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REVIEW

Corollary of Marine Eco-system Sustainability by Addressing the Issues of Bycatches

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ABSTRACT

The issue of bycatch in the fisheries sector has been a major concern for the marine biologists over few decades in terms of conservation of marine eco-system and sustainability of the fisheries sector and marine biodiversity. As far as the concept of Bycatch is concerned, these are the unwanted species having less commercial importance, which in most of the cases disposed into the seas onboard or are caught during fishing. The article discusses the social, economic, cultural, and environmental impacts in addressing the issue of bycatch. The concern regarding bycatch is relatively new in the horizon in Indian perspective though it is often considered a major issue faced by developed nations since long. The problem of bycatch poses a serious threat to livelihoods and food security as it acts as a precursor to depletion of the food sources for local consumption with adverse social, economic, cultural, and environmental impacts. The adverse effects of by-catch can be mitigated through commercialisation of bycatch and making profits from the sale of bycatch, opening up of new markets for bycatch species or products, uses of bycatch as fishmeal and application of bycatch reduction devices (BRDs). Reorientation of the present monitoring system to collect fisheries data, strengthening technical know-how, bringing effective policy intervention, efficient co-management, and sincere and honest efforts in reducing bycatch and discards may provide a considerable impact towards sustainability of marine eco-system.

1. Introduction

The issue of sustainability in fisheries has become a global concern and ecosystem-based approaches are getting wider recognition through-out the world [1,3,8]. The issue of bycatch in the fisheries sector has been a major concern for the marine biologists over few decades in terms of conservation of marine eco-system and sustainability of the fisheries sector. Heavy commercial fishing creates disequilibrium not only in terms of the catch of targeted species, but it also encompasses other species, which are incidentally caught along with the targeted species. The concept of bycatch can be stated as catching of non-targeted species having less commercial importance, which in most of the cases disposed into the seas onboard or at fish landing centres. It is estimated that 7.3 million tonnes of the catch are globally discarded as bycatch per annum [4] and 16.7 million tonnes in world shrimp fisheries. The term 'discards' is synonymously equated with the word 'bycatch' in different countries which describes a portion of the targeted catch that is discharged on boards or at landing cen-

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tres. There are different impacts of by-catch can be seen in the fisheries sector which are: overexploitation of target and non-target fish stocks, the environmental damage caused by fishing gears which are lost or discarded, "ghost fishing" and pollution as a consequence of discards, as well as the "carbon footprint". There are various effects on the function of the marine ecosystem due to removal of some species as a consequence of bycatch. The decrease in numbers of several predatory species such as dolphins, sharks and sea snakes may lead to sudden rise in populations of various prey species, whereas population of predatory species may decrease if there is a sudden decrease of prey species including many sea birds and marine mammals [5,9].

The article discusses the social, economic, cultural, and environmental impacts in addressing the issues of bycatch.

2. Impacts of Bycatch in Biodiversity

Threats of bycatch are perceived to be a major issue concerning marine biodiversity. However, the real magnitude of this problem is masked by the different estimates which may be misleading [5]. It may be introspected that, further in-depth study in the same line can reveal some additional facts.

The concern regarding bycatch is relatively new in the horizon in Indian perspective though it is often considered a major issue faced by developed nations since long [7].

The issue of bycatch became relevant in India after independence due to gradual mechanisation of fisheries sector due to introduction of trawl fisheries, which made a drastic change in fishing behaviour. Trawl fisheries were mainly driven by demands from export markets in comparison to artisanal fisheries, as it is prominent in different parts of the world. Trawl fisheries resulted in the capture of a large number of non-target species as bycatch which unfortunately the largest share of the catch. There is a compulsion to the fishers as they have limited space available for storing the bycatch as the major storage spaces are occupied by commercial catches. As a result of this fishers have to discard the whole quantity of bycatch altogether. In tropical countries like India with diverse characteristics of fisheries and social groups, the problems of bycatch are perceived to be the utmost factual and normative and the stakeholders frequently seek the in-depth implications of bycatch so as to bring equity and justice in fisheries. The problem of bycatch poses a serious threat to livelihoods and food security as it acts as a precursor to depletion of the food sources for local consumption with adverse social, economic, cultural, and environmental impacts. As delineated by Gibinkumar [2], the followings are some of the direct/indirect impacts of bycatch-

2.1 Environmental Impacts

Apart from impacts on fish populations, the upwelling of benthic organisms to the surface can cause habitat modifications. The reduction of oxygen level in water bodies due to the decomposition affects the benthic community. Discards may have positive effects on the scavengers' populations, by making inaccessible food available with minimal efforts or otherwise. Habitat modification resulting from discards may at times be confused with habitat modification resulting from the fishing gear itself or with unobserved fishing mortalities because habitat and individuals can be damaged but not brought to the surface. Thus the extent of discards versus gear induced mortalities may be difficult to quantify.

2.2 Economic Impacts

Economic impacts though have got much significance, is one of the least discussed and studied aspects of bycatch. Following are the classifications of the economic impacts:

(1) Mortalities of commercially important economic species during discard of bycatch

(2) Economic losses due to additional expenses associated with catching, sorting, and discarding of unwanted catch, higher fuel consumption, greater wear and tear of the fishing system, and as well as the associated opportunity costs.

(3) Costs associated with monitoring, surveillance, and control for reducing discards.

2.3 Socio-cultural Impacts

Socio-cultural impacts of bycatch are difficult to quantify as the significance of conservation of marine biodiversity equilibrium and management of commercially important fish stocks in the maintenance of livelihoods of the poorest sections of the fishing community, is having a relatively higher level of difficulties.

3. Possible Solutions

Though the problems and issues of bycatch are relevant in a global perspective, the thrusts towards reducing its impacts in the marine ecosystem have been seen as in the nascent stage. The following probable solutions are being discussed herewith to derive a concrete comprehension to get rid of the menace of bycatch, especially associated with the marine fisheries.

3.1 Endowing a Commercial Value to Bycatch

There is a silver lining as the positive trends of bycatch commercialisation are being noticed amidst the ongoing

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practice of discarding bycatch, even for some protected species. This trend is also conspicuous even in trawl fisheries. There is an utmost need for adoption of this trend in India due to the following reasons:

3.1.1 Diminishing Returns from Target Catch

Due to overfishing and depletion of target stocks near the shoreline in India, there is a considerable reduction in the profit margin for the fishing industries. To compensate for the reduction of profit margin, fishers are being compelled to sell the bycatch, which was earlier discarded. It is also beneficial for livelihood and food security, and sustainability of the marine ecosystem.

3.1.2 Emerging Markets for Selling of Bycatch Products

There is a changing pattern in food habit in India are being noticed. Increasing market demand for seafood provides a clear indication of this changing food habit. Thus, it helps to explore the marketing opportunity by utilizing the bycatches as potential target catch.

3.1.3 Uses of By-catch as Fishmeal

There is a prevalent practice of discarding bycatches at landing centres and seas, which is detrimental to the marine ecosystem. But presently by and large the major portion of trash fishes are being landed by trawlers, are being dried up for use as a fish meal in aqua and poultry industries.

3.1.4 Reluctance in the Usage of Bycatch Reduction Devices (BRDs)

There are several instances of reluctance in the usage of Bycatch Reduction Devices by fishing communities in different parts of the world. As an example, adoption of Turtle Excluder Devices (TED) faced strong resistance from some coastal states of India due to loss of other valuable fishes in the process of conserving endangered species of olive ridley turtle which is often discarded as bycatch [6]. Thus, commercialization of bycatch becomes more pertinent as an alternate solution for the adoption of BRDs as an innovation.

3.2 Reorienting the Present Monitoring System for Collection of Fisheries Data

For coping up with the loopholes in the present mechanism of fisheries data generation that only enumerates the fisheries data generated in landing sites but not taking into account the catches that are discarded onboard. It certainly gives the incomplete figures of bycatch and lot of endangered marine species are being pushed to the level of extinction without any clues, which is a tremendous threat to the marine ecosystem and biodiversity. Thus, it demands a reorientation of the present monitoring system for collection of error-free fisheries data.

3.3 Development and Adoption Relevant of Technological Innovations in Bycatch Reduction

It is allegedly said that the using bycatch reduction devises like, TEDs, SONAR etc. reduce the quantity of targeted catch, which resulted in the non-adoption of these devices. Hence, it is introspected as a need of the hour to strengthen the innovation development process for the conservation of marine ecosystem in general, and addressing the issues of bycatches in particular to make it more compatible with the demand of fishers.

3.4 Policy Implications

There are several rules, regulations and laws framed by different nations all over the world to address the issue of bycatch for the conservation of the marine ecosystem. But the lack of uniformity in the rules, regulations and laws is posing a major hindrance in implementation and enforcement. Even the honest intention by the different nation for implementation of laws is not at all conspicuous with their existing efforts. Moreover, lack of implementation and enforcement of rules and regulations and weak monitoring system in the Exclusive Economic Zone (EEZ) in addressing the issue of bycatch made it more complex. Thus, a global policy formulation, enforcement and strategy for surveillance is required to address the issue.

4. Conclusion

Hence, bycatch and discards are the common problems faced by all fisheries stakeholders globally, and it is a major component of the negative impacts of fishing in marine ecosystems. It is an extremely complex set of scientific and ecosystem-based issue and includes many economic, political, and moral factors. Bycatch is recognized as unavoidable in any kind of fishing, but the quantity varies according to the gear operated. Bycatch quantity also varies. Thus, efficient management in reducing bycatch and discards, and sincere and honest efforts to address the issue may provide a considerable impact towards sustainability of marine eco-system.

References

[1] Bhatal B, Pauly D. “Fishing down marine food webs”

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and spatial expansion of coastal fisheries in India, 1995-2000. Fisheries Research, 2008, 91: 26-43.

[2] Gibinkumar TR. Investigations on Hard Bycatch Reduction Devices for Selective Trawling (Ph.D. Thesis),Faculty of Marine Sciences, Cochin University of Science And Technology, 2008, available at (accessed 1 Nov. 2019): https://pdfs.semanticscholar.org/d9c1/78a68f77f7963f3ff33d2f22a37fd7ec7c3a.pdf

[3] Jennings S, Kaiser MJ. The effects of fishing on marine ecosystem. Advanced Marine Biology, 1998, 34: 201-352.

[4] Kelleher K. Discards in the world's marine fisheries, An update. FAO Fisheries Technical Paper, 2005, 470: 131.

[5] Lobo AS. Managing fisheries in an ocean of bycatch. Position Paper for CBD-COP 11. Dakshin Foundation, Bengaluru and Foundation for Ecological Security, Anand, 2012.

[6] Lobo AS, Balmford A, Arthur R, Manica A. Commercializing bycatch can push a fishery beyond economic extinction. Conservation Letters, 2010, 3: 277-285. https://doi.org/10.1111/j.1755-263X.2010.00117.x.

[7] Pauly D. 1996. Fleet-operational, economic and cultural determinants in Southeast Asia. Solving by-catch: considerations for today and tomorrow. University of Alaska, Sea Grant College Program, Report No. 90-03. Fairbanks, Alaska.

[8] Pikitch EK, Santora C, Babcock EA, Bakun A, Bonfil R, Conover DO, Dayton P, Doukakis P, Fluharty D, Heneman B, Houde ED, Link J, Livingston PA, Mangel M, MacAllister MK, Pope J, Sainsbury KJ. Ecosystem-based fishery management. Science, 2004, 305: 346-347.

[9] Raby GD, Colotelo AH, Blouin-Demers G, Cooke SJ. Freshwater commercial bycatch: an understated conservation problem, BioScience, 2011, 61(4):271–280. https://doi.org/10.1525/bio.2011.61.4.7

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ARTICLE

Prevalence of *Argulus* sp. in Indian Major Carps from Bhangore Block of South 24 Parganas District, West Bengal, India

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ABSTRACT

The study was carried out to find the prevalence and severity of *Argulus* sp. in Indian Major Carps (IMCs) collected from Bhangore block of South 24 Parganas district of West Bengal during November 2018 to August 2019. January (2019) recorded the highest parasitic prevalence (PFI, 83.33%) and the month of August (2019) recorded the lowest parasitic prevalence (PFI, 9%). The identities of selected parasites were further confirmed by molecular identification through 18S rDNA analysis. The study revealed that *Argulus* sp. infestations had great economic implications especially in the winter months and is one of the most prevalent problems in fresh water aquaculture systems.

1. Introduction

The aquaculture sector of India is a potent food producing sector providing nutritional security, supporting livelihoods, providing employment and contributing to a major portion of agricultural exports. West Bengal's complete water area supports the state's potential fish farming and provides a variety of nutritional and dietary resources. West Bengal is popularly known as "rice – fish society" for the love of the people towards fish. Farming of the IMCs mainly takes over the state's freshwater aquaculture system in promoting fish culture and development in West Bengal. The availability of safe and unique pathogen-free fingerlings and carp fry is of utmost importance. Literature cautioned that diseases, especially the parasitic diseases caused great damage to carps affecting their fry and fingerlings [1]. The intensity and severity of parasitic contamination in fishes show substantial variation within the distinctive environmental situations wherein they live [2]. Certain environmental conditions encourage diseases, among which water temperature is a crucial criteria [3]. Disease being the key problem for the fish culture causes a catastrophic effect both in economic and social growth. The upliftment and pro-

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motion of healthy and sustainable aquaculture practices face a number of problems, of which, emergent parasitic diseases pose a major threat to the production of fishes. Such emergent parasitic diseases endanger the economic, financial and nutritional security of the fish farmers and the consumers of the aquaculture products.

