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11. Application of genetic and evolutionary approaches in marine conservation and management (Case study: *PANGAS* project)

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Abstract: Contemporary studies of population structure and genetic diversity increasingly employ new analytical and technical tools that provide high-resolution genetic information. These tools, that include procedures such as polymerase chain reaction amplification (PCR) and automatic DNA sequencing, combine to provide unprecedented power to detect and interpret genetic variation in natural populations. There are numerous advantages to applying molecular tools for studying the structure and dynamics of marine ecosystems, especially for purposes of marine reserve design and sustainable fisheries management. For the latter, results of connectivity studies allow us to predict how populations are likely to respond to varying levels of extraction or protection by explicitly accounting for replenishment of populations through local or distant sources. The geographic scale of genetic structure helps guide decisions on the scale of management schemes. We are able to investigate the spatial genetic structure of certain targeted species (in this case species of fish and invertebrates that are of commercial interest) thanks to advances in molecular approaches - both regarding the development of new technologies as well as their increased availability (molecular techniques are becoming more affordable and available for population level studies that require large numbers of samples). Some of the procedures and tools that will be mentioned here are the following: DNA isolation; PCR amplification; Automatic DNA sequencing; Data (DNA sequence) analysis using various software packages which involved sequence alignment and analysis, phylogenetic reconstructions and population genetics.

Key words: DNA isolation; PCR amplification; DNA sequencing;

INTRODUCTION

Marine ecosystems are increasingly under a variety of pressures from human activities. Besides the historical and current effects of legal and illegal overfishing and destructive fishing methods, there is also the concern of increasing sea surface temperatures, ocean acidification and pollution [9]. All of these have negative impacts on marine biodiversity and ecosystem services to humans. The proliferation of ocean-related activities and growing demands for marine resources have given rise to increasingly complicated policies, rules and regulations that are meant to govern those activities. The widespread degradation of ocean resources necessitates ecosystem-based approaches to the management of marine resources [1].

Management goals must be framed with respect to the conservation of ecosystem services, i.e. ensuring that marine ecosystems can fully function in order to sustain the delivery of a wide range of services. For most, if not all, sectors of management, this constitutes a major shift in perspective [10]. Among the most visible and most exploited marine ecosystem services is the provision of seafood, primarily fish. Fishing represents an important source of protein, as well as income for much of the global population and has a long history. Nearly half of the world's marine fish stocks are fully exploited, and another quarter are overexploited. The declining production of capture fisheries due to overharvesting is a well-recognized problem [8]. Overexploited and collapsed fish stocks, poor recovery after fishing ceases, and altered interspecific interactions indicate that fisheries science and management are not accounting for all relevant factors that influence the dynamics of aquatic ecosystems [6]. Current goals inherently recognize that it is not possible to sustain humans without sustaining ecosystems over long time frames [10]. Ecological changes - including harvesting, pollution and climate change, to name a few that can directly be tied to human activities - cause phenotypic changes in natural populations [12].

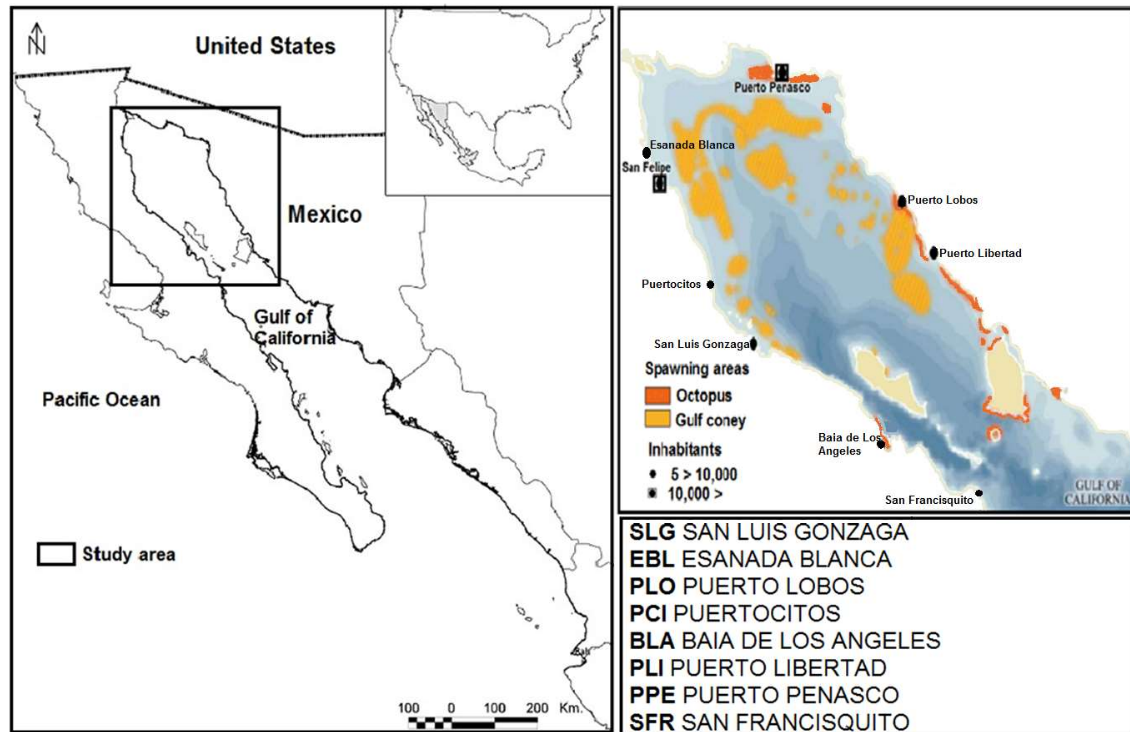
A number of recent studies have reported on the rapid evolution of morphological, physiological, behavioral and demographic traits over time scales of *generations* – which coincides with the time horizon of many conservation schemes [2]. Both population size and patterns of gene flow have been dramatically affected by human activities that cause increasingly fragmented environments resulting in increasing levels of genetic distinctness and loss of accessible genetic variation. From an evolutionary perspective, natural populations are threatened by three forces that interact to produce a downward spiral of evolutionary potential: reduction in genetic variation as a consequence of decreases in population size (affecting *in situ* evolution); reduction in gene flow preventing influx of genetic variants from another population; reduction in environmental heterogeneity that can lead to a decrease in adaptive capacity of a species as a whole [7; 5; 3]. However, ecologically significant evolution occurring over tens of generations or fewer is widely documented in nature [4]. The factors that influence evolution on ecological time scales such as phenotypic plasticity, maternal effect and gene flow have significant implications for population persistence, speciation, community dynamics and ecosystem functioning. The rate of environmental change is exceeding the capacity of many populations to adapt. One of its primary goals would be to identify the conditions that allow the recovery of declining populations. It should be added that human activities (harvesting, pollution etc.) constitute a significant part of what we call "environmental change".

