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First insights on the bacterial fingerprints of live seahorse skin mucus and its relevance for traceability

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ABSTRACT

Developing a technique to trace the geographic origin of live seahorses is paramount to increase trade regulation and foster conservation. The present study evaluated for the first time the suitability of using bacterial fingerprints present in live seahorse skin mucus to trace their origin. Bacterial 16S rDNA fragments were retrieved from seahorse mucus in a non-invasive and non-destructive way, with their profile (fingerprint) being determined using denaturing gradient gel electrophoresis (DGGE). Bacterial fingerprints were compared among seahorses: (1) originating from different geographic origins sampled at the same period; (2) originating from the same location but sampled one month apart; and (3) originating from specimens in the wild and after being stocked in captivity for 40 and 80 days. Similarities in bacterial fingerprints were determined

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using hierarchical cluster analysis. Results showed that geographic location affected the bacterial fingerprints of wild seahorses and that specimens sampled in the same location displayed a higher level of similarity. This finding supports that this methodological approach holds the potential to reveal local signatures and trace the origin of live seahorses. Bacterial communities from wild seahorses varied over short-time periods, with this natural variability being a potential constraint that may limit the comparison of specimens collected over long periods. Bacterial fingerprints displayed by wild specimens significantly shifted after 40 days in captivity, with a higher level of similarity being recorded for seahorses after 40 or 80 days in captivity, than when compared with those displayed in the wild. This stabilization of the bacterial community under captive conditions shows the potential that bacterial fingerprints may hold for aquaculture, as these can be used as unique signature to trace seahorses to their production facility.

Keywords: *Hippocampus*, Marine ornamentals, Microbiological barcodes, Bacterial communities

1 INTRODUCTION

Developing traceability protocols is paramount to foster seahorse conservation and contribute to a more sustainable trade. The global trade of dried and live seahorses is a multi-million dollars industry that targets several species within genus *Hippocampus* (Foster *et al.*, 2016). In 2004 all *Hippocampus* species were included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) to regulate their international trade and avoid the overexploitation of natural stocks (CITES, 2004). Nonetheless, these restrictive regulations appeared to have the opposite effect, as they have somehow contributed to an increase on the illegal trade of millions of specimens through black markets without any type of certification of origin (Cohen *et al.*, unpublished data). Therefore, it is important to improve trade regulations and promote sustainable fisheries and aquaculture practices that may contribute to protect wild seahorse populations (Cohen *et al.*, 2017). A reliable method to trace the origin of traded specimens is essential to control the blurry supply chains trading seahorses worldwide and promote a sustainable use of these highly-priced marine resources (Cohen *et al.*, 2013, 2017).

Establishing a reliable traceability protocol to trace the geographic origin of seahorses is a challenging task. It is unlikely that a single methodological approach may suffice to implement a reliable traceability protocol to cover the whole trade network of seahorses (Cohen *et al.*, 2017). Seahorses have two very distinct trades, one of dried specimens for Traditional Chinese Medicine, religion and curio; and another one targeting live specimens for marine aquaria (Foster *et al.*, 2016; Rosa *et al.*, 2011). This framework, *per se*, hampers the implementation of a single traceability protocol that suitably addresses the specificities of surveying both dried and live specimens. Some methodologies available to trace seafood for human consumption (reviewed in Leal *et*

al., 2015) may be suitable as well to trace the geographic origin of dried seahorses (Cohen *et al.*, 2017). However, tracing live seahorses traded to supply marine aquaria may be a more difficult task. The marine ornamentals industry trades pricey specimens demanding healthy and aesthetically pleasing (e.g. colour, size and shape) seahorses, which impairs the use of most traceability methods available for seafood or large-sized marine organisms traded live (Cohen *et al.*, 2013).

Microbiological barcodes from fish skin mucus may be one of the best options to trace the geographic origin of marine ornamentals in a non-invasive and non-destructive way (Cohen *et al.*, 2013). This method consists on the amplification and analysis of the 16S rRNA gene from the bacterial communities present in the fish mucus. The analysis is performed using non-culture dependent approaches (e.g., through a denaturing gradient gel electrophoresis - DGGE), to determine and compare the existence of significant differences in bacterial fingerprints that may be site-specific (Tatsadjieu *et al.*, 2010). Previous works employing this technique have focused on the seafood production chain and reported their successful use to trace the geographic origin of fish from the wild (Smith *et al.*, 2009; Tatsadjieu *et al.*, 2010), as well as captive-cultured fish (Le Nguyen *et al.*, 2008). To date, no study has addressed the use of this methodological approach to trace the geographic origin of small-sized live ornamental fish.