Carps are the mostly cultured fish species in West Bengal. One of the key problems for productive aquaculture is parasitic infestations of cultured freshwater carps causing severe economic losses in the industry. To study fish diseases, it is important to attain knowledge on different parasites, their biology and life cycle. Cultured carps are susceptible to myriad parasitic diseases of which *Argulus* sp. predominates.

The current study investigated the prevalence of *Argulus* sp. in IMCs from Bhangore block of South 24 Parganas District, West Bengal and the molecular identification of the same.

2. Methodology

2.1 Species and Area of Study

The study was carried out on Indian major carps namely Mrigal (*Cirrhinus mrigala*), Catla (*Catla catla*) and Rohu (*Labeo rohita*) for a period of 10 months from November' 2018 to August' 2019. The diseased fish samples were collected from Bhangore block [22°30'45.36"(N); 88°36'34.92"(E)] of South 24 Parganas District of West Bengal.

2.2 Sampling

100 numbers of each species were collected for a period of 10 months. A total of 300 species of Catla (*Catla catla*), Rohu (*Labeo rohita*) and Mrigal (*Cirrhinus mrigala*) were screened for the experiment of juvenile stage (Average 250 – 500 gm weight). The strategies used for collection and preservation of the samples for parasitic examination were followed as described by Soota, 1980 [4]. The fishes were examined immediately after collection. Prior to collect the affected fish samples, its behaviour and clinical signs were recorded.

2.3 Parasitic Prevalence Study

The length and body weight of the fishes along with date and site of collections were recorded. The gills and body surface were checked thoroughly. Microscopic examinations were done from the smears taken from gills and body surface & photomicrographs of ectoparasites were taken using Olympus microscope (model no. BX51, made of Japan) with in-built digital camera (top view version 3.5). Phenotypic characterizations of parasites have been studied as defined and described by Soulsby (1982) [5].

The Parasitic prevalence has been calculated with the aid of Parasitic Frequency Index (PFI, %), the formulae suggested and proposed by Margolis et, al. (1982) [6].

$$\text{Prevalence (\%)} = \frac{\text{No. of hosts infected}}{\text{No. of hosts examined}} \times 100$$

Srivastava (1980) [7] suggested that the frequency index could be further classified into rare (0.1-9.9%), occasional (10-29.9%), common (30-69.9%) and abundant (70-100%). Determination of the Severity of infection was characterized for assigning numerical qualitative value to severity grade of infections, surface infestations and disease syndrome severity, through the following scale suggested by Lightner (1993) [8]:

Table 1. The Scale by Lightner (1993)

Disease Syndrome Severity	Remarks
0.5	Non infective
1	Mild
2	Moderate
3	Infective
4	Excessive

2.4 Parasitic DNA Extraction and PCR Amplification of 18S rDNA Gene

Genotypic identification of selected parasitic isolates was done by 18S rDNA sequencing. The genomic DNA of parasitic isolates were extracted by using genomic DNA isolation kit (Macherey-Nagel, Germany) as per the manufacturer's protocol. The 18S rDNA gene was amplified through PCR reaction that was performed in a Master cycler Pro S system (Eppendorf, Germany). The universal primers (forward primer UEP-F and reverse primer UEP-R) of amplification size 1900 - 2100bp were used.

2.5 Agarose Gel Electrophoresis & DNA Analysis

The PCR products were analysed on 1.0%agarose (HiMedia, India) gels containing 0.5 µg/ml ethidium bromide in 1X Tris-acetate- EDTA (TAE) buffer. The parasitic isolates were randomly selected for further identification through 18S rDNA analysis. This assay involved DNA isolation, amplification and sequencing of the gene coding for 18S rDNA. The result of sequencing is yet to derive (awaiting results).

3. Results

The Prevalence, Frequency Index and severity of infection

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of the *Argulus* sp. (Figure 1) were presented in Table 2. The infestation reached its peak in the winter (from December to February) which was described as "abundant". January recorded the highest infestation showing a PFI % of 83.33% (abundant) and lowest in the month of August (PFI, 9%) which was "rare" in terms of frequency index. During the ten (10) months study period Argulosis was almost always present in IMCs, which was "common" in most of the cases (Table 2).

Table 2. Prevalence, Frequency Index and Severity of Infection of *Argulus* sp. in Indian Major Carps (IMCs)

Months	PFI (%)	Frequency Index	Severity of infection
November 2018	66.67 ± 11.30^{c}	Common	2
December 2018	75.00 ± 10.10^{d}	Abundant	2
January 2019	83.33 ± 11.21^{d}	Abundant	3
February 2019	72.30 ± 09.30^{d}	Abundant	2
March 2019	44.00 ± 10.30^{c}	Common	2
April 2019	41.68 ± 11.04^{c}	Common	2
May 2019	36.67 ± 10.62^{c}	Common	2
June 2019	33.33 ± 09.70^{c}	Common	2
July 2019	28.67 ± 10.43^{b}	Occasional	1
August 2019	09.00 ± 10.23^{a}	Rare	0.5

Note: Parasitic Frequency Index: a – rare (0.1-9.9%); b – occasional (10-29.9%); c – common (30-69.9%); d – abundant (70-100%).

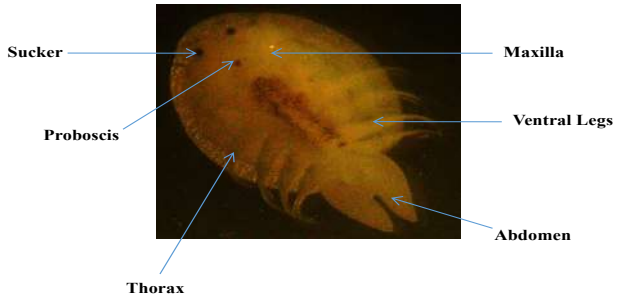

Figure 1. Identified Parasite – *Argulus* sp.

Following the research works of Lightner (1993) [8] severity grade of infection was calculated in *Argulus* sp. It was observed that severity of infection was "moderate" almost throughout the year (November, December, February-June). The month of January showed "infective" severity grade due to extreme seasonal variation. July and August recorded "mild" & "non-infective" severity grades respectively (Table 2).

Randomly selected *Argulus* sp. were further identified through 18S rDNA analysis. In 1% agarose gel electrophoresis, approximately 1.9 kbp bands were obtained with 18S universal primers for parasitic isolates through PCR (Figure 2).

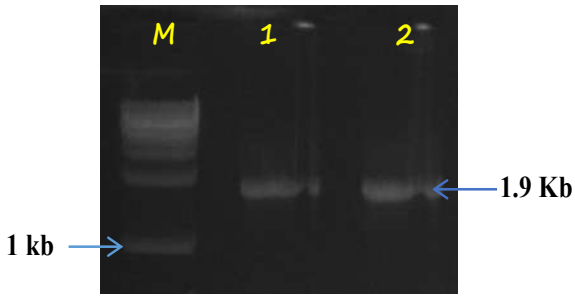

Figure 2. 18S rDNA PCR product from *Argulus* sp. [M-1kb ladder, 1 & 2-Samples]

4. Discussion

All fishes are potential hosts to different species of parasites. With the increase in the interests of the fisheries potential, the awareness of the fish farmers and their experience with parasites have also increased. Though meagre amounts of parasites are ubiquitous in the culture system, they do not pose serious threats. However, all piscine parasites have a tremendous reproductive potential and can, under ideal conditions, quickly overwhelm the host.

The parasitic infestation influenced by the seasons had been portrayed by many researchers. Due to changes in water temperatures and poor fish immunity during the months of Winter and Spring, parasitic infestations tends to increase. The results of the current study were in agreement with the works of Bhuiyan *et al*. and Hoole *et al*. [9,10], who worked on disease incidence in relation with seasonal variations in aquaculture. In this context it can be said that in winter due to fall in temperature, fish species were more susceptible to diseases. The period of November-February recorded the highest parasitic prevalence.

The identities of selected parasitic strains were further confirmed by molecular characterization through 18S rDNA analysis (Figure 2). Modern scientists have been using a variety of kits which provide better progress at molecular level and are highly sought in the field of academic research. This evolving field accomplish the needs of the both laboratory and clinical study.

A lot of works had been done by researchers based on parasitic infestations and their seasonal influences. Crustacean parasites dominated most of the seasons. The current study clearly indicated that the month of winter is the most vulnerable time for the fishes [9,10]. Fish parasites when in higher concentrations can cause heavy mortalities and huge economic loss. Argulosis is one of the problems in fish culture program in Bhangore, South 24 Parganas, West Bengal.

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5. Conclusion

Argulus sp. were more severe in winter. The current study highlighted the infestations of *Argulus* including their molecular identity. In winter lower temperature along with other factors made the fishes more vulnerable towards parasitic infestations.

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References

[1] Gopalkrisnan V. Observation on a new epidemical eye disease affecting the Indian Carp Catlacatla (Hamilton-Buchanan). Ind J Fish, 1961, 8: 222-232.

[2] Hossain MD, Hossain MK, Rahaman MH, Akter A, Khanom DA. Prevalence of ectoparasites of carp fingerlings at Santaher, Bogra. Univ J ZoolRajshahi-Univ, 2008, 27: 17-19.

[3] Banerjee S and Bandyopadhyay PK. Observation on prevalence of ectoparasites in carp fingerlings in two districts of West Bengal. Journal of Parasitic Diseases, 2010, 34: 44-47.

[4] Soota T. D. Collection and preservation of trematodes and cestodes. Proc. Workshop Tech. Parasitol. Zool. Surv. India, 1980: 27-29.

[5] Soulsby E. J. L. Helminths. In : Helminths, Arthropods and Protozoa of domesticated animals. Seventh edition. The English Language Book Soc. And Bailliere Tindall. London, 1982, 5: 354.

[6] Margolis L., Esch G. W., Holmes J. C., Schod G. A. The use of ecological terms in parasitology. Journal of Parasitology, 1982, 68: 131-133.

[7] Srivastava C.B. Estimation of helminthic infections. Proceedings Workshop Technology Parasitol. Zoological Survey of India, 1980: 29-31.

[8] Lightner D.V. Diseases of cultured penaeid shrimp. CRC handbook of mariculture, 1993, 1: 393-486.

[9] Bhuiyan A.S., Sarmin A., Musa G. M. Occurrenceof parasites in Labeo rohita (Hamilton) from Rajshahi, University journal zoology, Rajshahi University, 2007, 26: 31-34.

[10] Hoole D., Bucke D., Burgess P., Wellby I. Infectious diseases-parasites. Diseases of carp and other cyprinid fishes. Fishing News Books, UK., 2001: 63-122.

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ARTICLE

Halobacterium Identification in Saltworks of Gran Canaria (Canary Islands, Spain)

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ABSTRACT

This work analyzes bacterial communities present in evaporation ponds of solar salterns of Gran Canaria and reveals specific organisms through molecular techniques. Solar salterns are protected areas in Canary Islands where salt is produced from sea water by solarand windpowered evaporation. Salt was an important product for ancient islanders who used it for a broad field of purposes, but also has a great importance in recent time for its implications in the island economy. Based on amplifications with specific primers for 16S ribosomal DNA (16S rDNA) and subsequent nested-PCR approaches, different amplicons were obtained, and analyzed in silico. A taxonomic classification was carried out through phylogenetic trees. Results revealed different bacteria according to the evaporation grade of crystallizer ponds in saline works. It is worthstanding the presence of the genus *Halobacterium* in all crystallizer ponds. This opens an interesting framework for further studies and continuative molecular characterization approaches of bacterial communities in solar salterns of Gran Canaria.

1. Introduction

Traditional solar salterns still have a high socioeconomic impact due to their primary and secondary products. There are numerous ways of extracting salt from seawater, however this work is focusing on salt extraction from seawater using multipond solar salterns (salt works or salterns). This process involves the selective recovery of pure NaCl, whilst seawater is evaporated in artificial pond systems through natural evaporation driven by insolation and wind.

Multipond salterns create a gradient of salt concentrations which has an impact on the environment and their microbial population [1]. Ponds not only serve as refuge zones for migratory birds, but also host the halophilic unicellular green algae *Dunaliella* grown as a source of valuable chemicals [2]. There have been various studies regarding the microbial populations of crystallizer ponds around the world [3-5], the contribution of halobacterial pigments to the color of the water of crystallizer ponds [6,7] and the interrelationships between *Dunaliella* and halophilic prokaryotes in saltern crystallizer ponds [8], just to mention a few of them.

Furthermore the increase in salinity in different ponds is accompanied by a decrease in prokaryotic diversity, from the marine biota to the dense populations of halo-

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philic Archaea and bacteria [5]. High salinity and long-term selection pressure, as a result of evaporation, have resulted in specific metabolic mechanisms that render biologically active substances which are directly related to their halophilic behavior [9]. Halophilic bacteria and archaea are, for instance, useful biological sources of carotenoid pigments [10,11].

Evaporation of seawater also leads to crystallization of salts contained, process based on their varying solubility. The thin layer on the surface of the salt tide only forms in traditional salt works during the continuous evaporation and has to be harvested daily by hand. Provision of salt for consumption to the marketplace, "flower of salt" can be obtained from this thin layer. Even more, it is highly valuable and offers wealth and work to the local population.