MATERIALS AND METHODS

Through a review of literature during June – August 2015 we attempted to identify, describe and connect topics from genetics and evolutionary biology that we believe are of crucial importance to current and future fisheries management and conservation strategies. There will be explained most of the protocols that were used in the lab for obtaining data (primarily mtDNA fragment sequences), with some useful notes on various steps of the procedures. The described protocols are given exactly as there were performed in the lab, but of course, all labs differ in their approach, practice, and available equipment and therefore it cannot be claimed that these protocols are universally applicable.

Sites and sampling
The tissue samples were collected in the northern Gulf of California. Some examples of collection sites (**Fig. 1**) and their three-letter abbreviations used for labeling.

Fig. 1 Study area, spawning areas of two commercial species: *Octopus bimaculatus* and *Epinephelus acanthistius* (Gulf coney) and sample sites



The fish species included in the study: HGU *Hoplopagrus guentherii* Barred Pargo, EAC *Epinephelus acanthistius* Gulf Coney (Baqueta), MRO *Mycteroperca rosacea* Leopard Grouper, PAR/PLE *Paralictidae/Pleuronectidae* (a flatfish family), SPH (SPP) *Sphyrna spp.* Hammerhead shark,
The invertebrate species included in the study: HNI *Hexaplex nigritus* Black Murex, SPOCAL *Spondylus calcifer* Rock scallop, OCTBIM *Octopus bimaculatus* Spotted octopus

The focus of PANGAS[1] project is on marine species (fishes and invertebrates) captured by commercial and sports fishermen. The tissues collected were largely fin clippings or muscle tissue. In general, when collecting tissue for DNA extraction, it is good to choose one of the following:

- Tissue that contains smaller amounts of DNAases (such as testicles, thymus, brain tissue or blood in the case of non-mammals – birds and fishes). The drawback here is that the DNA is huge and hard to handle when extracted; it takes a long time to re-suspend.
- Tissue that contains a lot of DNA (such as liver cells, which are in a syncytium – many nuclei within a single membrane – the *enzymes: DNA* ratio is more favorable here)

One of the crucial things about collecting samples is how they are preserved (fixed) in the field. For the purpose of phylogenetic or population genetics studies, it is extremely important that the samples are fixed *as soon as possible* after being collected, so that the DNA in the samples remains intact, and can later be extracted and sequenced. The problem is that with time, cell membranes are getting degraded and DNAases present in the cell can start digesting the cell's own DNA. Fixing the tissue in formaldehyde, alcohol (95% ethanol, or at least 75%) or in salts, or by quickly drying it, inactivates the DNAases, giving us a good source of intact DNA.

