

## Research Article

## Origins of isolated populations of an Indo-Pacific damselfish at opposite ends of the Greater Caribbean

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**Citation:** Robertson DR, Dominguez-Dominguez O, Solís-Guzmán MG, Kingon KC (2021) Origins of isolated populations of an Indo-Pacific damselfish at opposite ends of the Greater Caribbean. *Aquatic Invasions* 16(2): 269–280, <https://doi.org/10.3391/ai.2021.16.2.04>

**Received:** 13 May 2020

**Accepted:** 26 October 2020

**Published:** 14 December 2020

**Handling editor:** Charles Martin

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### Abstract

The non-native Indo-West Pacific (IWP) damselfish *Neopomacentrus cyanomos* has two, recently discovered, isolated Northwest Atlantic (NWA) populations separated by ~ 3000 km of the Caribbean Sea. One of them spans the southern and northern Gulf of Mexico (GoMx) and the other is at Trinidad, in the southeast Caribbean. We compared DNA (CO1) sequences of 71 individuals from the southwest GoMx to those of 86 fish from Trinidad to determine similarities in their origins. In the native range, there are four distinct, largely allopatric haplogroups, and the two NWA populations are composed of individuals from the same two of those four haplogroups. Over 90% of individuals in each NWA population have six haplotypes shared between those populations, and no pairwise  $\Phi_{ST}$  differences in population structure (relative abundances of different haplotypes) were found between the GoMx and Trinidad populations. Levels of haplotype diversity and nucleotide diversity are similarly high in the two NWA populations. The existence of two isolated populations in areas with major, long-standing offshore oilfields is consistent with those fish being transported to those areas of the NWA by floating offshore infrastructure moved from the IWP. The strong population similarities and genetic diversity of the two NWA populations indicate that both resulted from the introduction of a substantial number of individuals from the same part of the native range. However, existing genetic and other data do not allow determination of the sequence of events that led to the establishment of those populations, and whether one established first then effectively “seeded” the other or both were established concurrently as a result of a single introduction.

**Key words:** *Neopomacentrus cyanomos*, DNA sequence, regal demoiselle, Gulf of Mexico, Trinidad, offshore petroleum

### Introduction

The regal demoiselle, *Neopomacentrus cyanomos* (Bleeker, 1856), which is native to coral reefs of the Indo-West Pacific (Allen 1991), was first discovered in the Atlantic in mid-2013, on inshore coral reefs of Mexico in the southwest corner of the Gulf of Mexico (GoMx) (González-Gándara and de la Cruz-Francisco 2014). Over the following two years, it was found on inshore and offshore reefs over most of the southwest GoMx, as well as

on offshore oil platforms in that area. By 2016, it was known from natural- and artificial reefs and oil platforms in the northern GoMx and was found across the western three-quarters of that shelf area by 2018 (Schofield and Neilson 2019). Between 2013 and early 2019, surveys in Venezuela, Colombia, Panama, Belize, Honduras, the Caribbean coast of Mexico, and southeastern Florida failed to find this species in any of those areas, indicating that it likely was restricted to the GoMx at that time.

Three main mechanisms have been proposed to account for the introduction of a species such as *N. cyanomos* to areas well outside its native range: (1) it was carried by commercial cargo shipping (in ballast water or in a sea-chest) from its native range (González-Gándara and de la Cruz-Francisco 2014); (2) it was released from aquaria, a process that has led to the introduction of many other non-native reef fishes to southern Florida (Schofield and Akins 2019); and (3) it was carried externally on the structure of offshore petro-platforms (or other pieces of infrastructure such as a drill ship or support vessel) immersed in the sea while being relocated from the species native range to oil and gas fields in the NWA (Robertson et al. 2016, 2018). Robertson et al. (2018) presented genetic and other data indicating that the most likely mode of introduction of *N. cyanomos* to the northwest Atlantic (NWA) was via such oilfield infrastructure relocation.