Moreover, secondary salts, so called bitterns, which are used for chemical industry can be obtained and betacarotenes and glycerol can be extracted from microalgae and halobacteria inhabiting pond systems [2]. Red bacteria of the *Halobacterium – Haloferax – Haloarcula* group contribute to the red color of saltern crystallizer ponds which increases light absorption by the brine. This leads to an increase of temperature and enhances the salt production process so that the microbial composition is of interest for operating solar salterns. Even purely aesthetic considerations make it interesting to study the highly diverse communities of microorganisms in salterns, since the broad range of red shades beautify the landscape and attracts tourism [2,7].

No screening of the bacterial community in solar saltworks of Gran Canaria (Spain) has been conducted until now. The hypothesis of this work is that halophilic bacteria can be characterized in order to thereupon isolate and identify specific species with biotechnological methods. Since the source of water, the Atlantic Ocean, as well as the conditions of wind and temperature are nearly the same for both solar salterns, it is presumed that the organisms contained in the crystallizer ponds of Vargas and Tenefé are equal. The aim of this work is to screen bacteria from crystallizer ponds featuring different salinities, fatty layers, and dried salt in order to characterize specific bacteria.

2. Methodology and Methods

2.1 Solar Salterns on Gran Canaria

The Canary Islands are located in the southeastern sector of the North Atlantic Ocean, approximately between 27 ° to 29 ℃N and 14 ° to 18 ℃W and are closed to the occidental African coast. They are highly influenced by the dominant northeast trade winds associated with the Azores High Pressure Area resulting in strong and frequent wind and a high number of hours of sunshine. Gran Canaria Island (Figure 1A), with its area of 1560 km^2, is located between 27.7°28.2°N and 15.3°15.9°W and features perfect conditions for solar salterns [12]. Samples processed in this work were taken on Gran Canaria southeast coast (Figure 1B) at the solar salterns of Vargas (Figure 1C) and Tenefé (Figure 1D).

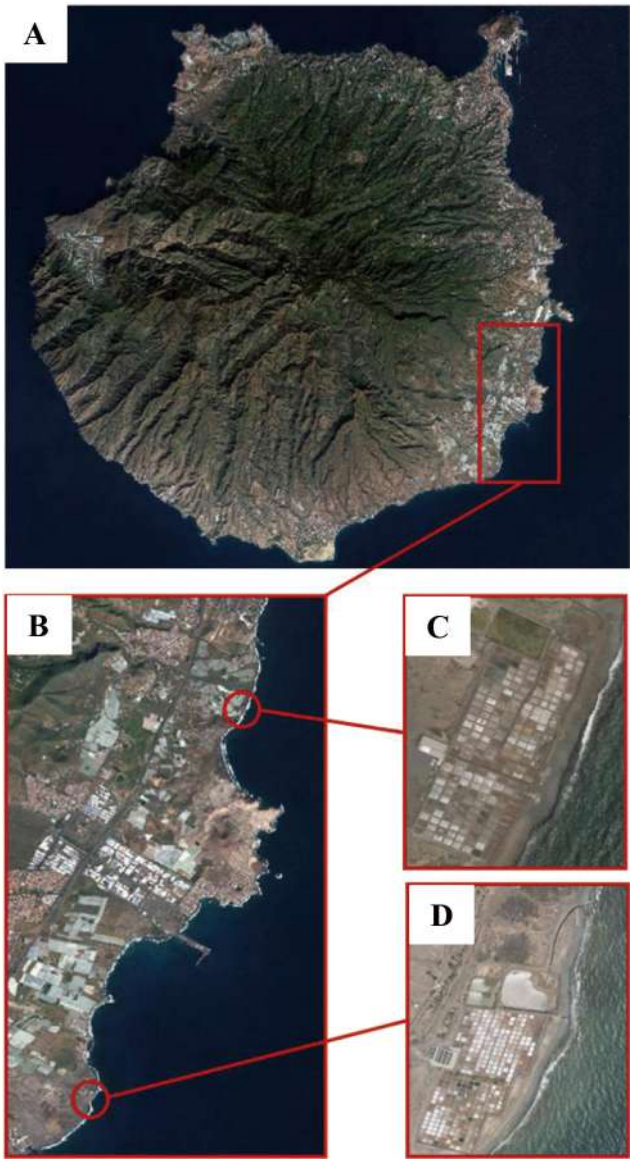

Figure 1. (A) Satellite images of Gran Canaria Island; (B) Southeast coast Gran Canaria Island; (C) The solar salterns of Vargas; (D) The solar salterns of Tenefé

2.2 Collection of Samples in Local Saltworks

Solar saltworks consist of a single series unconnected ponds. Ponds were selected according to their progress of

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evaporation, representing habitats of different specificity due to the changing salt concentrations. The selection and classification were carried out through previous visualization and photographic recording. These were ordered by increasing salinity and denoted as station 1 (ST1; Supplementary Figure 1A), station 2 (ST2; Supplementary Figure 1B), and station 3 (ST3). The latter was subdivided into station 3.1 (ST3.1; Supplementary Figure 1C) representing lipid layers on the surface and station 3.2 (ST3.2; Supplementary Figure 1D) representing water and lipids from the crystallizer pond. Moreover dried salt was also analyzed (SAL2; Supplementary Figure 1E)

One liter of water was taken at each sampling site and subsequently centrifuged (BECKMAN Avanti™ J-25) at 20000 rpm for 10 min. Pellets were then pooled and two grams (c.a.) were put into a 5 ml screw cap tube, filled up with lysis buffer (50 mM TRIS HCl, 2 mM EDTA, 0.1% SDS and 1% TRITON X-100) and afterwards stored at -20 °C until use.

2.3 Isolation of DNA

DNA extraction was performed following the previously described in Garcia-Jimenez et al. [13]. DNA from each of sampling site were separately isolated. This way, water was centrifuged and resulting pellet was homogenised in liquid nitrogen and then incubated in 800 µl of isolation solution containing 100 mM Tris-HCl (pH 8.2), 4 M NaCl, 20 mM EDTA, CTAB (2%, w/v), PVPP (0.1%, w/v), SDS (0.1%, w/v) and mercaptoethanol (2%) in a water bath at 65 ºC for 1 h. A volume of chloroform:isoamyl alcohol solution (24:1 v/v) was added and the samples were gently mixed by inversion at intervals of 20 s. The mixture was then centrifuged for 10 min at 3000 rpm in a Beckman Coulter Allegra X-22R centrifuge (Beckman Coulter Inc. Brea, CA USA). Successive washings with chloroform:isoamyl alcohol (24:1 v/v) solution were performed. The supernatant was then placed in a fresh tube and an equal volume of n-propanol (-20 °C) was added, mixed gently and centrifuged at 13000 g for 30 min. The resulting pellet, containing DNA, was washed with ethanol (80%, v/v, molecular grade), dried and suspended in sterile deionised water. DNA yield were assessed using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). All samples were in triplicate. Purity DNA was valued by smear absence migrating on a 0.8% (w/v) agarose gel.

2.4 Amplification with 16S rRNA and Specific Primers

DNA (90-95 ng) was amplified using oligonucleotide pairs, 16S rRNA-F as a forward primer, and 16S rRNA-R as a reverse primer (Table 1).

Table 1. Sequences of the forward (F) and reverse (R) primers, for 16S rRNA gene

Target	Direction	Primer Pair	Nucleotide Sequence 5' → 3'	Reference
Archaea	Forward	BF27	AGAGTTTGATCCTGGCTCAG	[14]
	Reverse	BR1462	TCCAGCCGCAGATTCCCCTAC	
Bacteria	Forward	BF27	AGAGTTTGATCCTGGCTCAG	[14]
	Reverse	B765R	CTGTTTGCTCCCCACGCTTTC	[15]
Archaea	Forward	B704F	GTAGCGGTGAAATGCGTAGA	[15]
	Reverse	BR1462	TCCAGCCGCAGATTCCCCTAC	[14]
Bacteria	Forward	B22F	ATTCCGGTTGATCCTGC	[15]
	Reverse	B1521R	AGGAGGTGATCCAGCCGCAG	
Archaea	Forward	BF8	TTGATCCTGCCGGAGGC-CATTG	[14]
	Reverse	BR1462	TCCAGCCGCAGATTCCCCTAC	

Amplification was performed in a GenAmp 2400 thermal cycler (PerkinElmer Inc., USA) with 30 cycles consisting at 95 °C for 1 min, 48 °C for 1 min, and 72 °C for 1.5 min, followed by a final extension step of 5 min at 72 °C. Each PCR reaction mixture contained 0.5 U Takara Ex Taq DNA polymerase (TaKaRa Shuzo Co., Shiga, Japan), 2.5 mM dNTP, 10 µl Takara Ex Taq PCR buffer with MgCl2, 10 µM each forward and reverse primers and DNA template.

Amplification products corresponding to 16S rRNA were purified through QIAEX agarose gel extraction kit (Qiagen Inc., Hilden, Germany) according to manufacturer. Sequences were used for designing new primer pair for nested PCR. The specific primer pairs used and annealing temperatures, including putative bacterial species associated, are shown in Table 2.

Table 2. Specific Primer Pairs design in this study with correlating Bacterial Species

Target	Direction	Primer Pair	Tm [°C]	Ta [°C]	Sequence 5' - 3'
Halobacterium salinibacter	Forward	BACTF	60,5	52	GTCCGGGGTAGGAGTGAAAT
	Reverse	BACTR	56,4		CCCGCCAATTCCTTTAAGTT
Halobacterium cutirubrum	Forward	HCF	70,7	51	ATTCCGGTTGATCCTGCCGGAGGTC
	Reverse	HCR	64,6		GATCCAGCCGCAGATTCCCC
Halorubrum salinum	Forward	HSALIF	60,5	52	GGTAGTCCTGGCCGTAAACA
	Reverse	HSALIR	60,5		AGGTCATCAACCTGGTCGTC
Salinobacter	Forward	SALIBF	58,4	51	CAGGAATAAGCACCGGCTAA
	Reverse	SALIBR	56,1		ACATGCTCCACCGCTTGT

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PCR products were visualised by agarose gel electrophoresis. In addition, amplification products were obtained and purified using the QIAEX agarose gel extraction kit. The fragments were then ligated to the pGEM-T-easy cloning vector (Promega, Wisconsin, USA) and cloned in JM109 cells according to the manufacturer's instructions (Promega). Plasmids were isolated using a plasmid purification kit (Qiagen Inc.). The insert in the plasmid was checked by PCR using primers M13F and M13R (Promega). The insert was then sequenced on both strands using an ABI-310 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA) and BigDye Terminator v3.1. Nucleotide sequences were submitted to NCBI GenBank BLAST search and identified through similarity values. Alignment of sequences was performed with ClustalX v.1.7 [16] using the default settings and was further refined by visual inspection. The alignment output was used to generate a phylogenetic tree based on the Maximum Likelihood method and General Time Reversibility model [17] as implemented in MEGA X [18]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed [19]. Branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates collapse. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [19]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences between sites (2 categories (+G, parameter = 0.1000)).

3. Results and Discussion

The saline waters of the solar salterns in Gran Canaria have a marine origin with a gradient of salt concentrations according to evaporation. The appearance of salt produced in Vargas and Tenefé (Gran Canaria) ranged from a pink color gradient to strong intense pink in relation to the salinity increment in different ponds. It has also been observed those ponds, whose color intensity is high, brine become oily and viscous.

Despite diverse microbial groups inhabit multi-pond solar saltworks, in which a gradient of salinities ranges from seawater to NaCl precipitation, little is known about bacterial communities in saltworks. Thus an approach to characterize them via 16S rDNA gene sequencing method was conducted. In order to determine the bacteria population in different ponds, we identified bacteria through 16S DNA-molecular technique. It is worth be aiming that the objective was only a 16S DNA-bacteria screening. An approach to study structure of microbial communities and biodiversity cultivation is needed onwards.

Amplified 16S rDNA gene sequences with a molecular size of approximately 1500 bp serve as genetic marker and can be used to study bacterial phylogeny and taxonomy by further processing combined with biocomputing analysis [20]. Universal primers (Table 1) have been validated for high-salinity bacteria on isolated DNA derived from crystallizer ponds in different stages of evaporation. Different universal primers match different positions of the genome, identifying different areas of the 16S region [21-23]. Since a broad range of primer combinations was already tested, successful results were obtained with all primer pairs in different sampling stations. In general, bacteria biodiversity detected in ponds is quite similar although a bacteria genus is remarkable over others.

Furthermore internal-PCR amplifications were conducted as consequence of combinations of sequences obtained by universal PCR and different specie-specific primer pairs (Table 2). Twenty-nine sequences of internal-amplifications were obtained and 18 out of them allowed successfully identifying. 16S rDNA-internal sequences ranged from 133 to 223 bp were most closely annotated to three genera namely *Halobacterium sp*., *Halorubrum sp*., and *Salinobacter sp*. All of three genus have been described as extremely halophilic bacteria that grows in saturated sodium chloride [24].

Bacterial community fluctuated depending on the progress of evaporation and concomitant change in salinity and environmental conditions. Moreover, the increasing salinity leads to a progressive specification of species being uninhabitable for unspecified organisms [25]. *Halobacterium* genus was always reported in all crystallizer ponds and salt whilst *Halorubrum sp*. and *Salinobacter sp*. were unreported in solid salt. *Halobacterium sp*. species get benefits from increasing salinity.

As this *Halobacterium* species have a cell membrane red-color [26], presumably the red shade in ponds comes from cell membrane. Additionally, since the red color is correlated to the presence of these kind of halophilic bacteria, an increasing habitability and improvement of living conditions can be assumed [6].

