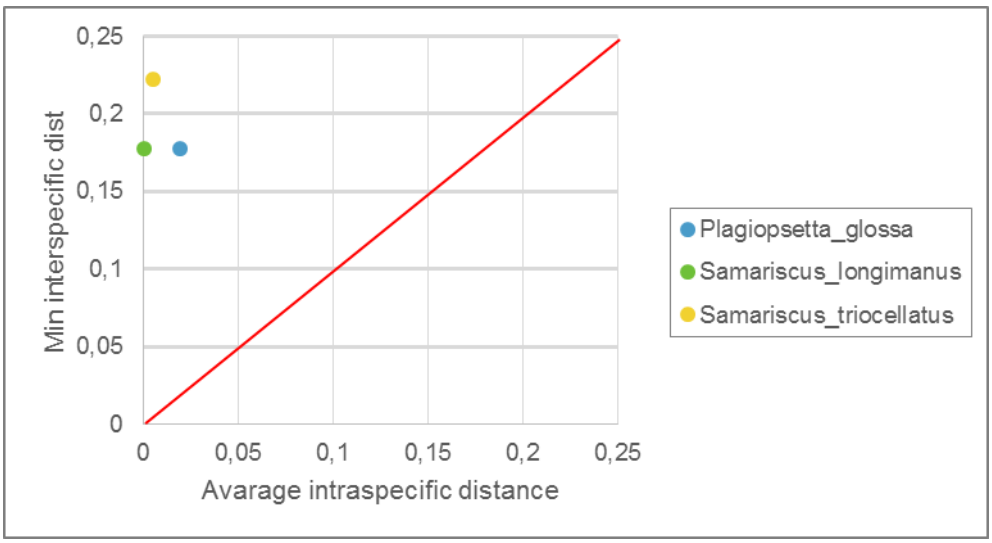

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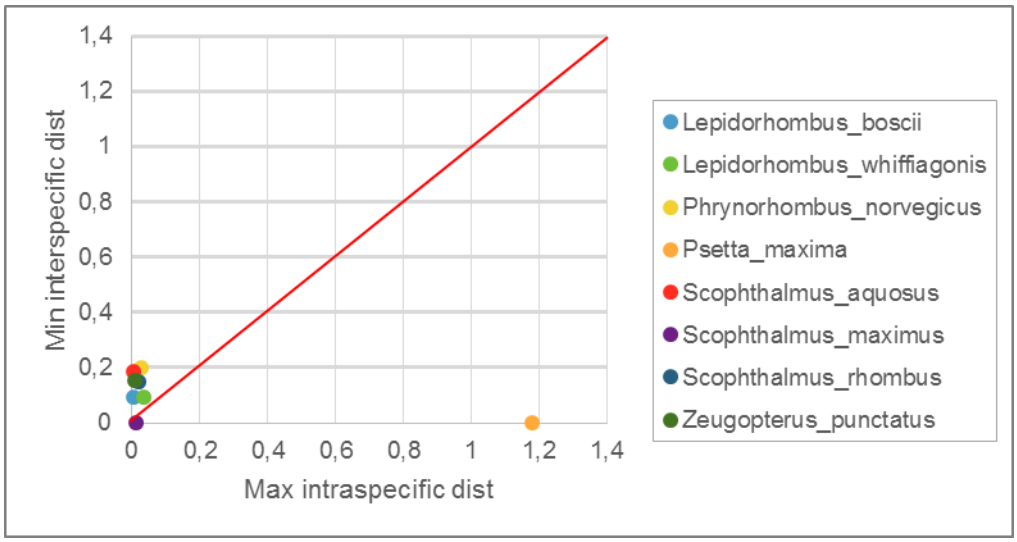