Protocol description

a) *DNA extraction*

1. Label fresh Eppendorf tubes for all samples.
2. Prepare the master solution for digestion of the tissue, and always be sure to prepare enough for at least one extra sample (n+1), the amounts per sample and order of mixing are as follows:
Lysis Buffer*………..613 µl
20 % SDS …………….30 µl (breaks membranes, destabilizes proteins: stops DNAase activity)
Proteinase K …………..7 µl (removes histones)
This means a total of 650 µl of master mix per sample, which we distribute in the labeled tubes.
*Lysis Buffer recipe:

- Tris…………10 mM
- NaCl……….400 mM
- Na_2EDTA…….2 mM

3. Add a small piece of tissue to the digestion solution in the vials
Cutting tissue from the original tissue samples that were collected:
In the case of the *PANGAS* species, most of the samples are fish, and the tissue was taken from the fins. There are two "schools" of tissue cutting – those that cut tissue from the very tip of the fin (the idea behind this choice being that the alcohol reaches the cell's DNAases faster during fixation, therefore the DNA is better preserved) and then, there are those that take tissue from the proximal part of the fin (there is more muscle tissue there, and thus more DNA). We use sterile razorblades and a pincette.
4. Secure tubes on rack by taping a piece of paper over them and place on the incubator rocker in a *horizontal position.* Incubate overnight at 55º C, rocking speed 8, for no less than 4 h and no longer than 17 h. After overnight incubation:
5. Add 375 µl 5 M NaCl slowly invert or vortex for several seconds wait 30 minutes centrifuge 30 minutes at 14000 rpm (or 13000 rpm, depending on the model)
6. Add 750 µl of chloroform to a fresh tube
Remove and keep the supernatant (take 800µl) and put into the tube containing the chloroform Vortex 2 seconds, the sample should look milky Wait 10 minutes, Centrifuge 10 minutes at 11000 rpm (or 10000).
7. Add 750 µl of *isopropanol* to a fresh tube
Remove and keep the supernatant (take 750 µl) and put into the fresh tube containing the isopropanol. When taking up the supernatant: use a *manual* pipette and make sure not to touch or take up the interface with the tip. It is better to take up less than 750 µl than to touch the interface. Vortex for 2 seconds and wait 30 minutes Centrifuge 30 minutes at 14000 rpm (or 13000).
8. Hold tightly when opening the cap – shaking ("popping" it open) might dislodge the pellet. Quickly and carefully remove the supernatant (pour it out into the sink). Invert the tube very briefly onto tissue paper to dry. Centrifuge just 5 seconds at 13000 rpm to separate pellet and leftover alcohol.
Remove alcohol with a manual micropipette (a P200 set at 120-150 µl) placing the pipette tip on the opposite side of the tube from the pellet. "Follow" the surface of the liquid down with the tip as you suck it up (image at right). Dry

[1] *PANGAS* is an interdisciplinary, integrative, bi-national project for *ecosystem-based research and management* of coastal fisheries. *PANGAS* stands for *"Pesca Artesanal del Norte del Golfo de California – Ambiente y Sociedad"* (Small-scale Fisheries in the Northern Gulf of California – Environment and Society).

the DNA in the vacuum dryer, using program no.1. Leave tubes open and balance the load (like for centrifugation). The DNA is dry when there is no droplet at the bottom of the tube (check this by holding it up against the light).
9. Add 100 µl of *milliQ* water to re-suspend. Label the tube as “DNA” and store in refrigerator.

b) PCR - the Polymerase Chain Reaction

PCR (*Polymerase Chain Reaction*) is a technique that allows researchers to amplify a sequence of interest, producing millions of exact copies from as little as one molecule of the original template (DNA extracted from the sample) in just a few hours. If it is successful, the product of the PCR can be used for sequencing the amplified fragment.

Primers are conserved regions bordering the fragment of interest, usually around 18 – 25 base pairs in length. Our goal here is to determine the *variable sequence* between the primers. Primers can be designed by a researcher and ordered from producers. The price of commercially synthesized primers is calculated per base pair. Finding primers for invertebrates is more difficult than for vertebrates (fishes) because they are less closely related (belonging to different phyla) and have been less studied.

For setting up the PCR we need a forward primer (borders the target sequence on the 5’ end; its name ends with “L”- for “light” chain - such as PROL) and a reverse primer (on the 3’ end of the target sequence; its name ends with “H” - for “heavy” chain - such as TPHEH).

Ammounts for one sample:

- Mastermix*………….. 11.25 µl
- Primer 1 (20µM)…….. 0.625 µl
- Primer 2 (20 µM)……. 0.625 µl

* Mastermix contains buffer, Taq polymerase and dNTPs

Total volume of master solution per sample is 12.5 µl.
Vortex briefly to mix.
Add DNA template………………0.5 µl
Total reaction volume per tube is 13 µl.

PCR cleanup is performed to remove unincorporated nucleotides, primers, enzymes and salts from the PCR reaction, leaving only the amplified DNA fragments.

The functioning principle of the QIAGEN columns: the negatively charged phosphate groups in the DNA bind to the positively charged clay in the column. Small molecules such as nucleotides and primers do not bind to the clay and will pass through the filter and be discarded.

QIAquick PCR Purification Kit Protocol (using a microcentrifuge)

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions.

Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifuge steps are at 13,000 rpm (~17,900 x *g*) in a conventional tabletop microcentrifuge

c) Sequencing reaction

The goal of this step is to separate the strands of the amplified DNA fragment and then perform the amplification with each of the primers in separate tubes.