There are three other areas of the tropical Atlantic with large offshore oil and gas fields that have connections with the IWP, with the GoMx offshore fields, and with each other via movements of floating infrastructure: (1) Trinidad, at the northeast corner of South America; (2) Central Brazil; and (3) various fields scattered along a 3000 km stretch of the West African shelf between Angola and Sierra Leone. Such relocation of offshore infrastructure has moved reef fishes across the Atlantic (Pajuelo et al. 2016), and could carry *N. cyanomos* between those oilfields, leading to the establishment of populations in the areas where they are located. To assess that possibility, in July 2019, Robertson and Kingon (2019), and Robertson et al. (*in press*) surveyed reefs and shipwrecks on the islands and peninsula of northwestern Trinidad that straddle the northern entrance of the Gulf of Paria, between Venezuela and Trinidad. There they found a substantial, evidently isolated population of *N. cyanomos*, 3,000 km from the population in the Gulf of Mexico.

In this paper, we describe the results of an assessment of genetic relationships between the GoMx and Trinidad populations of *N. cyanomos*, using Cytochrome Oxidase Subunit 1 (CO1) DNA sequence data, in order to try to answer two questions: (1) Are those two populations the result of an introduction from the same part of the IWP? (2) Were those two populations established separately or did one establish first then provide propagules that produced the other? To do so, we predicted that: (1) If those two populations resulted from introduction from the same part of

the IWP, then they should have very similar genetic signatures. (2) If the haplotypes of one population are a subset of those of the other population, that would indicate that the former produced propagules that established the latter. If that was not the case, there would be no indications of a directional, secondary introduction.

## Materials and methods

Published CO1 sequences from the GoMx are available for 65 individuals of *N. cyanomos* collected from reefs on Campeche Bank, in the southwest part of that gulf. Such sequences are also available from 54 individuals collected at 16 sites scattered throughout the species native, IWP range (Robertson et al. 2018).

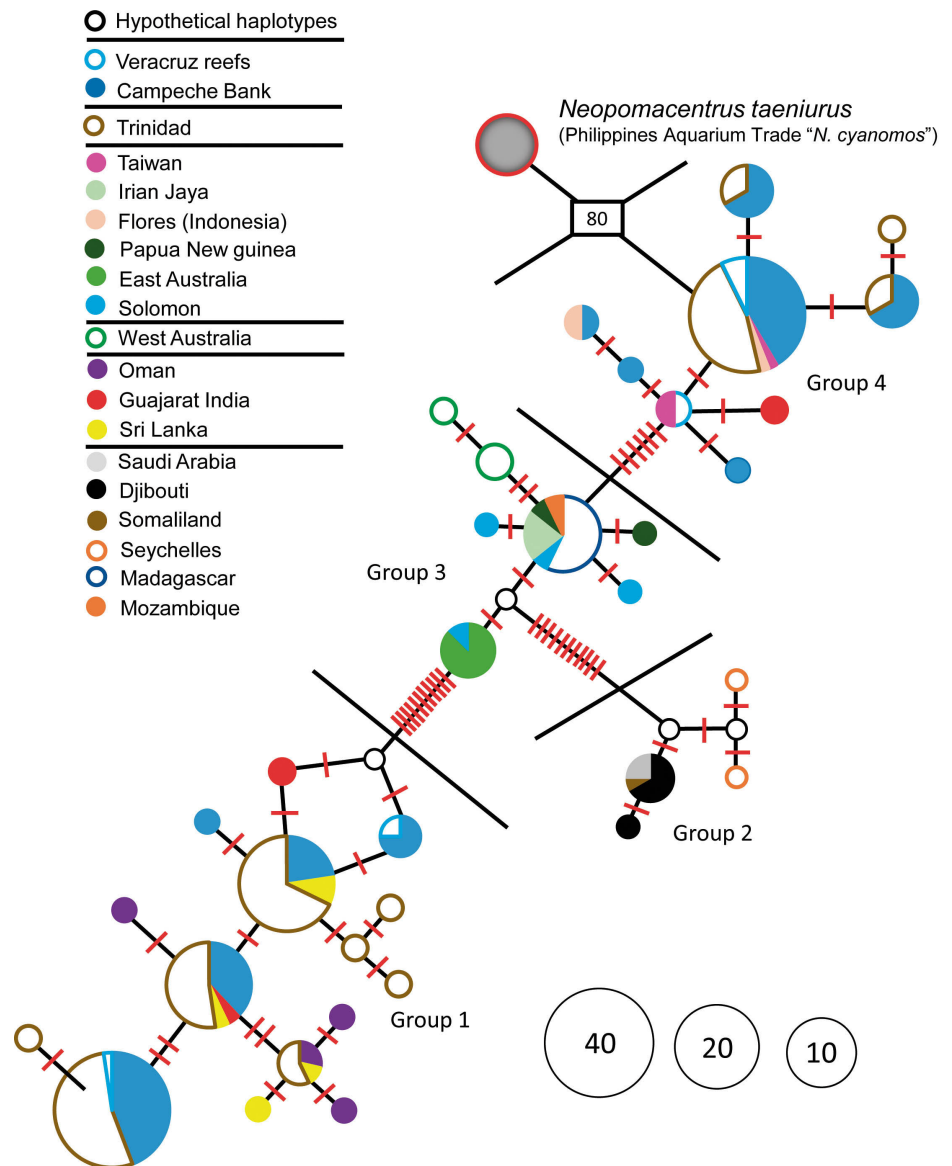
Those published sequences were supplemented by new sequences generated from six individuals collected by ODD on shoreline reefs of Veracruz state, on the western shore of the southwest GoMx, ~ 400 km west of the Campeche Bank reefs. In addition, DRR and KK collected 100 individuals of this species at Trinidad. In both cases, fish were collected on SCUBA, using the anesthetic clove-oil and hand nets. The fish were then euthanized with an overdose of that oil. Subsequently, a pectoral fin from each was preserved in 95% ethanol to provide a tissue sample for DNA sequencing. CO1 sequences were successfully obtained from all six of the Veracruz fish and 86 of the Trinidad fish.