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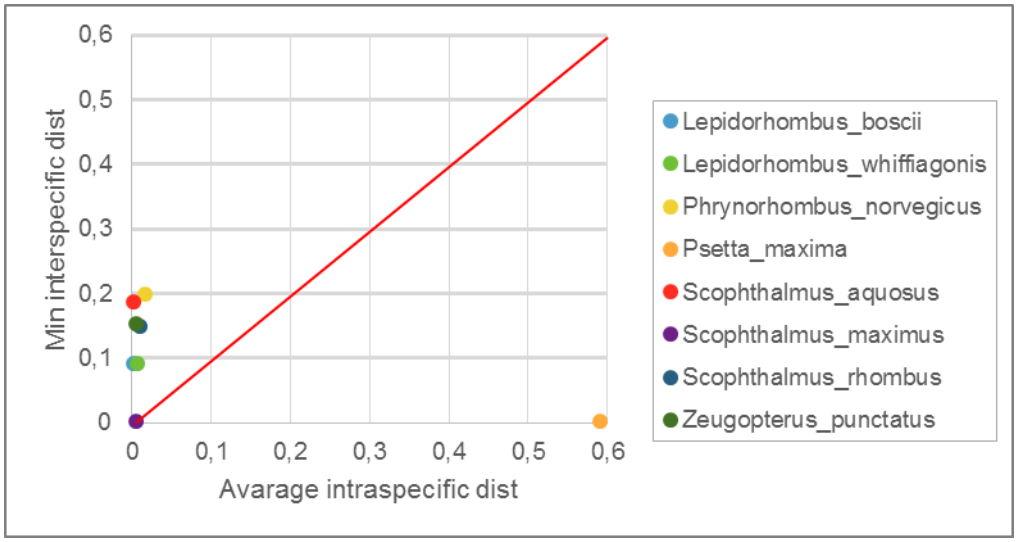
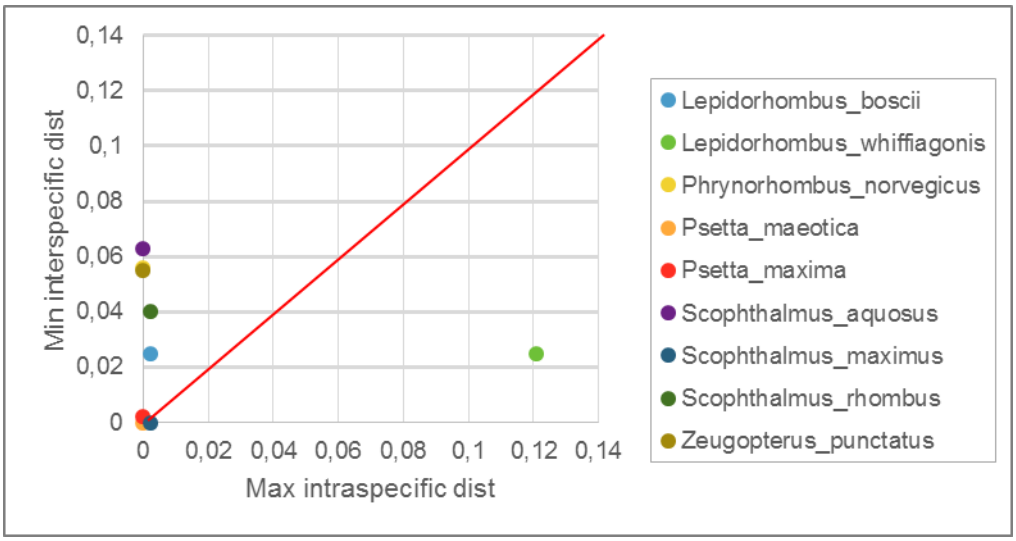
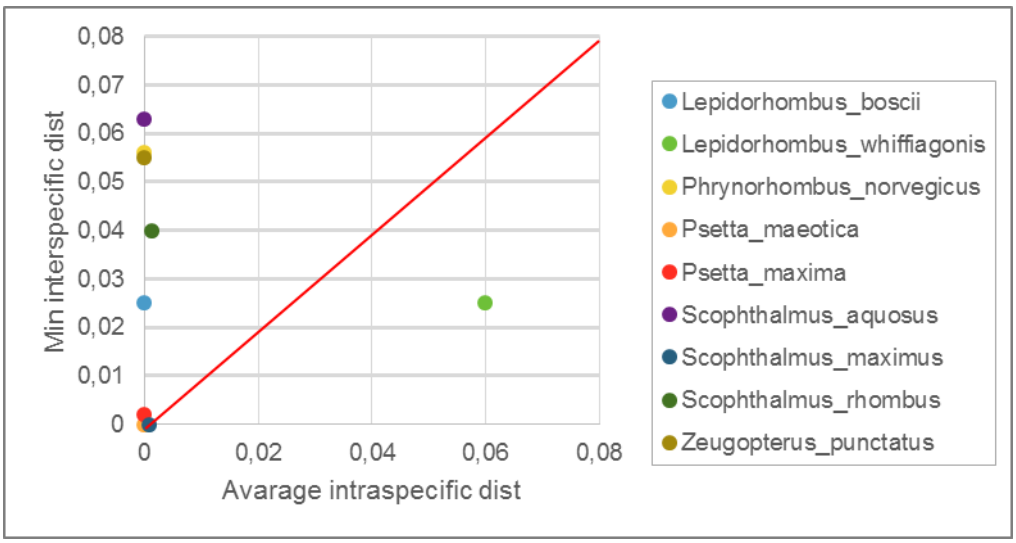