Prepare a master solution containing water, sequencing buffer, one of the primers and Bigdye® (prepare an amount enough for at least one extra sample), distribute to PCR tubes, and then add template (EL DNA).

The protocol

Preparing the master solution: (Amounts for one sample, to be added in this order)

- Milli-Q water ………….6 µl
- Sequencing buffer…..1.5 µl
- Primer (2µM)………….1 µl
- Bigdye®……………….1 µl

Centrifuge for several seconds. Total volume of master solution per sample is 9.5 µl, distribute to PCR tubes. Add DNA *EL* template, 0.5 µl (touch the bottom of the tube with tip). Total volume in PCR tube is 10 µl. Chose the Bigdye® run program on the PCR machine. The parameters of the program are:

- Reaction volume……..10 µl
- Number of cycles…….35

Sequence reaction cleanup

Cleanup of the sequencing reaction was done by sodium-acetate and ethanol precipitation. Ethanol and salt are added to the aqueous solution, which forces the precipitation of nucleic acid out of the solution. After precipitation,

the nucleic acids can be separated from the rest of the solution by centrifugation. The pellet is washed in 70% ethanol and after a further centrifugation step the ethanol is removed and the nucleic acid pellet is allowed to dry before being resuspended in formamide loading buffer.

Preparing precipitation solution (amounts per sample):

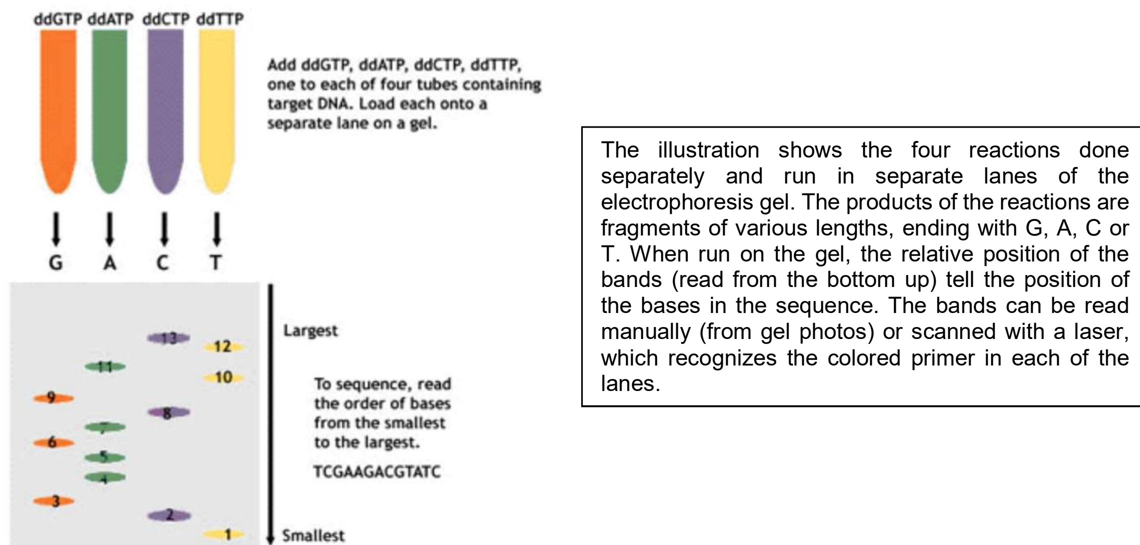
- Na-acetate 3M 3.0 µl
- 95% ethanol 72.7 µl
- Milli-Q water 4.3 µl

Total volume of precipitation solution per sample is 80 µl. Distribute to fresh 1.5 ml Eppendorf tubes Add 10 µl of MilliQ water to the PCR tubes, making 20 µl of the sample. Transfer the samples to the precipitation solution (pick up *all* 20 µl!). Vortex for 2 seconds. Wait 15 minutes. Centrifuge for 20 minutes at 13000 rpm. Remove the supernatant carefully, using a P200 manual pipette, and placing the pipette tip on the opposite side of the tube from the pellet. Add 250 µl 70 % ethanol (the water in it removes the salts). Centrifuge for 20 minutes at 13000. Quickly and carefully remove 250 µl of supernatant with a P1000 manual micropipette. Repeat if necessary, samples must be completely dry for sequencing. Add 14 µl of formamide to dry template and vortex briefly. Formamide separates the strands and keeps them linear, which is important for the next step – automatic sequencing.

d) *DNA sequencing*

Two of the better-known methods of sequencing, developed in the mid-1970s:

- the Maxham/Gilbert method – this is a chemical method that is used relatively rarely, because it is slow (can take weeks) and involves a lot of exposure to radioactivity. It is useful when the fragment we wish to sequence contains palindrome sequences (“hairpins”) that interfere with the more commonly used Sanger method;
- the Sanger method (**Fig. 2**) – is based on the use of di-deoxynucleotides (ddNTPs) in the sequencing reaction, which prevent further polymerization. The sequencing reaction is basically a PCR reaction, with the addition of ddNTPs, as well as dNTPs (regular deoxynucleotides).