The present study aims to perform the first evaluation on the suitability of using bacterial fingerprints present in seahorse skin mucus to trace their geographic origin. For this purpose, the following null hypothesis were tested: (1) geographic location does not affect the bacterial communities present in the skin mucus of wild seahorses; (2) bacterial communities present in the skin mucus of wild seahorses do not present significant variations over short-time periods; (3) the stocking of wild seahorses in

captivity does not promote a significant variation on bacterial communities present in their skin mucus.

2 MATERIAL AND METHODS

2.1 Experimental design

Mucus from adult seahorses *Hippocampus reidi* was collected in four locations in Brazil Southeast Coast (Fig. 1). Location and sampling period was dependent on the null hypothesis being addressed and are detailed below.

To test the null hypothesis "geographic location does not affect the bacterial communities present in the skin mucus of wild seahorses", mucus samples were collected from 10 seahorses sampled in three different islands of Rio de Janeiro (Fig 1; site 2, 3, and 4) in November 2015. All skin mucus samples were collected within two days: three samples from Ilha do Mantimento (Fig. 1; site 2), three from Ilha Comprida (Fig. 1; site 3), and four from Ilha do Cabo Frio (Fig. 1; site 4).

To test the null hypothesis "bacterial communities present in the skin mucus of wild seahorses do not present significant variations over short-time periods", a new sampling of five seahorse skin mucus was done at Ilha do Mantimento (Fig. 1; site 2), approximately one month (December 2015) after the first sampling event (when the mucus from 3 seahorses were sampled).

To test the null hypothesis "the stocking of wild seahorses in captivity does not promote significant variation on bacterial communities present in their skin mucus", the skin mucus of five wild seahorses was collected at Ubatuba, São Paulo (Fig. 1; site 1). Following this procedure, the same animals were transferred to the laboratory and additional mucus samples were collected after 40 and 80 days in captivity. These five seahorses were kept in a recirculated system operated with natural seawater (collected in Santos, São Paulo, and ~200 km south from the location where seahorses were

collected). The system was formed by four 80-L polyethylene barrels connected in parallel to a filter tank. The five seahorses were kept together in the same barrel, and no other animal was stocked in the system during this study. The filtration consisted of porous ceramics (biological filtration), a protein skimmer, two heaters (set at 27 °C) and an automatic osmoregulator dosing deionized freshwater to compensate for evaporation and keeping salinity stable at 28. Seahorses were fed twice a day *ad libitum* with frozen mysids, with uneaten food being siphoned from the tank to prevent decay in water quality.

2.2 Field sample collection

Authorized researchers manually collected each wild seahorse sampled using freediving gear and disposable gloves, one pair per each specimen collected. This procedure was employed to avoid potential cross-contamination of bacterial communities present in their skin mucus, with each diver putting on the gloves only when a seahorse was spotted. Skin mucus samples were collected from one seahorse at the time, with each specimen being removed from the water, slightly agitated to remove excess water and placed inside a disposable sterile sample bag (Labplas Twirl'EM^{TM}). Once inside the bag, the seahorse was gently rubbed from the outer side to stimulate the release of skin mucus. This procedure was done as fast as possible, taking approximately 30 s per specimen, to minimize any potential stress to the animals. Upon mucus collection, each specimen was immediately returned to the same place of collection. From each mucus sample collected, 200 µL were stocked in a screw cap microcentrifuge tube (DNA and RNA free) with 1000 µL of PBS (phosphate saline solution at pH 7.4). Samples were placed inside a thermic bag with ice for 24 h (maximum) before initiating bacterial DNA extraction in the laboratory.

2.3 DNA extraction and PCR amplification

Within 24 h after collection (maximum), bacterial DNA was extracted from each mucus sample using the QIAamp® mini DNA extraction kit (Qiagen, Hilden, Germany), according to manufacter's instructions for Gram-positive bacteria. At the end of the extraction procedure, bacterial DNA present in the skin mucus of each seahorse sampled was concentrated in a 200-µL buffer elution.

A nested polymerase chain reaction (PCR) was performed to amplify the V6–V8 region of 16S rRNA gene. PCR amplification was performed using the primer pairs 27F/1492R (Weisburg *et al.*, 1991) and 984GC(F)/1378R (Heuer *et al.*, 1997), for the first and second PCR, respectively. The amplification procedure followed Pimentel *et al.*, 2017 conditions. PCR amplicons were visualized by electrophoresis in an agarose gel at 2% (w/v) with TAE 1× buffer (40 mM Tris–HCL pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2-EDTA), at 90 V for 25 min.