(b)

Fig. 10.1: Barcode gap analysis for **Scophthalmidae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker COI

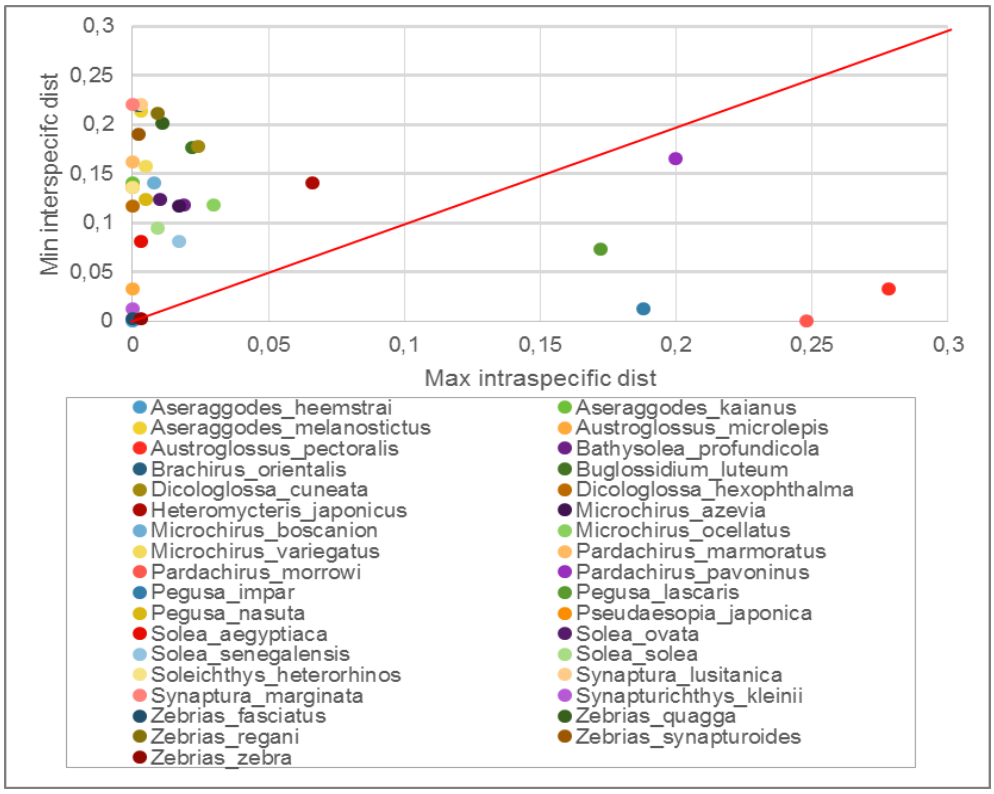


(a)