The illustration shows the four reactions done separately and run in separate lanes of the electrophoresis gel. The products of the reactions are fragments of various lengths, ending with G, A, C or T. When run on the gel, the relative position of the bands (read from the bottom up) tell the position of the bases in the sequence. The bands can be read manually (from gel photos) or scanned with a laser, which recognizes the colored primer in each of the lanes.

Fig. 2 The Sanger method

For the *PANGAS* project samples, sequencing was done with an automated sequencing machine, the ABI PRISM® 3100, owned by UC Santa Cruz. It is capable of running a wide variety of DNA electrophoresis applications for sequencing and fragment analysis—including microsatellite analysis, comparative genotyping, and SNP validation and screening—as well as *de novo,* and comparative sequencing. DNA fragments (up to 800np) can be sequenced using automated DNA sequencing machines. Complimentary DNA strands are made using starter primers and dideoxy-nucleotides (ddNTPs). When ddNTP's are incorporated, it terminates a growing DNA strand, thus we make many strands of different lengths, each with an end nucleotide fluorescently tagged different colors. All the strands undergo polyacrylamide gel electrophoresis through capillary tubes, which separate the DNA strands by size length. They are then scanned by fluorescent detectors and plotted out.

e) *Computer software packages*

The rapid infiltration of DNA-based technology has caused an infusion of computer software and online databases into the daily activities of many population geneticists. The identification of highly variable loci, discovered by a variety of techniques, has brought about a revolution in the way researchers view and analyze variation, as well as levels of variation. Computer programs and software packages can somewhat arbitrarily be divided and focus on two primary levels of enquiry:

- intra- and inter-population structure
- phylogenetic inference

The primary focus of population genetics programs is on the assessment of diversity within and among populations and determination of whether or not allelic associations exist at several different levels. At a genotypic level, at *individual loc*i, one can test for departure from Hardy-Weinberg equilibrium. At *two or more loci* linkage disequilibrium, can be tested, for example, as the departure of gametic frequencies from those expected under allelic independence among the loci. Finally, F-statistics for analyzing the distribution of genetic diversity within the data are used to test the null hypothesis that the presumptive populations are really samples from one large panmictic population. One can then base genetic distance estimates on the allele frequency distribution(s) to quantify any observed patterns of variation. Generally underlying all these analyses (of genetic diversity within the data) is the *characterization* of the alleles at a given locus, or more often, at multiple loci, followed by the *estimation* of the frequency distribution of these alleles among populations. In contrast, phylogenetic reconstruction programs are applied to molecular data to estimate the evolutionary history of: alleles within a species, homologous loci among several different species, or even entire genome comparisons (such as with DNA-DNA hybridization experiments). From these estimates, inferences are made about the evolutionary relationships among the species or populations from which the data were obtained, resulting in a *phylogenetic tree*. On the next page are some of the major features of the computer software packages for phylogenetic analysis that we used to analyze the DNA sequences we obtained from our *PANGAS* samples. These programs are, for the most part, readily available, actively supported and in widespread use.

RESULTS AND DISCUSSIONS

This is an exciting time for use of genetic methods in applied conservation and management, and genetic methods have become indispensable to twenty-first-century fisheries management. Several factors have come together to provide unprecedented opportunities in this field: technical advances in the laboratory have uncovered an essentially unlimited number of highly variable genetic markers that can be utilized to study natural populations, and available methods can extract DNA non-lethally from increasingly small amounts of biological material, which allows routine, non-invasive monitoring as well as retrospective analyses using historic samples. Further, numerous powerful analytical methods have been developed in the past decade that provide new opportunities to test hypotheses about contemporary evolutionary and ecological processes in populations. Finally, computational power continues to increase rapidly, and these increases have made feasible implementation of many of the new likelihood-based methods that are computationally demanding [11]. It can be expected that improvements in each of these areas will continue into the future, and will continue to refine and improve the applications of genetics to fishery management. The importance of genetic methods is likely to increase in the coming decades, particularly as genetic approaches become better integrated with more traditional ones. The applications of genetic markers take advantage of natural evolutionary processes that have occurred among and within populations and which produce a signal that can help inform fishery management. However, it is crucial that we start paying careful attention to the evolutionary changes to natural populations that are influenced by anthropogenic factors. Whereas the ecological consequences of anthropogenic changes on aquatic populations have received a great deal of attention, the evolutionary consequences of these changes have been relatively neglected. It is much more difficult to determine exactly what these evolutionary consequences are/will be, and how important they will be to conservation and management. Until recently conservation biology provided little more than reactive, short-term and small-scale solutions to environmental threats. Scientists are now advocating for a shift from saving *things,* i.e. the products of evolution, to saving *the underlying process* i.e. evolution itself.
The harsh truth is that ignoring evolutionary mechanisms and dynamics renders our conservation efforts (and sometimes successes) as temporary only [2]. To quote Woodruff [12] “Like it or not, evolutionary biologists have to recognize that the ultimate test of their science is not their ability to solve the riddles of the past and the origin of species, but rather to manage their viability and prevent their premature extinction, to manage the biosphere’s future. In this sense, if they turn around and face forward in time, evolutionary biologists become conservation scientists”.
The overall process of laboratory analysis involved DNA extraction, PCR amplification, sequencing and sequence alignment, followed by phylogenetic analysis. After DNA extraction, we proceeded with PCR amplification for which a first set of mitochondrial primers were tested:
Cytochrome oxidase I (COI) for *invertebrates*
If the primers worked, samples were sequenced. If they did not work, other CO1 primers were tested and/or conditions changed.
Control region (D-loop) for *fishes*
If the primers worked, samples were sequenced. If they did not work, cytochrome b (CYT B) primers were tested and/or conditions changed. We then proceeded with sequencing the samples, with a smaller number of individuals sequenced at first. Depending on the results, the strategy was as follows:

- If sequences did not work, PCRs were redone.
- If sequences worked, all samples of the species were sequenced.
- If sequences were not variable, a different gene was chosen.
- If sequences were variable, a phylogenetic tree was constructed and gene flow was calculated (a proxy for dispersal and connectivity).

Microsatellite primers were also tested for some key species.

Phylogenetic trees for some species (presented below) were obtained from the preliminary sets of mitochondrial sequences using the *Neighbor-Joining Method* implemented by the software package *PAUP*. Labels correspond to sampling localities.

Invertebrates - we worked on the following three species:
1. Rock scallop *Spondylus calcifer* (SPOCAL). DNA were successfully extracted but we had not yet been able to obtain any amplifications (we used two different sets of COI primers)
2. Black murex *Hexaplex nigritus* (HNI). DNA were successfully extracted but we had not yet been able to obtain any amplifications (we used two different sets of COI primers). We also obtained a microsatellite library. From this microsatellite library, we designed 40 primer pairs (loci). We ordered 15 primer pairs that would be tested in the next phase of laboratory work.
3. Octopus *Octopus sp. (possibly O. bimaculatus)* (OCT BIM). DNA extraction was successful. However, two students worked on several samples for one month without a single successful amplification. During the course, we tried two sets of primers, and the second set worked for two individuals. From this success, we proceeded to design new specific primers for future use (**Fig. 3**).

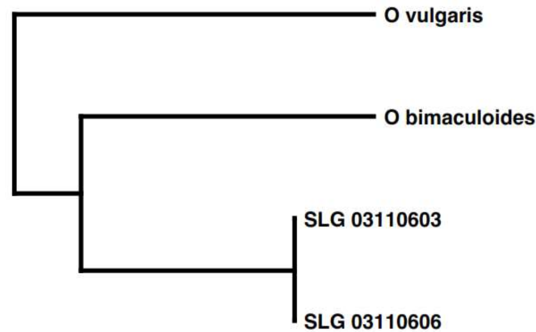


Fig. 3 Preliminary results from two Octopus sp. specimens from San Luis Gonzaga (SLG)

Fishes - we worked on the following five species:
1. Leopard Grouper *Mycteroperca rosacea* (MRO)
2. Hammerhead shark *Sphyrna spp.* - (SPHSPP)
3. Barred Pargo *Hoplopagrus guentherii* (HGU)
4. Gulf Coney *Epinephelus acanthistius* (EAC)
5. A flatfish family, *Paralictidae* or *Pleuronectidae* (PAR/PLE) halibut or flounder

DNA extraction for all species was successful. Control region (D-loop) amplification worked for all but Hammerhead and Leopard Grouper. Therefore, cytochrome b was used for these species and it worked well. A brief summary for species with few individuals:
Hammerhead shark appeared to be different in the Gulf and outside the Gulf (compared to GenBank sequences). Too few sequences were obtained of Leopard grouper to conclude anything. Importantly, though, this shows that the chosen approach does work, it is just a matter of time and sample sizes.
Halibut (the unidentified flatfish family) collected in San Luis Gonzaga showed to be different from California Halibut and different from *Paralychthys woolmani* (speckled flounder), a species from Bahia Magdalena (**Fig. 4**).