Other authors have also shown that *Halobacterium* imparts red coloration to the brine and favor evaporation of brine [27]. Likewise viscosity levels promote the formation of larger salt crystals and thereby improve the salt quality [27,28].

The phylogenetic analysis positioned the *Halobacterium* sequences in a clade within *Halobacterium salinarium* and *Halobacterium sp*. (Figure 2). Evolutionary analysis

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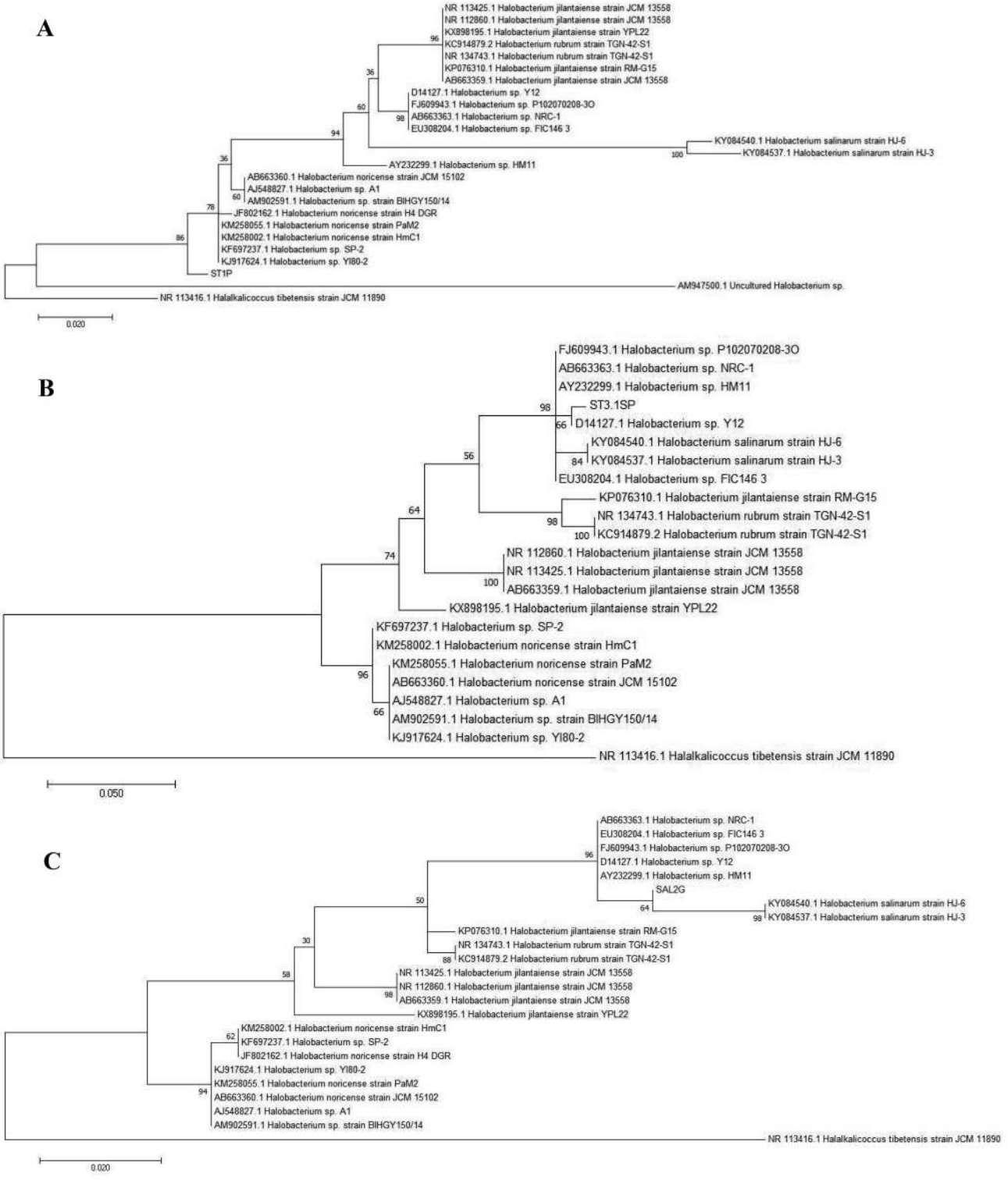

Figure 2. Phylogenetic trees created with MEGA X for nested PCR amplicon sequences. (A) ST1P; (B) ST3.1SP; (C) SAL2G

Note: The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model, with the percentage of trees in which the associated taxa clustered together shown next to the branches [17]. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site.

using Maximum Likelihood method involved 24 nucleotide sequences (Table 3).

Table 3. Sequences retrieved from NCBI database to design phylogeny tree of *Halobacterium*

Species	Strain	Accesion Number
Halalkalicoccus tibetensis	JCM 11890	NR 113416.1
Halobacterium jilantaiense	YPL22	KX898195.1
	RM-G15	KP076310.1
	JCM 13558	NR 113425.1
	JCM 13558	NR 112860.1
	JCM 13558	AB663359.1
Halobacterium noricense	JCM 15102	AB663360.1
	HmC1	KM258002.1
	PaM2	KM258055.1
	H4 DGR	JF802162.1
Halobacterium rubrum	TGN-42-S1	NR 134743.1
	TGN-42-S1	KC914879.2
Halobacterium salinarum	HJ-6	KY084540.1
	HJ-3	KY084537.1
Halobacterium sp.	Uncultured	AM947500.1
	NRC-1	AB663363.1
	BIHGY150/14	AM902591.1
	A1	AJ548827.1
	FIC146 3	EU308204.1
	HM11	AY232299.1
	P102070208-3O	FJ609943.1
	SP-2	KF697237.1
	Y12	D14127.1
	YI80-2	KJ917624.1

All in all, assuming the criterion for differentiating bacteria with a 16S rDNA gene sequence similarity value of over 85% [29], the phylogenetic trees of *Halobacterium* were consistent with the molecular characterization and their affiliations to the respective genus.

In conclusion, Vargas and Tenefé saltworks are only such biotope in Gran Canaria, which is done only for salt production, but there is unlimited scope for commercial exploitation of salts.

Nonetheless knowledge acquired in this work opens up an interesting framework for further biotechnological studies concerning revealed bacteria and the composition of the bacterial community, as well as for studies investigating hypersaline habitats of crystallizer ponds from Gran Canaria solar salterns. The commercial exploitation of putative bacterial rhodopsin could lead to value added by product.

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Author's contributions

P.G.J. conceived, designed and wrote the manuscript. M.C.A. conducted the phylogeny analysis. S.H. carried out microbiological assays. All the authors read and approved the manuscript.

Supplementary Figure

Pink color of the liquid phase
Salt in suspension

Orange/red color of the liquid phase
Beginning of salt precipitation without fats

Fatty supernatant on the surface

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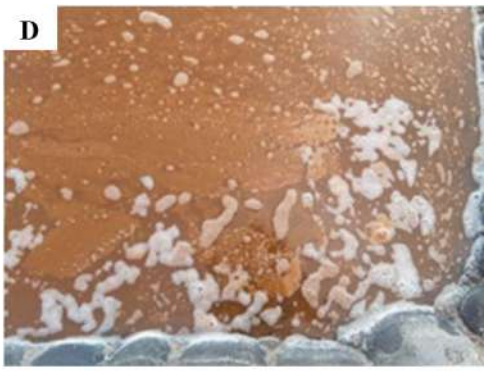

Fatty supernatant including water of the crystallizer

Dry salt from the corner of the crystallizer Pink color

Supplementary Figure 1. Sampling sites order by increasing salinity and their properties

Note: A) Station 1 (ST1); Station 2 (ST2); Station 3 with fatty supernatant on the surface (ST3.1); Station 3 with fatty supernatant including water of the crystallizer (ST3.2); Dry salt (SAL2).

References

[1] Rodriguez-Valera F Ventosa A Juez G Imhoff JF. Variation of environmental features and microbial populations with salt concentrations in a multi-pond saltern[J]. Microbial Ecology, 1985, 11: 107-115.

[2] Rocha RDM Costa DF Lucena-Filho MA Bezerra RM Medeiros DH Azevedo-Silva AM Araújo CN Xavier-Filho L. Brazilian solar saltworks-ancient uses and future possibilities[J]. Aquatic biosystems, 2012, 8: 8.

[3] Gómez-Villegas P Viagra J Léon R. Characterization of the Microbial Population Inhabiting a Solar Saltern Pond of the Odiel Marshlands (SW Spain)[J]. Marine Drugs, 2018, 16: 332.

[4] Benlloch S López-López A Casamayor EO Øvreås L Goddard V Daae FL Smerdon G Massana R Joint I Thingstad F. Prokaryotic genetic diversity throughout the salinity gradient of a coastal solar saltern[J]. Environmental Microbiology, 2002, 4: 349-360.

[5] Antón J Llobet-Brossa E Rodríguez-Valera F Amann R. Fluorescence in situ hybridization analysis of the prokaryotic community inhabiting crystallizer ponds[J]. Environmental Microbiology, 1999, 1: 517-523.

[6] Oren A Rodríguez-Valera F. The contribution of halophilic Bacteria to the red coloration of saltern crystallizer ponds[J]. FEMS Microbiology Ecology, 2001, 36: 123-130.

[7] Oren A Dubinsky Z. On the red coloration of saltern crystallizer ponds. II. Additional evidence for the contribution of halobacterial pigments[J]. International Journal of Salt Lake Research, 1994, 3: 9-13.

[8] Bardavid RE Khristo P Oren A. Interrelationships between Dunaliella and halophilic prokaryotes in saltern crystallizer ponds[J]. Extremophiles, 2008, 12: 5-14.

[9] Litchfield CD. Potential for industrial products from the halophilic Archaea[J]. Journal Industrial Microbiology and Biotechnology, 2011, 38(10): 1 635-1 647.

[10] Fang CJ Ku KL Lee MH Su NW. Influence of nutritive factors on C50 carotenoids production by Haloferax mediterranei ATCC 33500 with two-stage cultivation[J]. Bioresource Technology, 2010, 101(16): 6 487-6 493.

[11] Asker D Ohta Y. Haloferax alexandrinus sp. nov., an extremely halophilic canthaxanthin-producing archaeon from a solar saltern in Alexandria (Egypt)[J]. Journal Systematic and Evolutionary Microbiology, 2002, 52(3): 729-738.

[12] Luque A Martín JL Dorta P Mayer P. Temperature trends on Gran Canaria (Canary Islands). An example of global warming over the subtropical Northeastern Atlantic[J]. Atmospheric and Climate Sciences, 2014, 4: 20-28.

[13] Garcia-Jimenez P Carrasco-Acosta M Enrique-Payá C Alemán-López I Betancort-Rodriguez JR. Herrera-Melián JA. Composition and molecular identification of bacterial community in seawater desalination plants [J]. Advances in Microbiology, 2019, 9: 863-876.

[14] Deng Y Xu G Sui L. Isolation and characterization of halophilic bacteria and archaea from salt ponds in Hangu Saltworks, Tianjin, China[J]. Chinese Journal of Oceanology and Limnology, 2015, 33(4): 862-868.

[15] Barghouthi SA. A universal method for the identification of bacteria based on general PCR primers. Indian Journal of Microbiology, 2011, 51: 430-444.

[16] Thompson JD Higgins DG Gibson TJ. CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix

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Choice[J]. Nucleic Acids Research, 1994, 22: 4673-4680.

[17] Nei M Kumar S. Molecular Evolution and Phylogenetics[B]. Oxford University Press, New York, 2000.

[18] Kumar S Stecher G Li M Knyaz C Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms[J]. Molecular Biology and Evolution, 2018, 35: 1547-1549.

[19] Felsenstein J. Confidence Limits on Phylogenies: An Approach Using the Bootstrap[J]. Evolution, 1985, 39: 783-791.

[20] Janda JM Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls[J]. Journal of Clinical Microbiology, 2007, 45: 2761-2764.

[21] Fredriksson NJ Hermansson M Wilén BM. The choice of PCR primers has great impact on assessments of bacterial community diversity and dynamics in a wastewater treatment plant[J]. PLoS One, 2013, 8: e76431.

[22] Rohban R Amoozegar MA Ventosa A. Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran[J]. Journal of Industrial Microbiology & Biotechnology, 2009, 36: 333-340.

[23] Martínez-Cánovas MJ Quesada E Llamas I Béjar V. Halomonas ventosae sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium[J]. International Journal of Systematic and Evolutionary Microbiology, 2004, 54: 733-737.

[24] Bayley S Kushner D. The ribosomes of the extremely halophilic bacterium, Halobacterium cutirubrum[J]. Journal of Molecular Biology, 1964, 9: 654IN3-669IN4.

[25] Williams WD. Salinity as a determinant of the structure of biological communities in salt lakes[J]. Hydrobiologia, 1998, 381: 191-201.

[26] Kushwaha S Kates M Martin W. Characterization and composition of the purple and red membrane from Halobacterium cutirubrum[J]. Canadian Journal of Biochemistry, 1975, 53: 284-292.

[27] Haxby RE Tackaert W. Workshop report, Role of Artemia in salt operations: 291-293. In: Artemia research and its applications [R]. Sorgeloos P Bengtson DA Decleir W Jaspers E (Eds), Universa, Press, Wettern, Belgium, 1987, 3(556).

[28] Sorgeloos P. Brine shrimp Artemia in coastal salt works hydrobiological key to improved slat production, and inexpensive source of food for vertically integrated aquaculture[R]. International meeting Salt work Conversion for Aquaculture, Trapane, Italy, May 9-11. In Artemia Newsletter, 1986, 3: 50-53.