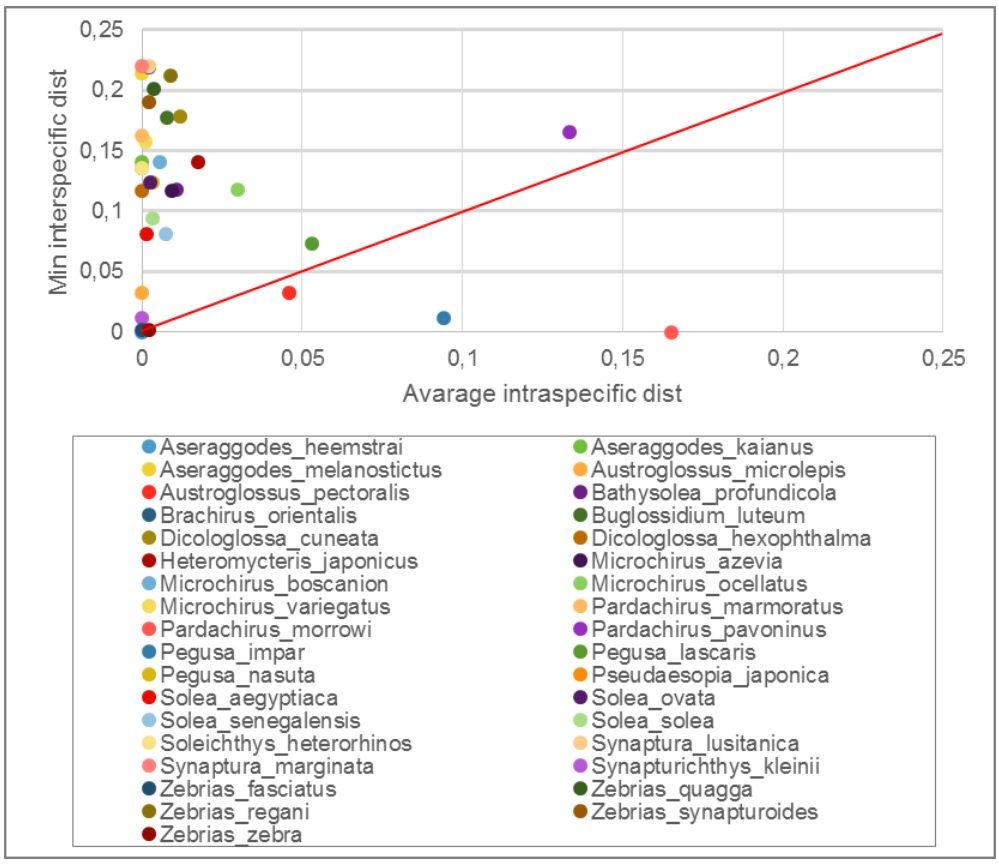


(b)

Fig. 10.2: Barcode gap analysis for **Scophthalmidae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker 16S rDNA