2.4 Denaturing gradient gel electrophoresis (DGGE)

Two DGGE fingerprints were performed according to procedures detailed in Pimentel *et al.* (2017). PCR amplicons from samples collected from Ilha do Mantimento, Ilha Comprida and Ilha do Cabo Frio (Fig. 1; site 2, 3 and 4) were used in the first DGGE fingerprint to compare bacterial communities present in the skin mucus of seahorses collected in different geographical locations and at different periods (Ilha do Mantimento only). In the second DGGE, PCR amplicons from mucus samples from seahorses collected from Ubatuba (Fig. 1; site 1) and subsequently sampled after being stocked in the laboratory for 40 and 80 days were used to compare their bacterial fingerprints. Prior to DGGE analysis, DNA present in all samples was quantified using Qubit® fluorometric quantitation method (LIFE technologies). This quantification allowed to standardize the amount of DNA used for each DGGE fingerprint, which allows to have a more reliable comparison of band intensities. The amount of PCR

product loaded to run the DGGE fingerprints were 130 and 77 ng of DNA for the first and second fingerprints performed, respectively.

2.5 Data processing and statistical analysis

Fingerprints formed by bacterial 16S rDNA fragments were analyzed using BioNumerics 6.6 (Applied Maths, Ghent, Belgium). This software processed the gel images to generate a matrix of intensity values for each band recorded. These values were processed to relative band intensity and transformed using $\log (X + 1)$, following the recommendations by Pimentel *et al.* (2017). Similarities in bacterial fingerprints were determined using hierarchical cluster analysis through the group average method and the Bray–Curtis measure, as well as similarity percentages (SIMPER) analysis to determine average similarity within groups and average dissimilarity between groups. Significant differences ($P < 0.05$) in bacterial fingerprints were determined using permutational multivariate analysis of variance (PERMANOVA), with subsequent pairwise comparisons being performed if required. Hierarchical cluster analysis and PERMANOVA were performed using PRIMER 6 with the add-on PERMANOVA+ (Primer-E Lta, Plymouth UK) following the recommendations of Anderson *et al.* (2008).

3 RESULTS AND DISCUSSION

The reduced number of specimens employed in the present study may be considered as a potential caveat to achieve a more robust data analysis. Nonetheless, it must be highlighted that the hierarchical cluster analysis performed revealed the existence of very consistent groups (see below) and that seahorses are a threatened group of marine fish currently listed under CITES Appendix II. Given the endangered status of all *Hippocampus* species, it would not have been responsible nor ethically acceptable to collect a larger number of specimens from the wild when investigating, for the first time

ever, the potential use of this traceability approach in seahorses. Results showed that geographic origin affects the bacterial fingerprints present in the skin mucus of wild seahorses, while specimens originating from the same geographical location display a higher similarity (Fig. 2 and Figure SI). No significant difference ($P = 0.12$) was found on the bacterial fingerprint present in the skin mucus of seahorses originating from the two closest locations sampled (Ilha do Mantimento and Ilha Comprida), but both differed significantly ($P = 0.03$) from those collected in the farthest location (Ilha do Cabo Frio). Although previous work reported that microbiological composition in seahorse skin mucus are significantly different from its surrounding water and live prey (Balcázar *et al.*, 2010), the difference in abiotic conditions and substrate may somehow affect the composition of bacterial communities present in seahorse skin mucus within different microhabitats. Therefore, the similarity recorded in the bacterial fingerprints displayed by seahorses collected in Ilha do Mantimento and Ilha Comprida may be explained by the fact of these adjacent islands being subjected to more similar abiotic conditions (e.g. weather, currents, etc.); it is also possible that seahorses may sporadically migrate between these two islands, namely at their juvenile stage (dispersion). Thus, the accuracy of this method to pin point the geographical location of a wild caught seahorse is possibly related to the abiotic conditions of the habitat and to the dispersion/migration capacity of seahorses. Previous works have confirmed that the bacterial profile displayed by fish skin mucus is a reliable approach to trace their geographical origin, such as for the marine fish *Merlangius merlangus* (Smith *et al.*, 2009) and the freshwater fish *Oreochromis niloticus* (Tatsadjieu *et al.*, 2010). Therefore, this higher similarity on bacterial fingerprints among seahorses from the same location is a good indicator that this methodological approach holds the potential to reveal local signatures and allow the tracing of the place of origin of live seahorses.