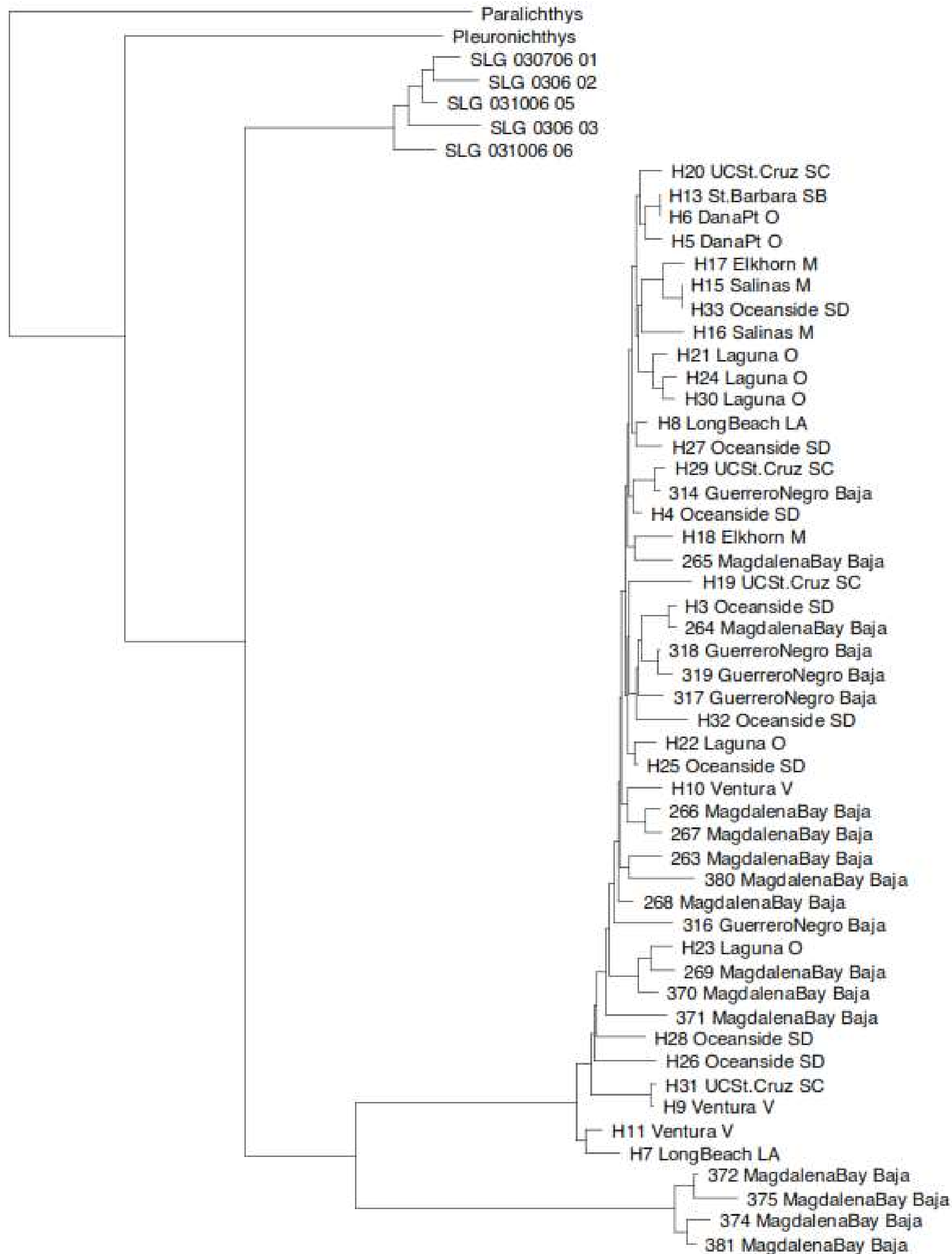


Fig. 4 Preliminary results from halibut collected in San Luis Gonzaga (SLG)

The Gulf Coney (*Epinephelus acanthistius)* was our star species, all samples worked right away. They showed high diversity. We had only sequenced one population at the time, but the samples looked very promising (**Fig. 5**).

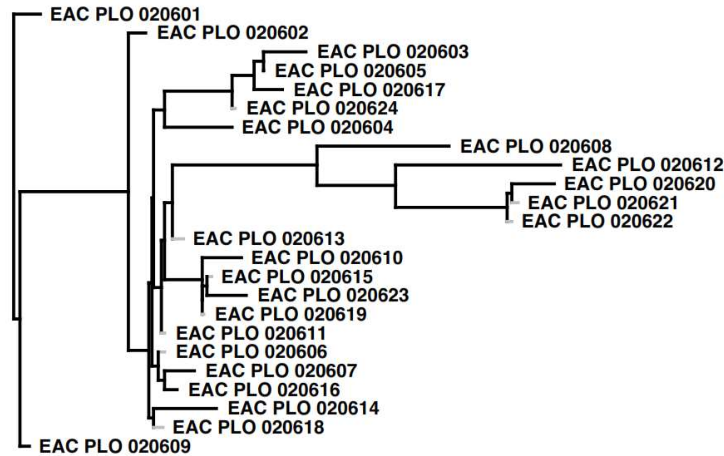


Fig. 5 Gulf Coney from Puerto Lobos (PLO)

Barred pargo samples also worked very nicely (**Fig. 6**). They showed, at the D-loop level, a huge diversity - "among the highest diversity recorded for any of the fish" (Giacomo Bernardi, personal communication, 2006). First values (gene flow, connectivity) were, in some cases, very high, suggesting a low level of gene flow between populations, thus explaining how high diversity may be maintained.