### *DNA sequencing*

Total genomic DNA was extracted using the conventional proteinase K/phenol/chloroform method (Hillis et al. 1996). PCR amplifications were performed in volumes of 12.5 µl composed of 6.25 µl of 10% trehalose, 2 µl of ultra-pure water, 1.25 µl of 10× PCR buffer (10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM Tris-HCl (pH 8.8), 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100), 0.625 µl of MgCl<sub>2</sub> (50 mM), 0.125 µl of each of two primers (0.01 mM; Fish 1F and Fish 1R (Ward et al. 2005)), 0.0625 µl of dNTP (10 mM), 0.0625 µl of Taq DNA polymerase (New England Biolabs) and 2 µl of template DNA. The PCR amplification was performed using denaturation at 94 °C for 30 seconds, annealing at 52 °C for 40 seconds, an extension at 72 °C for one minute, and a final extension at 72 °C for 10 min. PCR products were visualized electrophoretically on 1.5% agarose gel and submitted to MACROGEN Korea for sequencing.

### *Haplotype network construction and population structure*

Nucleotide sequences were edited and manually aligned in Mega v.6.06 (Tamura et al. 2013) and examined with a chromatogram. A final alignment of 582 base pairs was used in the analyses. Genbank Accession numbers for the 92 sequences that have not been previously published are GenBank