Further genetic information on these wild populations of seahorses, obtained through non-lethal sampling approaches (e.g., sampling dorsal fin or skin filaments as described by López et al. (2015)), will be important to validate this hypothesis in future studies.

The present study also showed that bacterial communities present in the skin mucus of seahorses in the wild may vary over short-time periods (Fig. 2). When comparing samples from Ilha do Mantimento collected in November and December, it was possible to confirm that bacterial fingerprints changed significantly ($P = 0.03$) from one month to the other; moreover, the differences recorded were higher in samples collected in the same location over two consecutive months than those found among specimens originating from different geographic regions (Fig. 2). It is likely that some environmental shifts may have significantly affected local conditions and consequently promoted a shift in the bacterial communities present in seahorse mucus within this period. The temporal variability recorded in the bacterial fingerprint of fish mucus has been previously reported in freshwater fish sampled during rainy *vs.* dry season in wild lakes (Tatsadjieu *et al.*, 2010), as well as in earth ponds (Le Nguyen *et al.*, 2008). This variability in bacterial fingerprints may limit the period during which the approach addressed herein can be used to trace the place of origin of wild seahorses. This constraint may be overcome if the place of origin of seahorses is accessed through the bacterial fingerprinting shortly after their collection (e.g., up to one-week post-collection). Nonetheless, it is important to confirm if this temporal shift in bacterial fingerprints occurs periodically (e.g., monthly) during the whole year or rather displays a seasonal pattern. Periods during which environmental changes are less pronounced may contribute to a stabilization of the fingerprints displayed by the bacterial communities in seahorse mucus. Additionally, it is important to highlight that at least some bacterial bands recorded in the DGGE analysis using the skin mucus of *Pangasius*

remained stable throughout different seasons (Le Nguyen *et al.*, 2008). Therefore, further DNA sequencing studies may reveal if some bacteria taxa are more stable over a wider temporal scale than others, and thus may still allow to reliably trace the geographical origin of live seahorses.

The stocking of wild seahorse in captivity, even when placed in a recirculated system operated with natural seawater, promoted a shift in the bacterial communities associated with their skin mucus (Fig. 3 and Figure SII). Bacterial fingerprints from wild specimens changed significantly after only 40 days ($P < 0.01$) in captivity (Fig. 3). Unfortunately, as seahorses were not individually tagged (see Cohen et al. 2018), it was not possible to determine if this shift was more pronounced in some specimens than in others. Future studies should aim to follow these shifts in seahorses bacterial fingerprints at an individual level to reveal the existence of any potential intraspecific variability. From collection to final costumer, marine ornamentals may pass through several aquarium systems in exporters, importers, wholesalers, and retailers facilities (Cohen *et al.*, 2013). Therefore, our results showed that if specimens are stocked for one month in a system throughout the supply chain they may change the bacterial signature of their skin mucus. This issue may limit the chances to accurately trace their place of origin. Nonetheless, future studies should investigate if specimens originating from different geographical locations, and therefore displaying different bacterial fingerprints, exhibit similar bacterial fingerprints after being stocked in captivity for at least 40 days. If this is not the case and a high level of differentiation persists in the bacterial fingerprints of seahorses originating from different locations, the present approach may still be used to reliably indicate if all specimens originate from the same location or not. Additionally, DNA sequencing studies can provide insights on which bacterial communities remain stable after the stocking of wild seahorses in captivity.

These studies may allow researchers to refine their analysis, focus detection on those specific bacterial communities and more reliably assess the place of origin of target seahorses.

The bacterial fingerprints displayed by seahorse skin mucus after 80 days in captivity also changed significantly ($P < 0.01$) from those displayed by specimens collected after 40 days. However, it must be highlighted that the longer seahorses were kept in captivity the more similar were the bacterial fingerprints displayed by in their skin mucus. Indeed, the SIMPER analysis revealed that average similarity percentages of bacterial fingerprints recorded in seahorse skin mucus increased from only 9.4% in specimens collected in the wild, to 32.0% and 50.7% after 40 and 80 days in captivity, respectively. These results suggest that bacterial communities in seahorse skin mucus may undergo a potential stabilization after specimens are stoked in captivity for only a few weeks. This stabilization of bacterial fingerprints was likely promoted by the conditions experienced by seahorses stoked in an indoor closed recirculated aquaculture system, as water parameters are much more stable in this system than in the wild or an outdoor aquaculture system, namely if operated in a flow-through regime. In this way, it is possible that bacterial fingerprints in seahorse skin mucus may remain stable after a prolonged stocking period (e.g., few months). This assumption is also supported by SIMPER values on average dissimilarity percentages between groups, with these values being lower when comparing the bacterial communities in seahorse skin mucus stocked in captivity for 40 or 80 days (78.5%), than when comparing wild specimens with those stocked in captivity for 40 days (93.3%). This stability in bacterial communities under captive conditions was also reported for cultured soft corals (Pimentel *et al.*, 2016) and may hold the potential to establish a unique bacterial signature for each production system. If different systems display different bacterial signatures, in theory, it can be