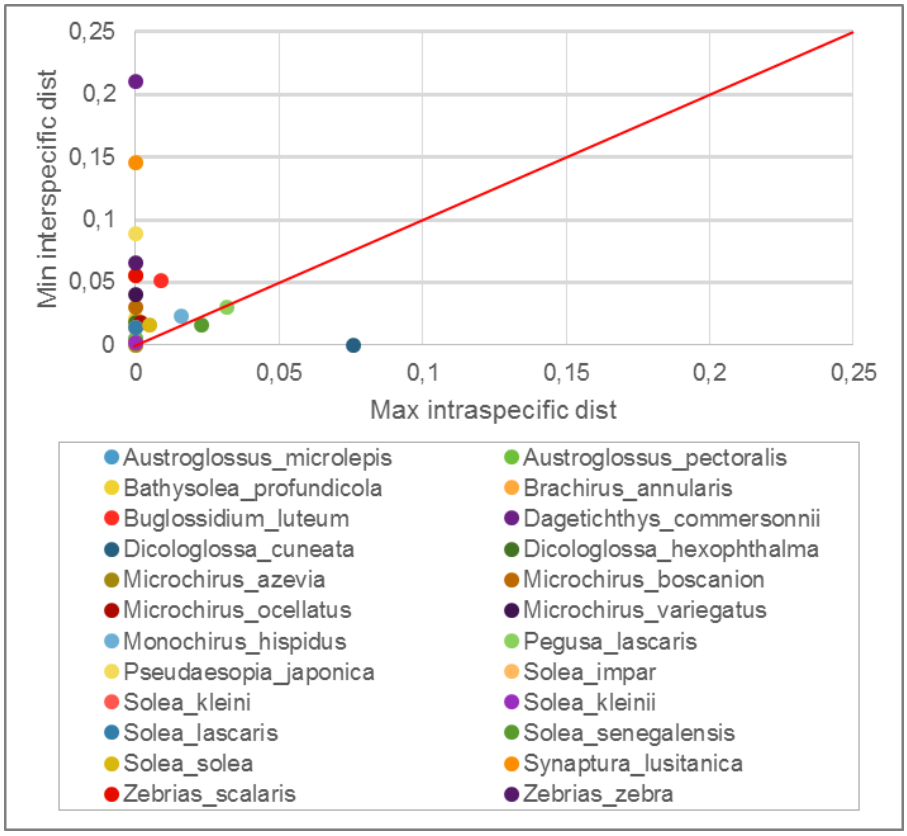


(a)

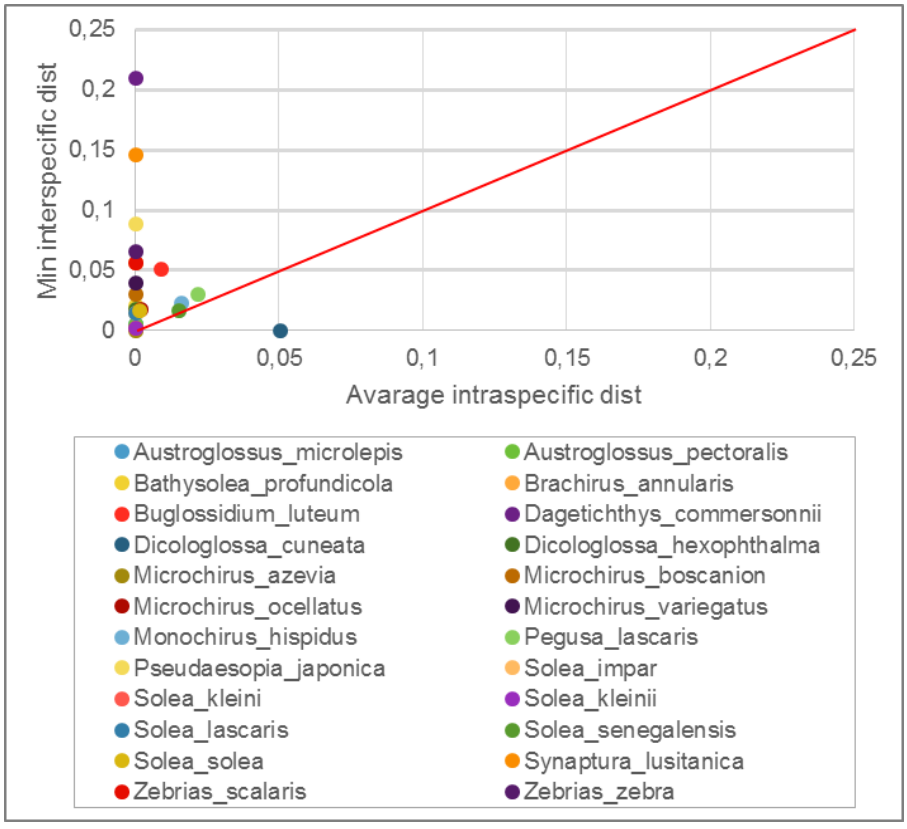


(b)

Fig. 11.1: Barcode gap analysis for **Soleidae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker COI