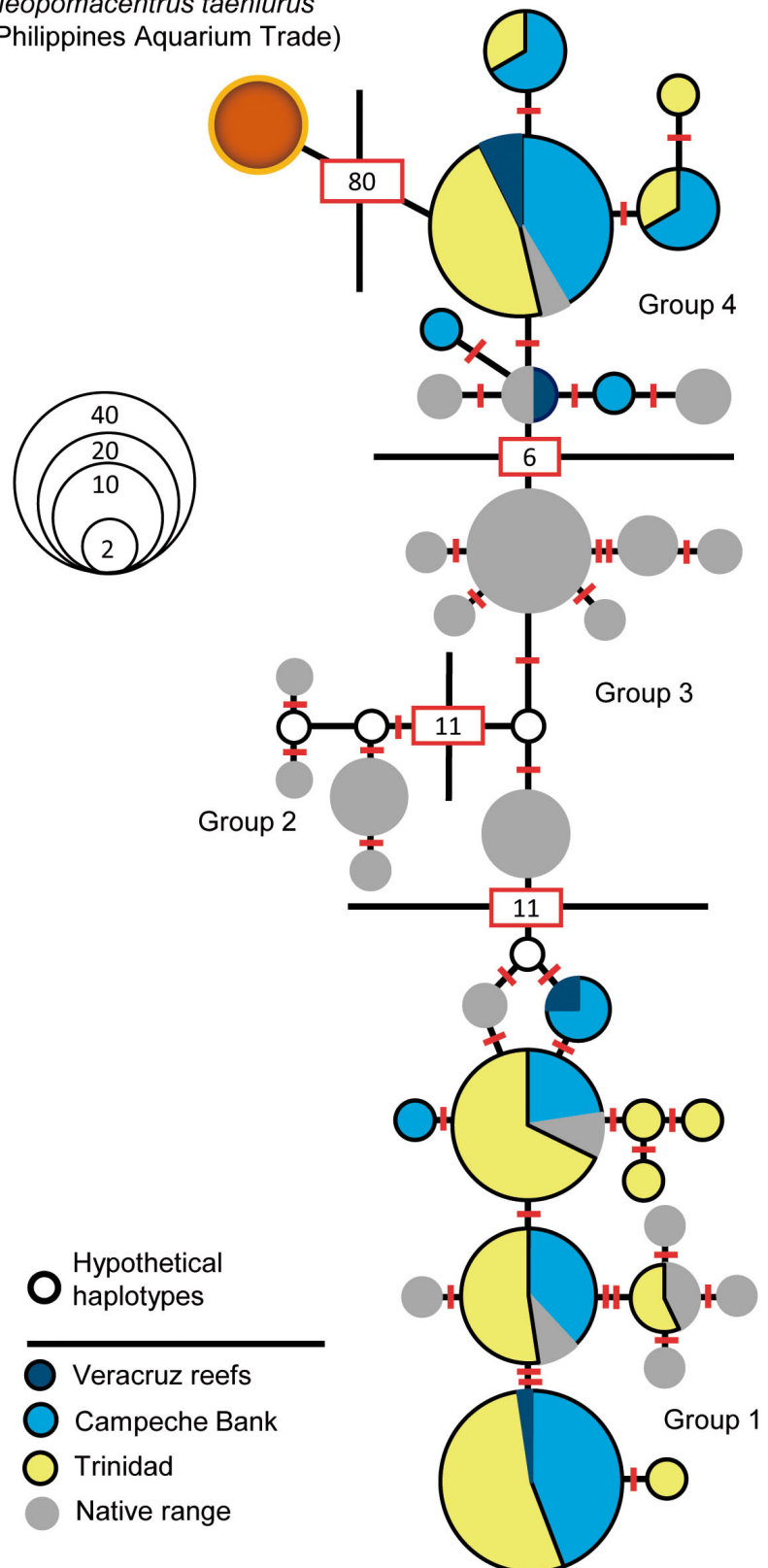


**Figure 1.** Full-color, complete haplotype network of *N. cyanomos* from 16 separately colored sites in the Indo-West Pacific and three sites in the Northwest Atlantic: Campeche and Veracruz in the Gulf of Mexico, and Trinidad in the southeast Caribbean. Relative circle sizes indicate numbers of individuals with each haplotype, with relative abundance at each location is indicated by pie slices. Red lines indicate number of mutation steps (all single-base substitutions) separating haplotypes. Modified after Figure 1 of Robertson et al. (2018), see methods.

MT155702 to MT155792. The haplotype network figured here was constructed using PopART (available at <http://popart.otago.ac.nz/downloads.shtml>) and applying the Median-Joining method (Bandelt et al. 2000).

Two versions of the resultant haplotype network are presented here, a comprehensive version differentiating haplotypes from each of the three NWA (Campeche, Veracruz, and Trinidad) locations and each of the 16 native-range locations (Figure 1), and a simplified version in which only haplotypes from the three NWA locations are identified (Figure 2). Those are intended to allow comparison of genetic diversity among native-range and NWA locations and between NWA populations, respectively.

*Neopomacentrus taeniurus*  
(Philippines Aquarium Trade)



**Figure 2.** Simplified haplotype network of *N. cyanomos*. Haplotype network from sites in the Indo-West Pacific (all grey) and three, separately colored sites in the Northwest Atlantic, Campeche and Veracruz in the Gulf of Mexico, and Trinidad in the southeast Caribbean. Relative circle sizes indicate numbers of individuals with each haplotype, with relative abundance at each location is indicated by pie slices. Red lines indicate number of mutation steps (all single-base substitutions) separating haplotypes.

The similarity of the NWA populations to each other and to the native-range population was evaluated using pairwise  $\Phi_{ST}$  tests (tests with haplogroups 1 and 4 separately and both haplogroups combined). These analyses were carried out in ARLEQUIN 3.5.1.2 (Excoffier and Lischer 2010), and the statistical significance of  $\Phi_{ST}$  values was evaluated by performing a randomization test of 10,000 replications with a significance level of  $\alpha = 0.05$ .