[29] Stackebrandt E and Goebel BM. Taxonomic Note: A Place for DNA-DNA Reassociation and 16s rRNA Sequence Analysis in the Present Species Definition in Bacteriology[J]. International Journal of Systematic Bacteriology, 1994, 44: 846-849.

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ARTICLE

Enriched Artemia Nauplii with Commercial Probiotic in the Larviculture of Angelfish *Pterophyllum scalare* Lichtenstein (1823)

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ABSTRACT

This study evaluated the effect of enriched artemia nauplii with commercial probiotic for angelfish larvae determining growth perfomance, survival and modulation of the intestinal microbiota. Therefore, it experiment occurred in completely randomized design with five treatments (T1- 0.0, T2- 1.5, T3- 3.0, T4- 4.5 and T5- 6.0g of commercial probiotic) and four replaces during 20 days. After larvaculture, post larvae passed by biometric procedures to determine productive performance and then microbiological analysis. Occurred reduction of total heterotrophic bacteria while increased lactic acid bacteria in the intestinal tract from the post larvae for treatments T3, T4 and T5. The commercial probiotic also increased the survival and performance as final weight, weight gain and specific growth rate. For these reasons, the use of 3g of commercial probiotic promotes greater performance and intestinal modulation for angelfish larvae.

1. Introduction

Ornamental fish trade around the world has becomes a profitable activity moving approximately US 15 million [1,2]. Currently, it market look for several fish species with highest quality, different shape and colors [3,4]. Among the native fish species from Amazon, the angelfish *Pterophyllum scalare* has economic potential into national and international market due to this varied patterns and colors required by the ornamental market [4,5,6].

In freshwater ornamental fish, the larvaculture remains as the most problematic phase of production with highest mortality rate caused by management, inadequate nutrition and stocking density [4,7,8]. These factors can reduces its productive performance and health of the larvae [4,5,9].

The larval phase, live feed have an important paper to supply the nutritional requirement and its development [8]. Currently, the ornamental fish sector use widely artemia nauplii due to the size, protein and lipid profile as well as enzymes that aid its digestion process [10,11]. In the last de-

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cade, fish farmers has applied different strategies for larvaculture to improve the performance and health [8,12,13,14]. Among the new strategies, enriched artemia nauplii with probiotic showed nice results about the productive performance, survival, improvement of immunological system and intestinal modulation [9,15,16,17,18,19].

In front this, enriched diets for ornamental fish larvae becomes an efficient strategy in captivity rearing. Nonetheless, still missing scientific data about the use of probiotic for larval diet. Thus, this study evaluated the effect of enriched artemia nauplii with commercial probiotic for angelfish larvae *Pterophyllum scalare* determining performance, survival and intestinal modulation.

2. Material and Method

2.1 Experimental Design

This study used larvae of angelfish *Pterophyllum scalare* (1.10±0.01 mg and 4.70±0.25 mm) from natural reproduction, placed 10 fish per polyethylene tanks (total capacity for one liter). Therefore, it experiment occurred in completely randomized design with five treatments (C- 0.0, T1- 1.5, T2- 3.0, T3- 4.5 and T4- 6.0 g of commercial probiotic) and four replaces during 20 days.

The feeding management have four daily frequencies (08, 11, 14 and 17 hours) providing one hundred artemia nauplii per larvae [5]. After the last daily feeding, it was carried out water exchange (30%). The water quality parameters were: temperature (28.6±0.35 ºC), dissolved oxygen (5.89±0.11 mg.L^{-1}) pH (6.56±0.42) total ammonia (0.18±0.04 mg.L^{-1}) and electric conductivity (168±28.32 µS.cm^{-1}).

2.2 Biological Culture and Enrichment Process

To obtain the artemia nauplii, it used cyst 1g into the tank (1 liter) with constant aeration during 24 hours [9]. After hatching time, artemia nauplii placed in becker (50mL) received commercial probiotic *Lactobacillus acidophilus* (1.1 x 10^8 CFU.g^{-1} and *Enterococcus faecium* 7.7x 10^7 CFU.g^{-1} during 40 minutes before feeding management [20].

2.3 Intestinal Microbiota

For determine bacterial amount in the intestine from post larvae, its intestinal tract (pools of five larvae) macerated with sterile saline solution 0.65% passed by serial dilution (1:10 factor). An aliquot (100 µL) from each of three dilutions (10^{-1}, 10^{-3} and 10^{-5}) was used to inoculate petri dishes containing either Triptone Soy Agar (TSA - incubated at 30 ºC for 24 hours) to obtain counts of total heterotrophic bacteria, or MRS Agar (incubated at 30 ºC for 48 hours) to obtain counts of lactic acid bacteria [19].

2.4 Growth Perfomance

At the end of experiment, all post larvae passed per biometric procedure to determine weight and length evaluating: Total length (TL), final weight (FW), weight gain (WG), specific growth rate (SGR), survival (S), uniformity (U) [21] and relative condition factor (Kr) [22].

2.5 Statistical Analysis

Data was tested for normality (Shapiro-Wilk) and homoscedasticity (Levene's). Subsequent analysis of variance tests were performed applying post hoc Tukey tests for separation of means ($P<0.05$) [23]. Microbiological counts showed non-parametric characteristics and were therefore log transformed [logX10 (x+1)] and arc sen square root (x).

3. Result

At the end of experiment, occurred reduction ($p<0,05$) of total heterotrophic bacteria (T2= 3.41±0.061; T3= 3.43±0.078 and T4= 3.36±0.04 Log CFU.g^{-1}), while increased ($p<0,05$) lactic acid bacteria in the intestinal tract from the post larvae for treatments T2 (3.81±0.06 Log CFU.g^{-1}), T3 (3.95±0.08 Log CFU.g^{-1}) and T4 (3.95±0.12 Log CFU.g^{-1}), compared to control treatments (Figure 1).

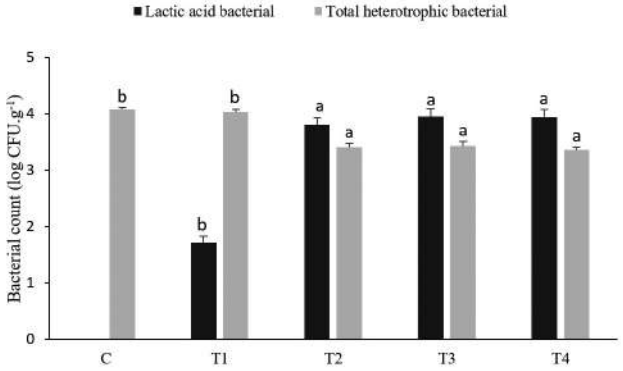

Figure 1. Heterotrophic and lactic acid bacterial counting from intestinal tract of angelfish larvae *Pterophyllum scalare*

Note: Different letters mean statistical difference ($p<0.05$) among the treatments.

After 20 days, With regard to the productive performance, larvae fed with the enriched artemia nauplii with T2, T3 and T4 treatment, increased ($p<0,05$) final weight (FW), weight gain (WG) and specific growth rate (SGRw) for when compared to the control (table 1). Furthermore, larvae mortality significantly increased ($p<0,05$) in the control (72,0±8,5) (table 1).

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Table 1. Productive performance and survival from angelfish larvae *Pterophyllum scalare* fed with Artemia sp. without enrichment (C) and enriched with commercial probiotic (T1: 1.5 g; T2: 3.0g; T3: 4.5g and T4: 6.0g) during larviculture

Treatments	TL (mm)	FW (mg)	WG (mg)	SGR_W (%)	SGR_L (%)	UNI (%)	Kr	S (%)
C	12,48±0,35 a	38±1,1 b	35±1,1 b	23,2±0,17 b	5,74±0,03 a	66.0±16,2 a	0,99±0,02 a	72,0±8,5 b
T1	12,47±0,32 a	39±1,2 b	35±1,2 b	23,4±0,18 b	5,71±0,16 a	75,8±12,4 a	0,99±0,02 a	84,5±12,5 a
T2	12,83±0,24 a	42±1,3 a	39±1,8 a	24,8±0,32 a	5,90±0,20 a	78,1±15,0 a	1,00±0,01 a	92,0±9,4 a
T3	12,86±0,48 a	41±1,7 a	40±2,1 a	25,0±0,47 a	5,77±0,23 a	76,2±14,8 a	0,99±0,01 a	95,5±8,8 a
T4	12,71±0,18 a	42±1,9 a	39±1,6 a	25,2±0,53 a	5,83±0,23 a	75,0±12,8 a	0,99±0,02 a	94,0±8,2 a

Note: Mean values ± standard deviation from productive performance, different letters in column mean statistical difference ($p<0.05$).

4. Discussion

The use of bacteria with probiotic potential has showed positive results to the ornamental aquaculture [9,17,24,25]. Nonetheless, still missing reports about their use for angelfish larvae on productive performance, intestinal modulation and survival.

Colonize the intestinal tract stands out as the main aspect to determine its probiotic potential [19,26]. For this study, the artemia nauplii works as transporter of probiotic bacterium to the host. Its intestinal tract reduced the heterotrophic bacteria amount with the increases of probiotic bacterium due to the competition for space, nutrients and releasing of bactericides [15,19,27].

[17] reported it modulator effect in the intestine for angelfish adult, as also to *Carassius auratus* [24] and *Xiphophorus helleri* [25]. In addition, probiotic act as immune stimulant forming barriers against pathogenic bacteria and increasing its defense cell numbers [16,19,25,28]. Thus, it increases above 80% of survival could be related to the effects of probiotic bacterium included in the exogenous diet during larvaculture.

The larval performance improved in this study with the enrichment of artemia nauplii promoting greater weight gain, specific growth rate and survival. It benefits would be related to the intestinal modulation that provides increased intestinal villi and better nutrient absorption [9,19]. According to [29], they observed similar result with increased performance for angelfish larvae Pterophyllum scalare using a different commercial probiotic.

For these reasons, enrichment of artemia nauplii becomes an efficient strategy to ornamental fish farming considering the probiotic amount to reach its benefits results. However, its microorganisms could influenced by environmental factors, age, development phase and physiological aspects [19,30,31,32].

5. Conclusion

The enriched artemia nauplii with commercial probiotic (3g/L) can modulates the intestinal tract, increases the performance and survival. Thus, it enriched diet can be used as a new strategy for larvaculture of the angelfish larvae *Pterophyllum scalare*.

Conflicts of interest

The authors have no conflicts of interest to declare.

Reference

[1] Zuanon, J.A.S.; Salaro, A.L.; Furuya, W.M. Produção e nutrição de peixes ornamentais. Revista Brasileira de Zootecnia, 2011, 40: 165-174.

[2] FAO – Food And Agriculture Organization of the United Nations. FAO Aquacultura Newsletter. Nº 56 (April), 2017: 64.

[3] Abe, H.A., DIAS, J.A.R., Cordeiro, C.A.M., Ramos, F.M., Fujimoto, R.Y. Pyrrhulina brevis (steindachner, 1876) como uma nova opção para a piscicultura ornamental nacional: larvicultura. Boletim do Instituto de Pesca, 2015, 41: 113-122.

[4] Abe HA, Dias J.A, Reis R.G., Sousa N.C., Ramos, F.M, Fujimoto, R.Y. Manejo alimentar e densidade de estocagem na larvicultura do peixe ornamental amazônico Heros severus. Boletim do Instituto de Pesca, 2016, 42: 514-22.

[5] Pereira, S.L., Gonçalves-Júnior, L.P., Azevedo, R. Diferentes estratégias alimentares na larvicultura do acará-bandeira (Peterolophyllum scalare, Cichlidae). Acta Amzonica, 2016, 46: 91-98. DOI: 10.1590/1809-4392201500472

[6] Fujimoto, R.Y., Santos, R.F.B., Dias, H.M., Ramos, F.M., Silva, D.J.F., Honorato, C. A. Feeding frequency on the production viability of production and quantitative descriptors of parasitism in angelfish. Ciência Rural, 2016, 46: 304-309. DOI: 10.1590/0103-8478cr20141704

[7] Gonçalves-Junior, L. P., Mendonça, P.P., Pereira, S.L., Matielo, M.D., AMORIM, I.R.S. Densidade de estocagem durante a larvicultura do kinguio. Boletim do Instituto de Pesca. 2016, 40: 597-604.

[8] Couto, M.V.S.D., Sousa, N.D.C., Abe, H.A., Dias,

J.A.R., Meneses, J.O., Paixão, P.E.G., Cunha, F.S., Ramos, F.M., Maria, A.N., Carneiro, P.C.F., Fujimoto, R. Y. Effects of live feed containing Panagrellus redivivus and water depth on growth of Betta splendens larvae. Aquaculture research, 2018, 49: 2671-2675.
DOI: 10.1111/are.13727

[9] Azevedo, R.V., Fosse-Filho, J.C., Pereira, S.L., Andrade, D.R., Júnior, V.M. Prebiótico, probiótico e simbiótico para larvas de Trichogaster leeri (Bleeker, 1852, Perciformes, Osphronemidae). Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 2016, 68: 795-804.
DOI: 10.1590/1678-4162-8580

[10] Malla, S., Banik, S. (2015). Production and application of live food organisms for freshwater ornamental fish Larviculture. Advances in Bioresearch, 6: 159–167.