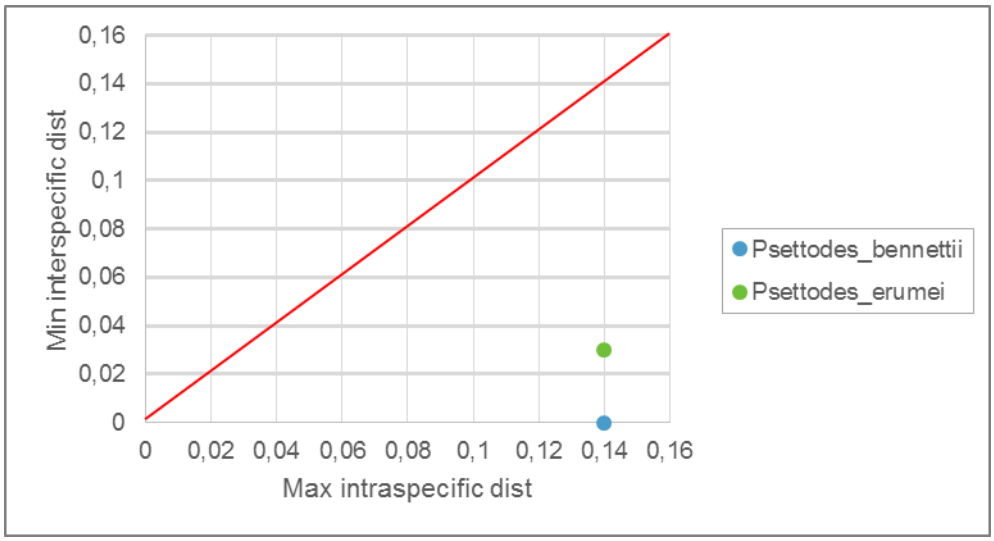


(a)

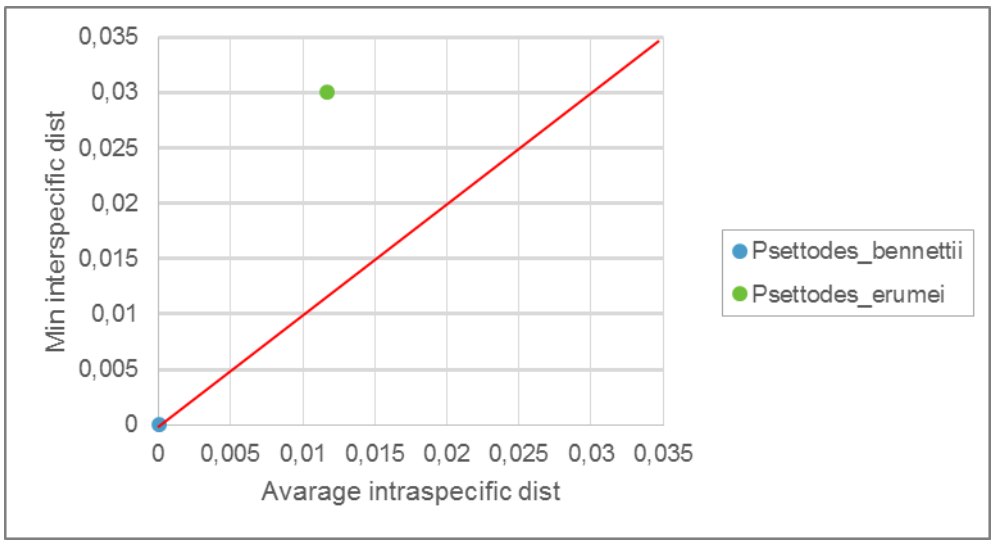


(b)

Fig. 11.2: Barcode gap analysis for **Soleidae** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker 16S rDNA



(a)



(b)

Fig. 12.1: Barcode gap analysis for **Psettodoidei** (a) maximum intraspecific distance vs minimum interspecific distance, (b) average intraspecific distance vs minimum interspecific distance, using the molecular marker COI