#### *Diversity indices and historical demography*

Diversity indices were calculated for each genetic group among both the GoMx and Trinidad populations, and for a combination of those populations, using the program DNAsp v5 (Librado and Rozas 2009). A comparison between the diversity of the two NWA populations and equally large samples from a set of local native-range populations in each of haplogroups 1 or 4 would have provided information about local-population diversity in the native range versus that in the NWA. However, no such local-population samples from the IWP currently exist.

To detect signatures of demographic changes in populations of each of the two major native-range lineages of *N. cyanomos* found in the NWA populations (those belonging to groups 1 and 4 in the haplotype network of Figures 1 and 2), Tajima's  $D$  (Tajima 1989), and Fu's  $F_s$  (Fu and Li 1993) neutrality tests were calculated in DNAsp v5 (Librado and Rozas 2009). Evidence of an expanding population of a lineage would be assumed if significant negative values of Tajima's  $D$  and Fu's  $F$ -statistics were obtained (Mousset et al. 2004). Additionally, a pairwise mismatch distribution was computed for each of the GoMx and Trinidad populations in DNAsp v5 (Librado and Rozas 2009). Historically stable populations have been predicted to have multimodal mismatch distributions, whereas those that have undergone a recent expansion are predicted to be unimodal (Slatkin and Hudson 1991). To test if the observed mismatch distributions predicted a stable or an expanding population model, differences between observed and expected (multimodal) distributions were evaluated using the Harpending's raggedness index (Harpending et al. 1993) and the  $R_2$  statistic (Ramos-Onsins and Rozas 2002).

## **Results**

The haplotype network (Figures 1 and 2) for the two NWA populations and group of IWP populations generated by the inclusion of additional data on six fish from Veracruz and 86 individuals from the Trinidad population shows that there are clear, strong similarities in the genetic structure of the GoMx and Trinidad populations of the study species. Both NWA populations have only haplotypes from the same two of the four haplogroups (groups 1 and 4) known from the IWP (Figure 2). Together

the two NWA populations have 17 haplotypes, *vs.* 23 currently known from all four haplogroups in the IWP native-range and 12 currently known from groups 1 and 4 in that area. However, the sample size of native-range fish in haplogroups 1 and 4 is very small compared to that from the NWA (18 individuals *vs.* 157 in the NWA). More haplotypes can be expected to occur in the native-range areas occupied by haplogroups 1 and 4. Both NWA populations have a higher proportion of group 1 haplotypes: 56% of fish in the GoMx and 73% of Trinidad's fish. Six haplotypes are shared by > 90% of individuals in each of the GoMx and Trinidad populations, with similar proportions of fish with each of those six in the two populations and with four of those accounting for the great majority (77% in the GoMx and 86% in Trinidad) of haplotypes in both populations.

The GoMx population, with a total of 11 haplotypes, contains five unique, minor haplotypes not present in the Trinidad population, and the latter, with a total of 12 haplotypes, contains six unique, minor haplotypes not present in the GoMx population. Further, four of those minor haplotypes found in Trinidad and four in the GoMx have not been found in the native range, and haplogroups 1 and 4 in the IWP samples contain seven unique haplotypes not found in the two NWA populations. The six fish from Veracruz have four haplotypes, including three shared with both Campeche and Trinidad, and one individual with a Group 1 haplotype that is also found in Taiwan (Figure 1), but not in the Campeche or Trinidad samples. The six haplotypes found in Trinidad but not the GoMx include a Group 4 haplotype also found in Oman and Sri Lanka, but not in the GoMx samples. Thus, between them the two NWA populations expanded the IWP area over which their haplotypes are known to occur, west in the Indian Ocean to Oman, and north in the Pacific to Taiwan.

Pairwise  $\Phi_{ST}$  tests (considering Haplogroups 1 and 4 separately and combined in each case) detected no statistically significant differentiation between the Trinidad *vs.* GoMx populations, or between each of those populations *vs.* the native-range populations of those two haplogroups ( $\Phi_{ST}$  values range from 0.19 to 0.185,  $p > 0.05$  in all cases). Neutrality tests (Tajima's  $D$  and Fu's  $F_s$ ) were statistically insignificant for all NWA populations (Table 1) as they were for the native-range populations (Table 1 here; Robertson et al. 2018). The GoMx and Trinidad populations both showed a multimodal mismatch distribution, with a lack of statistical significance for both Harpending's raggedness index and the  $R_2$  statistic (Table 1). Thus, neither of the NWA populations showed evidence of having passed through a bottleneck due to a founder effect.