[11] Loh, J.Y., Ting, A.S.Y. Effects of potential probiotic Lactococcus lactis subsp. lactis on digestive enzymatic activities of live feed Artemia franciscana. Aquaculture international, 2016, 24: 1341-1351.
DOI: 10.1007/s10499-016-9991-2

[12] Abe, H.A., Dias, J.A.R., Sousa, N.D.C., Couto, M.V.S.D., Reis, R.G.A., Paixão, P.E.G., Fujimoto, R.Y. Growth of Amazon ornamental fish Nannostomus beckfordi larvae (Steindachner, 1876) submitted to different stocking densities and feeding management in captivity conditions. Aquaculture Research, 2019, 50: 2276-2280.
DOI: 10.1111/are.14108

[13] Fabregat, T.E.H.P., Wosniak, B., Takata, R., Miranda Filho, K.C., Fernandes, J.B.K., Portella, M.C. Larviculture of siamese fighting fish Betta splendens in low-salinity water. Boletim do Instituto de Pesca, 2017, 43: 164-171.
DOI: 10.20950/1678-2305.2017v43n2p164

[14] Ahmadifard, N., Aminlooi, V. R., Tukmechi, A., Agh, N. Evaluation of the impacts of long-term enriched Artemia with Bacillus subtilis on growth performance, reproduction, intestinal microflora, and resistance to Aeromonas hydrophila of ornamental fish Poecilia latipinna. Probiotics and antimicrobial Proteins, 2018, 11: 957–965.
DOI: 10.1007/s12602-018-9453-4

[15] Mouriño, J.L.P., Vieira, F.D., Jatobá, A.B., Silva, B.C., Jesus, G.F.A., Seiffert, W.Q., Martins, M.L. Effect of dietary supplemenation of inulin and W. cibaria on haemato-immunological parameters of hybrid surubim (Pseudoplatystoma sp). Aquaculture Nutrition, 2012, 18: 73-80.
DOI:10.1111/j.1365-2095.2011.00879.x

[16] Mouriño, J.L.P., Vieira, F.N., Jatobá, A., Silva, B.C., Pereira, G.V., Jesus, G.F.A., Martins, M.L. Symbiotic supplementation on the hemato-immunological parameters and survival of the hybrid surubim after challenge with Aeromonas hydrophila. Aquaculture Nutrition, 2015, 23: 276-284.
DOI: 10.1111/anu.12390

[17] Azimirad, M., Meshkini, S., Ahmadifard, N., Hoseinifar, S. H. The effects of feeding with synbiotic (Pediococcus acidilactici and fructooligosaccharide) enriched adult Artemia on skin mucus immune responses, stress resistance, intestinal microbiota and performance of angelfish (Pterophyllum scalare). Fish & shellfish immunology, 2016, 54: 516-522.
DOI: 10.1016/j.fsi.2016.05.001

[18] Vázquez-Silva, G., Ramírez-Saad, H.C., Aguirre-Garrido, J. F., Mayorga-Reyes, L., Azaola-Espinosa, A., Morales-Jiménez, J. Effect of bacterial probiotics bio-encapsulated into Artemia franciscana on weight and length of the shortfin silverside (Chirostoma humboldtianum), and PCR-DGGE characterization of its intestinal bacterial community. Latin american journal of aquatic research, 2017, 45: 1031-1043.
DOI: 10.3856/vol45-issues5-fulltext-18

[19] Sousa, N. C, do Couto, M. V. S., Abe, H. A., Paixão, P. E. G., Cordeiro, C. A. M., Monteiro Lopes, E., Ready, J. S., Jesus, G. F. A., Martins, M. L., Mouriño, J. L. P., Carneiro, P. C. F., Maria, A. N., & Fujimoto, R. Y. (2019). Effects of an Enterococcus faecium□ based probiotic on growth performance and health of Pirarucu, Arapaima gigas. Aquaculture Research, 50, 3720–3728.
https://doi.org/10.1111/ are.14332

[20] Vázquez-Silva, G., Castro-Mejía, J.J., Sánchez de la Concha, B., González-Vázquez, R., Mayorga-Reyes, L., Azaola-Espinosa, A. Bioencapsulation of Bifidobacterium animalis and Lactobacillus johnsonii in Artemia franciscana as feed for charal (Chirostoma jordani) larvae. Revista Mexicana de Ingeniería Química, 2016, 15: 809-818.

[21] Furuya, W.M., Souza, S.R.D., Furuya, V.R.B, Hayashi, C. Ribeiro, R.P. Pelletized and extrused diets for reversed nile tilapia (Oreochromis niloticus L.) males, in finishing phase. Ciência Rural, 1998, 28, 483–487.
DOI:10.1590/S0103-84781998000300022

[22] Le Cren, E. D. The length-weight relationship and seasonal cycle in gonad weight and condition in the perch (Perca fluviatilis). Journal of Animal Ecology, 1951, 20: 201- 219.

[23] Zar, J. H. Biostatistical Analysis. 5th. Edition, Pren-

DOI: https://doi.org/10.30564/jfsr.v2i1.1569

tice-Hall, Inc., Upper Saddle River, New Jersey, USA. 2009: 576.

[24] Mehdinejad, N., Imanpour, M.R., Jafari, V. Combined or Individual Effects of Dietary Probiotic Pediococcus acidilactici and Nucleotide on Growth Performance, Intestinal Microbiota, Hemato-biochemical Parameters, and Innate Immune Response in Goldfish (Carassius auratus). Probiotics and antimicrobial proteins, 2017, 10: 558–565.
DOI: 10.1007/s12602-017-9297-3

[25] Hoseinifar, S.H., Roosta, Z., Hajimoradloo, A., Vakili, F. The effects of Lactobacillus acidophilus as feed supplement on skin mucosal immune parameters, intestinal microbiota, stress resistance and growth performance of black swordtail (Xiphophorus helleri). Fish & shellfish immunology, 2015, 42: 533-538.
DOI:10.1016/j.fsi.2014.12.003

[26] Feng, J., Li, D., Liu, L., Tang, Y., Du, R. Characterization and comparison of the adherence and imune modulation of two gut Lactobacillus strains isolated from Paralichthys olivaceus. Aquaculture, 2019, 499: 381-388.
DOI: 10.1016/j.aquaculture.2018.08.026

[27] Balcázar, J.L., Vendrell, D., Blas, I., Ruiz-Zarzuela, I., Muzquiz, J.L., Girones, O. Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. Aquaculture, 2008, 278: 188–191.
DOI: 10.1016/j.aquaculture.2008.03.014

[28] Lazado, C.C., Caipang, C.M.A. Mucosal immunity and probiotics in fish. Fish & Shellfish Immunology, 2014, 39: 78–89.
DOI: 10.1016/j.fsi.2014.04.015

[29] Farahi, A., Kasiri, M., Sudagar, M., Alamshahi, F. Angelfish (Pterophyllum scalare Schultze, 1823) Larvae. Journal of Animal and Veterinary Advances, 2011, 10: 2305-2311.

[30] Meidong, R., Doolgindachbaporn, S., Sakai, K., Tongpim, S. Isolation and selection of lactic acid bacteria from Thai indigenous fermented foods for use as probiotics in tilapia fish Oreochromis niloticus. Aquaculture, Aquarium, Conservation & Legislation-International Journal of the Bioflux Society (AACL Bioflux), 2017, 10: 455-463.

[31] Rendueles, O., Ferrières, L., Frétaud, M., Bégaud, E., Herbomel, P., Levraud, J.P., Ghigo, J.M. A new zebrafish model of oro-intestinal pathogen colonization reveals a key role for adhesion in protection by probiotic bacteria. PLoS pathogens, 2012, 26:e1002815.
DOI: 10.1371/journal.ppat.1002815

[32] Bledsoe, J.W., Peterson, B.C., Swanson, K.S., Small, B.C. Ontogenetic characterization of the intestinal microbiota of channel catfish through 16S rRNA gene sequencing reveals insights on temporal shifts and the influence of environmental microbes. PloS one, 2016, 15: e0166379.
DOI: 10.1371/journal.pone.0166379

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ARTICLE

Molecular Identification of Hammerhead Shark Trunks from the Southern Gulf of California using Multiplex PCR

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ABSTRACT

The demand for shark fins in Asiatic markets has resulted in excessive increases in shark catches, even for species that may be under protection or subject to management. As such, it has been necessary to develop and promote monitoring efforts for exploited species and taxonomic groups in order to improve fishing management strategies for elasmobranchs. Identifying species from landings is one of many fishing management problems because landed organisms have usually already been processed and are therefore incomplete, which makes identification problematic, impedes the generation of proper species records, and leads to poor fishery assessments. Tools that can correctly identify species, such as various molecular techniques, have become essential for accurate fishery assessments. In this study, 30 hammerhead trunks from artisanal fisheries from the southern portion of the Gulf of California were identified using multiplex PCR (17 *Sphyrna lewini* and 13 *Sphyrna zygaena*). The total fee to identify each trunk with this technique was ~ $3.80 and the procedure required 2 to 5 days. When compared with other widely-used methods, such as PCR-RFLP or barcoding, multiplex PCR is fast, efficient, low-cost, and easy to implement in a laboratory.

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1. Introduction

Shark landings have increased in recent decades due to the demand for shark fins in Asiatic markets and shark meat in locations, such as Hong Kong, China, and Korea [1-6]. Shark meat is a relatively inexpensive protein source and is consumed by both humans and domesticated animals [7]. Sharks fins are a traditional ingredient in Chinese cuisine and are considered to possess beneficial properties[8]. Moreover, shark fins continue to be viewed as status symbol due to the associated exclusiveness and exoticism of the product and shark fins are judged based on the length, thickness, and texture of their fin needles, or ceratotrichia [8]. Not all the larger shark species possess the most attractive fin needles, but a particular high demand exists for the cartilaginous ceratotrichia found on of the Sphyrnidae family, specially in the dorsal fin and lower lobe of the caudal fin[8-10].

In Mexico, shark fisheries represent an important economic activity. Along the Mexican Pacific, hammerhead sharks comprise up to 30% of elasmobranch catches [11]. The scalloped hammerhead, *Sphyrna lewini*, and the smooth hammerhead, *Sphyrna zygaena*, make up the majority of catches for this group. The distributions of these species primarily overlap along the coast of Sinaloa [12]. Although these two species are morphologically distinct as adults (*S. lewini* has an extra notch in the center of the head), juveniles and shark trunks are difficult to identify in the field and in landings [3,13]. Since 2014, both *S. lewini* and *S. zygaena* have been listed in CITES Appendix II with the specification that their commercial exploitation must not be detrimental to the survival of their populations [11]. Therefore, accurate fishery assessments are necessary for the effective management and conservation of these species and depend on the correct identification of landed specimens [14].

In developing countries few records of species-specific shark catches are available as official statistics only report landings based on common or group names (i.e., a species complex). Moreover, the lack of biological data and the misidentification of organisms and body parts has hindered the proper identification of species in landings [15]. For example, hammerhead fisheries often land sharks without heads, trunks, or fins or eviscerated sharks, making species identification problematic [1,16,17]. These factors have led to questionable fishery assessments and the biological characteristics of a species are generally not considered in management plans [1,16,18]. As such, the implementation of new tools to identify species in landings, such as the use of molecular markers, is essential for reliable fishery assessments and effective management plans. Molecular markers are species-specific and may be used by any molecular lab with the necessary equipment to identify organisms within hours or days given that their use requires following established protocols [3,19,20,21,22]. Furthermore, the use of low-cost molecular tools in developing countries where resources are often limited may facilitate the proper management and conservation of fishery resources [3,23].

Molecular methods of organism identification are diverse, yet the most common method is DNA barcoding, which is comprised of a PCR and the subsequent sequencing of a DNA fragment (COI and/or rDNA[15,22,24]). Techniques like PCR-RFLPs that involve a PCR, enzyme digest, and agarose electrophoresis [25] reduce costs and sample processing times. Furthermore, multiplex-PCR allows for the identification of multiple species in a single PCR reaction using a primer set that amplifies target sequences from multiple species at an affordable cost [3,23,26]. Although some multiplex PCR primers have been validated, multiplex-PCR is not commonly used for the identification of shark trunks or fins in shark fishery assessments [26,27].

Multiplex PCR is an accurate, rapid, and simple technique that may be implemented in any molecular biology lab to identify pelagic shark species [2]. The technique consists in a single PCR reaction with a primer set to simultaneously discriminate between the DNA of different species. The primer design is derived from DNA sequences that have been previously identified for each species. Each primer only hybridizes target DNA and additional primer design involves amplifying fragments of different sizes for each species. Therefore, a subsequent agarose or polyacrylamide electrophoresis is enough to identify the species present in the sample without a further manipulation of amplicons by restriction endonuclease digestion or sequencing [2].

Species of Sphyrnidae family are of the most caught sharks worldwide, and a large percentage of the fins sold in Asian markets come from this family [3,21,28,29]. Given their importance, identification protocols using molecular techniques have been implemented for sphyrnid species and have primarily consisted of DNA barcoding and multiplex PCR[3,19,21,27,29-37]. In fact, species-specific primers and a five-primer multiplex PCR have been developed and extensively tested worldwide for *S. lewini*, *S. mokarran*, and *S. zygaena* [3].

This work aimed to identify the trunks of hammerhead sharks from artisanal fisheries in the southern portion of

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the Gulf of California in Mexico using multiplex-PCR. This technique has been shown to identify sphyrnid species correctly, efficiently, and reliably from a variety of tissue types [3] and is thus amply suited for the identification of hammerhead sharks from Mexican artisanal fisheries. The results of this study provide an efficient solution to the identification problems associated with landed sphyrnid organisms as well as samples from ichthyological collections. A proper record of sphyrnid catches in Mexico will improve landing records and consequently improve fishery assessments and management in the country.