possible to trace the origin of traded specimens to different farms. Indeed, a previous study addressing the freshwater fish *Pangasius* was able to pinpoint their farm of origin through the use of bacterial fingerprints present in the skin mucus (Le Nguyen *et al.*, 2008). The possibility to trace captive seahorses to its producers would be paramount to support reliable certification programs and favour a more conscientious and sustainable trade (Cohen *et al.*, 2013, 2017). For example, certification agencies or programs, such as the Seafood Watch, could inform end-costumers on which producer is following the most sustainable culture practices (Monterey Bay Aquarium; available at: http://www.montereybayaquarium.org/conservation-and-science/our-programs/seafood-watch; accessed in January 2017). The ability to positively discriminate farms promoting sustainable culture practices would also be an important marketing strategy to producers that would be able to differentiate their product. To validate this approach, future studies should compare bacterial fingerprints of the skin mucus of wild-caught seahorses stocked in captivity with those displayed by specimens fully bred under captive conditions. Additionally, it would also be important to study bacterial fingerprints on seahorse skin mucus from farms with different production systems (e.g., closed *vs.* open systems).

Overall, the present study supports that bacterial fingerprints displayed by seahorse skin mucus holds the potential to trace the geographic origin of live seahorses, either if specimens are collected from the wild or farmed under controlled conditions. The variability displayed by bacterial communities over a short-time period in the same geographic location in the wild, as well as their shift when transferred from wild to captive conditions, are the main issues that need further research to support the use of this method for traceability. Considering the positive results reported in the present study, future research may now advocate the need to increase the number of samples to

be collected from wild seahorses to increase statistical power. Researchers may also now conscientiously choose to employ more expensive DNA sequencing techniques (namely next generation sequencing, NGS) to gain an in-depth knowledge on which bacterial taxa play the most relevant role on geographic and temporal variability displayed by bacterial fingerprints. The use of high-throughput sequencing techniques will likely make possible to discriminate between wild, captive raised and captive bred live seahorses.

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5 FUNDING

Supplementary data

Supplementary material

Supplementary **figure I.** PCR-DGGE 16S rDNA banding fingerprints from bacterial communities present in the mucus of seahorses *Hippocampus reidi* from three Islands in Southeast Brazil (Ilha do Mantimento, Ilha Comprida, and Ilha do Cabo Frio; Fig. 1) in November (Nov.) and December (Dec.) 2015.

Supplementary **figure II.** PCR-DGGE 16S rDNA banding fingerprints from bacterial communities present in the mucus of seahorses *Hippocampus reidi* from the same specimens after 40 and 80 days in captivity.

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Figure 1. Map of site collections of *Hippocampus reidi* in Southeast Brazil. Site 1: Ubatuba, São Paulo; Site 2: Ilha do Mantimento, Rio de Janeiro; Site 3: Ilha Comprida, Rio de Janeiro; Site 4: Ilha do Cabro Frio, Rio de Janeiro. Map made with ArcMap^{TM} 10.2.

Figure 2. Cluster analysis of bacterial 16S rDNA fingerprints from bacterial communities present in the skin mucus of *Hippocampus reidi* from three Islands in Southeast Brazil (Ilha do Mantimento, Ilha Comprida, and Ilha do Cabo Frio; Fig. 1) in November (Nov.) and December (Dec.) 2015.

Figure 3. Cluster analysis of bacterial 16S rDNA fingerprints from bacterial communities present in the skin mucus of *Hippocampus reidi* collected from the wild (Ubatuba, São Paulo) and after being stocked in captivity in an indoor closed

recirculated aquaculture system for 40 and 80 days (skin mucus samples were collected from the same individuals).

HIGHLIGHTS

- First experimental evidence that bacterial fingerprints present in seahorse skin mucus hold potential to trace geographic origin;
- Bacterial fingerprints of wild seahorses vary geographically and with time;
- Bacterial fingerprints of wild seahorses shift when these are stocked in captivity;
- Bacterial fingerprints of seahorses become stable after longer stocking periods in captivity.