## Discussion

Given those similarities in genetic structure between the two NWA populations, the simplest explanation concerning their origins is that both resulted from introduction(s) from the same part of the broad stretch of

**Table 1.** Genetic diversity parameters for different haplogroups of *N. cyanomos* in two populations from the North-West Atlantic and from the Indo-West Pacific native range (see Figures 1, 2).

Genetic group	N	S	H	$\pi$	$h$	D	F <sub>s</sub>	R <sub>2</sub>	r
Gulf of Mexico Group 1	40	5	5	0.00291	0.6859	1.11	1.189	0.167	0.085
Trinidad Group 1	63	20	8	0.00422	0.73169	-1.47	0.678	0.071	0.094
Gulf of Mexico + Trinidad Group 1	103	22	10	0.0037	0.721	-1.561	-0.417	0.056	0.062
Native-range Group 1	13	8	8	0.00397	0.91026	-0.406	-3.114	0.113	0.065
Gulf of Mexico Group 4	31	7	7	0.00185	0.57204	-1.125	-2.413	0.076	0.061
Trinidad Group 4	23	4	4	0.00086	0.32016	-1.497	-1.445	0.115	0.196
Gulf of Mexico + Trinidad Group 4	54	8	8	0.00143	0.468	-1.4134	-3.663	0.053	0.13
Native-range Group 4	5	4	4	0.00309	0.9000	-0.4101	-1.195	0.213	0.13
Gulf of Mexico + Trinidad Groups 1+4	157	41	18	0.0171	0.818	NA	NA	NA	NA
Native-range: Groups 1+4	18	26	12	0.0156	0.948	NA	NA	NA	NA
Native-range: all four Groups combined	54	41	23	0.0162	0.918	NA	NA	NA	NA

N = sample size; S = no. polymorphic sites; H = no. haplotypes;  $\pi$  = nucleotide diversity;  $h$  = haplotype diversity; D = Tajima's D; F<sub>s</sub> = Fu's F<sub>s</sub> statistic (all values of D and F<sub>s</sub> were non-significant at  $p > 0.05$ ); R<sub>2</sub> = Ramos-Onsins and Rozas' R<sub>2</sub> statistic; r = Harpending's raggedness index; Native-range = Indo-West Pacific native range; NA, not applicable.

the native range occupied by haplogroups 1 and 4. Both these highly isolated populations in the NWA are found at the only two sites in the NWA with offshore oil and gas fields and haplogroups 1 and 4 are in IWP areas with large offshore oilfields. This combination provides support for the hypothesis that this species was introduced to the NWA by floating offshore infrastructure brought from the IWP (Robertson et al. 2018). However, the geographic area covered by the two haplogroups that contributed to the NWA populations is very large, spanning 8,000 km from Oman in the northwest Indian Ocean to Taiwan in the northwest Pacific. More precise identification of the origin of the NWA population in the IWP may be possible when larger sample sizes that establish the extent of local variation in genetic structure across a range of sites between those limits become available. Robertson et al. (2018) pointed out that the presence of both haplogroups off Gujarat, northwest India, where there is a large offshore oilfield, could be due to haplogroup 4 fish being carried by mobile oilfield infrastructure from the west Pacific to the Indian Ocean. If that is the case, it may never be possible to establish precisely the location(s) from which the NWA fish originated.

Given the very high densities of *N. cyanomos* that can be found on oilfield platforms (up to 100/m<sup>2</sup>) (Simoes and Robertson 2016) and sunken ships in the GoMx and its ability to live in wrecks with or without a cover of fouling organisms (Robertson et al. 2019), it seems quite possible that a single item of floating offshore infrastructure could have carried a sufficient number of individuals to establish a diverse population at the end of its voyage. It also seems possible that both NWA populations resulted from the same introduction. If that is the case, how were two such isolated populations established? Although this species was only discovered recently (2013) in the NWA, its abundance at both the GoMx and Trinidad indicates that both populations have been established for many years. Although the GoMx population occupies a large area, the current



geographic range of the Trinidad population seems to be distinctly smaller (Robertson et al. *in press*). Offshore infrastructure does sometimes stop off at Trinidad during voyages from the IWP to the GoMx (e.g., BPCLC 2011). Such connections indicate that it is possible that both populations were established concurrently by a single offshore vessel (ship or drilling rig) carrying large numbers of fish that stopped at both sites during the same voyage long enough to produce larvae that populated each area.