2. Material and Methods

Thirty hammerhead trunk samples from the Mexican artisanal fisheries of Mazatlán (23°12'18.66''N, 106°24'36.35''W) and Teacapán (22° 31'45.44''N, 105°44'09.53''W) in Sinaloa, and from Santa Rosalia (27°20'13.72''N, 112°15'45.79''W) in Baja California Sur (Figure 1) were collected. Some of the sample trunks were identified by fishers using local species names (hammerhead, *Sphyrna lewini*, and the black hammerhead, *S. zygaena*).

Figure 1. Location of sample sites in the Gulf of California

Tissues from Sinaloa were collected in 2017 and kept in salt-saturated DMSO (SSD: saturated NaCl, ethylenediaminetetra-acetic acid, and dimethylsulfoxide 20%) and preserved at room temperature before being placed in long-term storage at 4 °C. Tissues from Baja California Sur were collected in 2011 and stored in ethanol at room temperature. A Student´s t-test was used to compare DNA quality and quantity between the different preservation methods (salt-saturated DMSO or ethanol; Table 1 [38]).

Table 1. Concentration and quality of hammerhead shark DNA. DNA quality is measured by the absorbance rate A260/A280. Sample IDs are composed of species abbreviations (SZY= S. *zyganea*; SLE= S. *lewini*), sample location (IP= Isla de la Piedra; TE= Teacapán; SR= Santa Rosalía), and consecutive number. Bold sample IDs correspond to the samples used in Figure 2. Results of the t-test to detect differences among preservation methods: DNA quality (t= 1.57; p= 0.13) and concentration (t= -0.46; p= 0.64)

ID sample	Sample year	Preservation Solution	Quality	Concentration (ng/µl)
SLETE-25	2017	SSD	1.95	774.2
SLETE-26	2017	SSD	1.98	1292.6
SLETE-27	2017	SSD	2.01	844.1
SLETE-28	2017	SSD	**1.98**	**222.8**
SLETE-29	2017	SSD	1.98	565.5
SLETE-30	2017	SSD	1.94	1560.1
SLETE-31	2017	SSD	1.99	392.9
SLETE-32	2017	SSD	**1.59**	**2055.7**
SLETE-33	2017	SSD	2.05	532.8
SLETE-34	2017	SSD	**1.97**	**742.9**
SLETE-35	2017	SSD	2.06	433.8
SLEIP-29	2011	SSD	2.03	173.0
SLEIP-30	2011	SSD	2.00	53.8
SLEIP-31	2011	SSD	2.06	206.8
SLEIP-32	2011	SSD	**2.1**	**184.8**
SLEIP-33	2011	SSD	2.08	201.1
SLEIP-34	2011	SSD	2.00	156.5
SZYIP-23	2011	SSD	1.98	185.6
SZYIP-24	2011	SSD	2.07	120.7
SZYSR-1	2011	ethanol	2.08	335.9
SZYSR-2	2011	ethanol	2.04	228.4
SZYSR-3	2011	ethanol	1.99	360.7
SZYSR-4	2011	ethanol	**2.05**	**151.0**
SZYSR-6	2011	ethanol	**1.99**	**239.1**
SZYSR-7	2011	ethanol	**1.98**	**417.2**
SZYSR-8	2011	ethanol	2.01	478.6
SZYSR-9	2011	ethanol	**1.91**	**655.8**
SZYSR-10	2011	ethanol	2.00	161.5
SZYSR-11	2011	ethanol	2.04	134.0
SZYSR-12	2011	ethanol	1.98	85.8
Average			2.00	464.9

Genomic DNA was extracted from 50 mg of tissue using standard proteinase K digestion and purified with a lithium chloride salting-out protocol, followed by organic extraction using chloroform-isoamyl alcohol and subsequent ethanol precipitation [39]. Nuclear ribosomal DNA

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(rDNA) was amplified. We used a PCR assay with three optimal species-specific primers (*S. lewini*, *S. zygaena*, and *S. mokarran*) combined in a 5-primer (pentaplex) reaction [3]. The pentaplex PCR included: shark universal primers FISH5.8S-F (5′-TTAGCGGTGGATCACTCG-GCTCGT-3′) and FISH28S-R (5′-TCCTCCGCTTAG-TAATATGCTTAAATTCAGC-3′ [23]) to amplify an rDNA fragment containing the entire nuclear ribosomal DNA internally transcribed spacer (*ITS*2) region plus short portions of the flanking *5.8S* and *28S* ribosomal RNA genes, and the species-specific primers for *S. lewini* (with a *ITS*2 fragment of 445bp; ScHH401F 5′-GGTAAAG-GATCCGCTTTGCTGGA-3), *S. mokarran* (with a *ITS*2 fragment of 782bp; GtHH123F 5′-AGCAAAGAGCGT-GGCTGGGGTTTCGA-3′), and *S. zygaena* (with a fragment of 249bp; SmHH630 5′-TGAGTGCTGTGAGGG-CACGTGGCCT-3′ [3]).

Nuclear rDNA has been extensively tested as a shark species diagnostic tool and has proven to be 100% accurate for identification [2,3,23]. The advantage of using intergenic regions, such as *ITS*2, is the presence of multiple insertion and deletions (indels), which are rare and reduce erroneous identification between DNA samples of different species because each species is tagged with a numeric profile of fragment lengths.

All PCR reactions contained 0.2 mM dNTPs, 1x PCR buffer (10 mM Tris HCL, 50 mM KCl and 1.5 mM $MgCl_2$), 0.4 µM of each primer, 0.5 U of Taq DNAPol (NEB, Ipswich, MA), and 10 ng of template DNA in a total volume of 10 µl. Amplifications were performed with the following thermal profiles: 94 ºC for 2 min, 35 cycles of 95 ºC for 1 min, 65 ºC for 1 min, and 72 ºC for 2 min, followed by a final incubation at 72 ºC for 10 min.

Positive DNA controls of previously identified sphyrnids (*S. lewini* and *S. zygaena*) and negative controls with no target shark species were included in all PCRs. The sphyrnid specimens that were used as positive controls were collected from an artisanal fishery in Mazatlán. These specimens were complete specimens and were positively identified morphologically by a specialist. The *ITS*2 region of the DNA samples collected from these specimens was sequenced to confirm their morphological identification. These samples were used as positive control in each PCR multiplex to identify thirty hammerhead trunk samples.

3. Results and Discussion

A total of 30 samples were successfully identified to the species level using the pentaplex method proposed by Abercrombie [3]. The amplified samples presented 2-3 rDNA fragments (Fig. 2). Bands of ~900 bp were complete fragments of the *ITS*2 region for both species, while the smaller bands were species-specific. The rDNA fragments of 249 bp pertained to *S. zygaena*, while fragments of 445 bp pertained to *S. lewini*. In some samples, we observed bands of ~2000 bp, which represented the unspecific amplification of a region of rDNA. Of the 30 samples analyzed, 17 pertained to *S. lewini* (6 from Mazatlán and 11 from Teacapán) and 13 to *S. zygaena* (2 from Mazatlán and 11 from Santa Rosalia). The DNA samples of the adult *S. lewini* and *S. zygaena* organisms that had been correctly identified and used as the positive controls in the multiplex PCR were used to confirm the corresponding *ITS*2 fragments and multiplex PCR results, as has been done in previous studies [3,35].

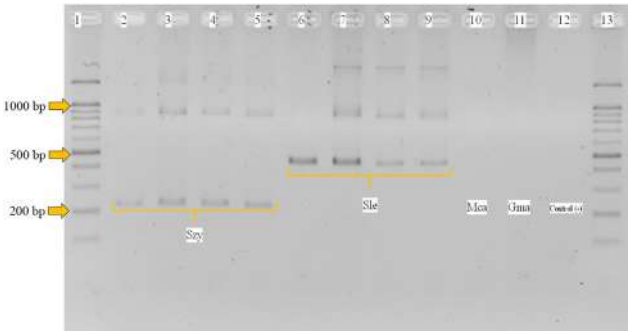

Figure 2. Agarose electrophoresis (2%) showing multi lex PCR results. Lane (1) DNA ladder, lanes (2-5) rDNA of *S. zygaena* (Szy), lanes (6-9) rDNA of *S. lewini* (Sle), lanes (10-11) samples no target (Mca=*Mustelus californicus* and Gma= *Gymnura marmorata*), lane (12) negative control, lane (13) DNA ladder. Amplicons of universal primers were~900 bp.

Multiplex PCR and electrophoresis allowed us to identify hammerhead shark species quickly, accurately, and at low cost. Furthermore, the advantages associated with multiplex PCR allowed us to make proper use of the samples collected along the Mexican Pacific, which may not have been possible with other methods. Our results, and those of previous studies, indicate that multiplex PCR can serve as a useful monitoring tool to identify hammerhead shark meat and fins in landings, markets, and in mislabeled products worldwide [3,19,40]. This application is particularly important because *S. lewini*, *S. zygaena*, and *S. mokarran* are listed in CITES Appendix II and their commercialization is regulated. In Mexico, the molecular identification of hammerhead sharks using multiplex PCR during the seasonal elasmobranch fishing ban (from May 1st to July 31st; DOF 2007) will support law enforcement and resource management efforts. Given the results of our study, we suggest that multiplex PCR should be used to identify hammerhead species in future evaluations of

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hammerhead landings in the Mexican Pacific and elsewhere.

Although a higher concentration of DNA was observed when samples were preserved with SSD than with ethanol, a statistical comparison between the preservation methods found no significant differences in either DNA concentration or quality (Table 1). In the absence of an experimental test for the preservation efficiency of target tissues, the use of either ethanol or the SSD buffer is appropriate as neither method compromises the effectiveness of amplification [41]. This is also true for samples that have been stored for several years.

The multiplex PCR results agree with the distributions of both species near the mouth of the Gulf of California. Artisanal fisheries land both *S. lewini* and *S. zygaena* in this region and particularly in Sinaloa [42,43]. The organisms collected in Tecapán were *S. lewini* juveniles, which also agree with previous studies that have designated the Tecapan lagoon system as a possible nursery area for this species [42,44]. The organisms collected in Santa Rosalia were *S. zygaena* individuals, which coincides with the distribution of this species in temperate zones [45]. As expected, *S. mokarran* was not present among the sampled tissues. This is likely because there have been few records of *S. mokarran* in the Mexican Pacific over the last two decades [46] and there have only been a few catch records in the southern Mexican Pacific [12,47]. While *S. media* and *S. tiburo* showed high catch rates in 1960s in the Gulf of California, no catch records for these species are available for the last decade. As such, these species have likely been extirpated from the Gulf of California [12,46]. According with this historic data, we did not expect to find these species among landed specimens.

Multiplex PCR produces accurate and efficient results after the designation of species-specific molecular markers, which requires a preliminary study involving the comprehensive sampling of organisms and loci. The disadvantages to using multiplex PCR include the initial investment costs required to develop the primers and that it can only be currently used to identify a small group of hammerhead species [2,3,19]. By taking into account species distributions and regional fishing records, it is possible to generate protocols for a given region. This implies that shark species identification protocols using multiplex PCR can be generated to identify species among samples, and that only those samples that produce questionable results may need to be sequenced[27]. A potential disadvantage to multiplex PCR is the possible presence of inhibitory substances in the DNA template for PCR. Similarly, errors in setting up the reaction may result in false negatives and the erroneous interpretation of the absence of target species. Even though preliminary studies involve an additional investment, multiplex PCR remains the best approach to rapidly identify species of importance to conservation efforts or commercial interests at an overall low cost.

We evaluated the time and costs associated with multiplex PCR and found that the generation of results requires 2-5 days, depending on the DNA extraction method, at an expense of ~ $3.80 (reagent cost) per sample. When comparing multiplex PCR with other methods, such as PCR-RFLPs or DNA barcoding, the associated costs are lower for multiplex PCR. For example, PCR-RFLPs require DNA extraction, a PCR ($3.80 per sample), and an enzyme digest, which carries an additional charge of 0.60 cents per enzyme and adds an additional day to the time required for the analysis. The DNA barcoding method requires DNA extraction and a PCR ($3.80 per sample), but it also requires Sanger sequencing, which brings an additional fee of $8.00 (http://www.macrogen.com) and 2-3 additional days are required to send sample by mail and have it processed in an external laboratory. Furthermore, PCR-RFLPs are generally inefficient because some enzymes can produce similar banding, making it necessary to use more than one enzyme [48], which is reflected in increased laboratory costs. Likewise, barcoding requires the use of external sequencing services to obtain the final result, which implies not only extra cost but also additional time.

Identifying species from landings is one of many common fishing management problems. Landed organisms have usually already been processed and are therefore incomplete, which makes identifying species highly problematic. Hammerhead sharks in landings are particularly difficult to identify morphologically as they are often landed without heads. Therefore, molecular tools that can correctly identify species, such as multiplex PCR, have become essential for better catch records. Given that the species-specific primers used in our study have been extensively tested and have been proven to be reliable[3,19,27], we were able to identify 30 hammerhead trunks from artisanal fisheries from the southern portion of the Gulf of California using multiplex PCR (17 *Sphyrna lewini* and 13 *Sphyrna zygaena*).

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Authors Contribution

TAAR, JJRH and VOG manuscript preparation and data analysis, ECOG and ODD data collection and manuscript preparation, and NCSS manuscript preparation and funding.