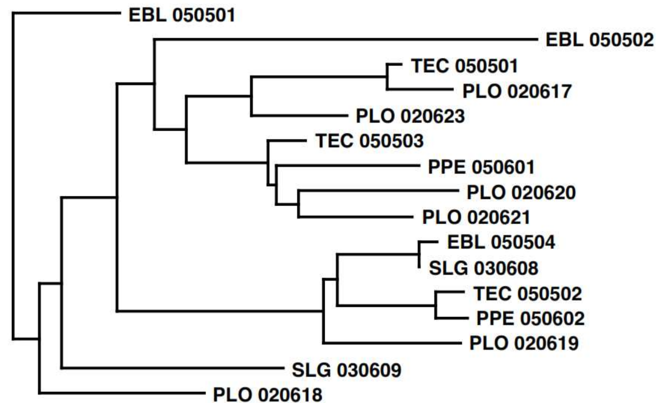


Fig. 6 Barred pargo from Tiburon (EBL, TEC), Puerto Lobos (PLO), and San Luis Gonzaga (SLG)

CONCLUSIONS

1. Invertebrates are technically more difficult than fishes
2. Results so far were encouraging and surprising. One would never have imagined such a high level of diversity and population structure.

Possible directions of further laboratory work: Deciding which species to focus on (if any), which species to drop (if any); Considering our results, will would want to get other microsatellite libraries (Grouper, Coney, Octopus…); Once we obtained more results, we might want to refine our sampling design, to get more information in specific regions that seem more genetically diverse, or different habitats. Overall, knowledge about the connectivity of marine populations is likely to be gained only through integrated, multidisciplinary efforts in which genetic methods can and should play an important part. Although the "big" picture afforded by indirect estimates of gene flow should always be considered, direct genetic methods are likely to take precedence in future studies focusing on ecological time scales and processes. With respect to future genetic surveys, they should include more detailed spatial and temporal sampling and employ analyses of DNA sequence data that can reveal the signatures of natural selection and historical changes.

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REFERENCES

1. COCHRANE (K.L.) (ed.), 2002 - A fishery manager's guidebook. Management measures and their application. FAO *Fisheries Technical Paper*. No. 424. Rome, FAO. 231p.
2. FERRIÈRE (R.), DIECKMANN (U.), COUVET (D.D.) (eds.), 2004 – Evolutionary Conservation Biology. *Cambridge Studies in Adaptive Dynamics*, Cambridge University Press.
3. GARZA (J.C.), WILLIAMSON (E.G.), 2001 - Detection of reduction in population size using data from microsatellite loci, *Molecular ecology*, vol. 10, Issue 2, pp. 305–318.
4. HOFFMANN (A.), GRIFFIN (P.), DILLON (S.), CATULLO (R.), RANE (R.), BYRNE (M.), JORDAN (R.), OAKESHOTT (J.), WEEKS (A.), JOSEPH (L.), LOCKHART (P.), BOREVITZ (J.), SGRÒ (C.), 2015 - A framework for incorporating evolutionary genomics into biodiversity conservation and management. *Climate Change Responses*, 2:1.
5. LANDE (R.), BARROWCLOUGH (G.F.), 1996 - Effective population size, genetic variation, and their use in population management, chapter 6 in: *Viable Populations for Conservation* edit. Michael E. Soulé, Cambridge University Press, 120 p.
6. LAUGEN (A.T.), ENGELHARD (G.H.), WHITLOCK (R.), ARLINGHAUS (R.), DANKEL (D.J.), DUNLOP (E.S.), EIKESET (A.M.), ENBERG (K.), JØRGENSEN (C.), MATSUMURA (S.), NUSSLÉ (S.), URBACH (D.), BAULIER (L.), BOUKAL (D.S.), ERNANDE (B.), JOHNSTON (F.D.), MOLLET (F.), PARDOE (H.), THERKILDSEN (N.O.), UUSI-HEIKKILÄ (S.), VAINIKKA (A.), HEINO (M.), RIJNSDORP (A.D.), DIECKMANN (U.), 2014 - Evolutionary impact assessment: accounting for evolutionary consequences of fishing in an ecosystem approach to fisheries management. *Fish and Fisheries*, vol. 15, pp.65-96.
7. MASATOSHI (N.), TAKEO (M.), RANAJIT (C.), 1975 - The Bottleneck Effect and Genetic Variability in Populations, *Evolution, Published by Society for the Study of Evolution,* vol. 29, no. 1, pp.1-10.
8. MILLENNIUM ECOSYSTEM ASSESSMENT. 2005. Ecosystems and Human Well-being: Synthesis. Island Press, Washington, DC.
9. NELLEMANN (C.), HAIN (S.), ALDER (J.) (eds.), 2008 - In Dead Water – Merging of climate change with pollution, over-harvest, and infestations in the world's fishing grounds. *United Nations Environment Programme, GRID-Arendal*, Norway, 189 p.
10. ROSENBERG (A.A.), MCLEOD (K.L.), 2005 - Implementing ecosystem-based approaches to management for the conservation of ecosystem services. *Marine Ecology Progress Series*, 300, pp. 270-274.
11. WAPLES (R.), NAISH (K.A.), 2009 - Genetic and Evolutionary Considerations in Fishery Management: *Research Needs for the Future*. Publications, Agencies and Staff of the U.S. Department of Commerce, 457 p.
12. WOODRUFF (D.S.), 2001 - Decline of biomes and biotas and the future of evolution, *Proceedings of the National Academy of Sciences* 98, pp. 5471-5476.