References

[1] Bonfil R. Overview of world elasmobranch fisheries[R]. FAO Fisheries Technical Paper No. 341. Roma, 1994.

[2] Shivji M, Clarke S, Pank M, Natanson L, Kohler N, Stanhope M. Genetic identification of pelagic shark body parts for conservation and trade monitoring[J]. Conservation Biology, 2002, 16: 1036-1047. DOI: 10.1046/j.1523-1739.2002.01188.x

[3] Abercrombie D, Clarke SC, Shivji MS. Global-scale genetic identification of hammerhead sharks: Application to assessment of the international fin trade and law enforcement[J]. Conservation Genetics, 2005, 6: 775-788. https://doi.org/10.1007/s10592-005-9036-2

[4] Lack M, Sant G. Trends in global shark catch and recent developments in management[M]. TRAFFIC International, 2009: 30.

[5] Musick JA, Musick S. Sharks[R]. FAO Fisheries and Aquaculture Reviews and Studies. FAO. Rome, 2011.

[6] Dent F, Clarke S. State of the global market for shark products[R]. FAO Fisheries and Aquaculture Technical Paper No. 590. FAO. Rome, 2015: 187.

[7] Dulvy NK, Simpfendorfer CA, Davidson LNK, Fordham SV, Bräutigam A, Sant G, Welch DJ. Challenges and Priorities in Shark and Ray Conservation[J]. Current Biology, 2017, 27, 11: R565-R572. https://doi.org/10.1016/j.cub.2017.04.038

[8] Clarke SC, Milner-Gulland EJ, BjØrndal T. Social, Economic, and Regulatory Drivers of the Shark Fin Trade[J]. Marine Resource Economics, 2007, 22, 3: 305-327.

[9] Musick JA. Shark utilization. In Musick J and Bonfil R (eds) Elasmobranch fisheries management techniques[R]. FAO Fisheries Technical Paper. No. 474. Rome, 2005.

[10] Steinke D, Berdard AM, Horn RL, Hilton P, Hanner R, Shivij MS. DNA analysis of traded shark fins and mobulid gill plates reveals a high proportion of species of conservation concern[J]. Scientific Reports, 2017, 7(9505). https://doi.org/10.1038/s41598-017-10123-5

[11] Castillo-Géniz JL, Tovar-Ávila J. Tiburones mexicanos de importancia en la CITES. [R]. Instituto Nacional de la Pesca. México, 2016: 11-15.

[12] Pérez-Jiménez JC. Historical records reveal potential extirpation of four hammerhead sharks (Sphyrna spp.) in Mexico Pacific waters[J]. Reviews in Fish Biology and Fisheries, 2014, 24: 671–683. https://doi.org/10.1007/s11160-014-9353-y

[13] Castro JI. The Sharks of North American Waters[M]. Texas: Texas A&M University Press, College Station, 1983: 180.

[14] Dudgeon CL, Blower DC, Broderick D, Giles JL, Holmes BJ, Kashiwagi T, Krück NC, Morgan JAT, Tillett BJ, Ovenden JR. A review of the application of molecular genetics for fisheries management and conservation of sharks and rays[J]. Journal of Fish Biology, 2012, 80: 1789-1843. DOI: 10.1111/j.1095-8649.2012.03265.x

[15] Holmes HH, Steinke D, Ward RD. Identification of shark and ray fins using DNA barcoding[J]. Fisheries Research, 2009, 95, 2–3: 280-288. https://doi.org/10.1016/j.fishres.2008.09.036

[16] Castro JI, Woodley C, Brudek R. A preliminary evaluation of the status of shark species[R]. FAO Fisheries Technical, Roma, 1999, 380: 72.

[17] INP. Plan de Manejo de la Pesquería de Tiburones y Rayas del Pacífico Mexicano[R]. Dirección General Adjunta de Investigación Pesquera en el Pacífico. México: Instituto Nacional de Pesca, 2012.

[18] Smith E, Au D, Show C. Intrinsic rebound potentials of 26 species of Pacific sharks[J]. Marine and Freshwater Research, 1998, 49, 7: 663 - 678. https://doi.org/10.1071/MF97135

[19] Clarke SC, Magnussen JE, Abercrombie DA, McAllister MK, Shivji MS. Identification of shark species composition and proportion in the Hong Kong shark fin market base on molecular genetics and trade records[J]. Conservation Biology, 2006, 20: 201-211. DOI: 10.1111/j.1523-1739.2005.00247.x

[20] Liu SYV, Chan CLC, Lin O, Hu CS, Chen CA. DNA barcoding of shark meats identify species composition and CITES-listed species from the markets in Taiwan[J]. PLOS ONE, 2013, 8, 11: e79373. https://doi.org/10.1371/journal.pone.0079373

[21] Chuang PS, Hung TC, Chang HA, Huang CK, Shiao

JC. The species and origin of shark fins in Taiwan´s fishing ports, markets, and customs detention: a DNA barcoding analysis[J]. PLOS ONE, 2016, 11, 1: e0147290.
https://doi.org/10.1371/journal.pone.0147290

[22] Feitosa LM, Barbosa-Martins AP, Giarrizzo T, Macedo W, Monteiro IL, Gemaque R, Silva-Nuñes JL, Gomes F, Schneider H, Sampaio I, Souza R, Sales JB, Rodrigues-Filho LF, Tchaicka L, Carvalho-Costa LF. DNA-based identification reveals illegal trade of threatened shark species in a global elasmobranch conservation hotspot[J]. Scientific Reports, 2018, 8: 3347.
https://doi.org/10.1038/s41598-018-21683-5

[23] Pank M, Stanhope M, Natanson L, Kohler N, Shivji M. Rapid and simultaneous identification of body parts from the morphologically similar sharks Carcharhinus obscurus and Carcharhinus plumbeus (Carcharhinidae) using multiplex PCR[J]. Marine Biotechnology, 2001, 3: 231–240.
https://doi.org/10.1007/s101260000071

[24] Chapple DG, Ritchie PA. A retrospective approach to testing the DNA barcoding method[J]. PLOS ONE, 2013, 8, 11: e77882.
https://doi.org/10.1371/journal.pone.0077882

[25] Mendonça FF, Hashimoto DT, Porto-Foresti F, Oliveira C, Gadig OB, Foresti F. Identification of the shark species Rhizoprionodon lalandii and R. porosus (Elasmobranchii, Carcharhinidae) by multiplex PCR and PCR-RFLP techniques[J]. Molecular Ecology Resources, 2009, 9: 771-773.
DOI: 10.1111/j.1755-0998.2009.02524.x

[26] Caballero S, Cardeñosa D, Soler G, Hyde J. Application of multiplex PCR approaches for shark molecular identification: feasibility and applications for fisheries management and conservation in the Eastern Tropical Pacific[J]. Molecular Ecology Resources, 2012, 12: 233-237.
DOI: 10.1111/j.1755-0998.2011.03089.x

[27] Sebastian H, Haye PA, Shivhi MS. Characterization of the pelagic shark-fin trade in north-central Chile by genetic identification and trader surveys[J]. Journal of Fish Biology, 2008, 73: 2293–2304.
DOI: 10.1111/j.1095-8649.2008.02016.x

[28] Clarke SC, McAllister MK, Milner-Gulland EJ, Kirkwood GP, Michielsens CGJ, Agnew DJ, Pikitch EK, Nakano H, Shivji MS. Global estimates of shark catches using trade records from commercial markets[J] Ecology Letters, 2006, 9: 1115-1126.
DOI: 10.1111/j.1461-0248.2006.00968.x

[29] Sembiring A, Pertiwi NPD, Mahardini A, Wulandari R, Kurniasih EM, Kuncoro AW, Cahyani NKD, Anggoro AW, Ulfa M, Madduppa H, Carpenter KE, Barber PH, Mahardika GN. DNA barcoding reveals target fisheries for endangered sharks in Indonesia[J]. Fisheries Research, 2015, 164: 130-134.
https://doi.org/10.1016/j.fishres.2014.11.003

[30] da Silva Rodrigues-Filho LF, da Rocha TC, Sena do Rêgo P, Schneider H, Sampaio I, Vallinoto M. Identification and phylogenetic inferences on stocks of sharks affected by the fishing industry off the Northern coast of Brazil[J]. Genetics and Molecular Biology, 2009, 32, 2: 405-413.
DOI: 10.1590/S1415-47572009005000039

[31] Jabado RW, Al Ghais SM, Hamza W, Herderson AC, Spaet JLY, Shivji MS, Hanner RH. The trade in sharks and their products in the United Arab Emirates[J]. Biological Conservation, 2015, 181: 190-198.
https://doi.org/10.1016/j.biocon.2014.10.032

[32] Vélez-Zuazo X, Agnarsson I. Shark tales: a molecular species-level phylogeny of sharks (Selachimorpha, Chondrichthyes)[J]. Molecular Phylogenetics and Evolution, 2011, 58, 2: 207-217.
https://doi.org/10.1016/j.ympev.2010.11.018

[33] Bineesh KK, Gopalakrishnan A, Akhilesh KV, Sajeela KA, Abdussamad EM, Pillai NGK, Basheer VS, Jena JK, Ward RD. DNA barcoding reveals species composition of shark and rays in the Indian commercial fishery[J]. Mitochondrial DNA Part A, 2016, 28(4): 458-472.
DOI: 10.3109/19401736.2015.1137900

[34] O´Bryhim JR, Parsons ECM, Lance ST. Forensic species identification of elasmobranch products sold in Costa Rica markets[J]. Fisheries Research, 2017, 186, 1: 144-150.
https://doi.org/10.1016/j.fishres.2016.08.020

[35] Almerón-Souza F, Sperb C, Castillo CL, Figueiredo PICC, Gonçalves LT, Machado R, Oliveira LR, Valiati VH, Fagundes JR. Molecular identification of shark meat from local markets in Southern Brazil base on DNA Barcoding: evidence for mislabeling and trade of endangered species[J]. Frontiers in Genetics, 2018, 9: 138.
https://doi.org/10.3389/fgene.2018.00138

[36] Appleyard SA, White WT, Vieira S, Sabub B. Artisanal shark fishing in Milne Bay Province, Papua New Guinea: biomass estimation from genetically identified shark and ray fins[J]. Scientific Reports, 2018, 8, 1: 6693.
DOI: 10.1038/s41598-018-25101-8

[37] Kuguru G, Maduna S, da Silva C, Gennari E, Rhode C, Bester-van der Merwe A. DNA barcoding of chondrichthyans in South African fisheries[J]. Fisheries Research, 2018, 206: 292-295.

DOI: https://doi.org/10.30564/jfsr.v2i1.1685

https://doi.org/10.1016/j.fishres.2018.05.023

[38] Zar JH. Biostatistical analysis[M]. 3rd ed. Prentice Hall Internationl. London, 1996: 944.

[39] Aljanabi SM, Martinez I. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques[J]. Nucleic Acids Research, 1997, 25(2): 4692–4693.
https://doi.org/10.1093/nar/25.22.4692

[40] Chapman D, Abercrombie D, Douady C, Pikitch E, Stanhope M, Shivji M. A streamlined, bi-organelle, multiplex PCR approach to species identification: application to global conservation and trade monitoring of the great white shark, Carcharodon carcharias[J]. Conservation Genetics, 2003, 4: 415–425.
https://doi.org/10.1023/A:1024771215616

[41] Gaither MR, Szabó Z, Crepeau MW, Brid CE, Toonen RJ. Preservation of corals in salt-saturated DMSO buffer is superior ethanol for PCR experiments[J]. Coral Reefs, 2011, 30: 329–333.
https://doi.org/10.1007/s00338-010-0687-1

[42] Bizzarro JJ, Smith WD, Castillo–Géniz JL, Ocampo-Torres A, Márquez–Farías JF, Hueter RE. The seasonal importance of small coastal sharks and rays in the artisanal elasmobranch fishery of Sinaloa, Mexico[J]. Pan-American Journal of Aquatic Sciences, 2009, 4(4): 513-531.

[43] Corro-Espinosa D. La importancia pesquera de los tiburones incluidos en el apéndice II de la CITES en aguas de México. Sinaloa[M]. In: Castillo-Géniz JL and Tovar-Ávila J (eds) Tiburones mexicanos de importancia en la CITES. Instituto Nacional de Pesca, Mexico, 2016: 29 – 93.

[44] Coiraton C, Amezcua F, Salgado-Ugarte IH. Estructura de longitudes de las capturas del tiburón martillo común (*Sphyrna lewini*) en el Pacífico mexicano[J]. Ciencia Pesquera, 2017, 25: 27-40.

[45] Castro JI. Sharks of North America[M]. Oxford University Press USA, 2011: 613.

[46] Saldaña-Ruíz LE. The artisanal shark fishery in the Gulf of California: Historical catch reconstruction and vulnerability of shark species to the fishery[D]. Doctor of Science thesis. Centro de Investigación Científica y de Educación Superior de Ensenada. Ensenada, Baja California, México, 2017: 73.

[47] Tovar-Ávila J, Gallegos-Camacho R. Oldest estimated age for *Sphyrna mokarran* (Carcharhiniformes: Sphyrnidae) in the Mexican Pacific[J]. Hidrobiológica, 2014, 24(2): 163-165.

[48] Cocolin L, D'Agaro ED, Manzano M, Lanari D, Comi G. Rapid PCR-RFLP method for the identification of marine fish fillets (Seabass, Seabream, Umbrine, and Dentex)[J]. Journal of Food Science, 2000, 65: 1315-1317.
DOI: 10.1111/j.1365-2621.2000.tb10604.x

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