In each NWA population, about half the haplotypes are unique, uncommon haplotypes not shared with the other population. This difference may simply reflect an inadequate sampling of the NWA populations. The fact that, among the six fish collected at Veracruz, one had a haplotype not found in either the Campeche or Trinidad populations indicates that haplotype diversity in the GoMx may be even higher than is currently indicated and that the lack of sharing of uncommon haplotypes between the GoMx and Trinidad could well be a sampling artifact. However, the presence of a group of unique haplotypes at both GoMx and Trinidad could also indicate that those populations arose from separate introductions from the same general source area. The fact that there are large differences in the apparent sizes of the GoMx and Trinidad populations (Robertson et al. *in press*) is consistent with multiple introductions, at different times. Regardless of whether there were one or two introductions, the IWP source(s) have yet to be identified with any precision due to a lack of geographically widespread, intensive population sampling across the IWP areas occupied by haplogroups 1 and 4.

Another alternative is that one NWA population was established first then, subsequently, produced a secondary introduction to the other. Neither NWA population has a genetic structure indicating that its haplotypes are a subset of the other's. Thus, there is no genetic signal of directionality in movement between the two populations. As offshore infrastructure moves in both directions between the GoMx and Trinidad oil and gas fields (BPCLC 2011; Bloomberg 2016), any secondary introduction could have gone in either direction.

There is one case of identifiable directional carriage of a reef-fish from its native range in one of those Atlantic oilfield areas to the other. The chaenopsid tube-blenny *Protemblemaria punctata* (Cervigon, 1966) is a Caribbean reef-fish endemic to a small area of eastern Venezuela that includes the Paria Peninsula on the northwestern side of the Gulf of Paria (see Robertson and Van Tassell 2019) and islands on the Trinidadian side of the Gulf of Paria (iNaturalist 2016), within several km of the area where offshore infrastructure in transit between oilfields or storage is often "parked" (e.g., Bloomberg 2016; Robertson et al. *in press*). Like other reef-dwelling tube blennies, *P. punctata* lives closely associated with benthic reef organisms (worm tubes, barnacles) likely to accumulate on floating offshore infrastructure. A single individual of this species was collected

near Tampa, Florida, on the eastern side of the GoMx in 2017 (Schofield and Brown 2019). Prior to that collection this species had never been recorded in the extensively sampled GoMx, or anywhere between the GoMx and eastern Venezuela. CO1 sequences of that fish and conspecifics from Venezuela show that they are from the same population (B Victor, *pers. comm.* to DRR, December 2019). Further, in 2018 Argüelles-Jiménez et al. (2020) collected multiple individuals of this same species on reefs at Veracruz, on the western side of the GoMx. Thus, it is evident that *P. punctata* now has established populations in two areas with extensive offshore oil and gas field activity, and that it was introduced to the GoMx from its native range in the other area. This discovery supports the hypothesis that sufficient numbers of fish can be carried by floating offshore infrastructure from Trinidad to establish a population in the GoMx that, in the case of *P. punctata*, now spans the GoMx.

Another reef-fish candidate for possible transport from southern Caribbean offshore oil and gas fields to the GoMx is the blenniid *Hypsoblennius invemar* Smith-Vaniz and Acero 1980. This species lives in barnacle tests in very shallow water and is often encountered on offshore platforms. It is thought that the native range of this species likely was restricted to the southern Caribbean (and lesser Antilles) and Brazil and that it could have been carried from there to the GoMx (Schofield 2020). It was first found in the northern Gulf of Mexico beginning in the late 1970s, and now is a common inhabitant of oil platforms in that area, and on such platforms in the southwest GoMx (authors *personal observations*). However, it is unclear if it was naturally present in the GoMx but undiscovered there before that date due to its unusual habitat preference (Smith-Vaniz 1980). Genetic comparisons of northern and southern populations of this species might help resolve this issue. None of the information we have about *N. cyanomos* populations at Trinidad and the GoMx rules out the possibility of a Trinidad to GoMx secondary introduction in this species, and the fact that this most likely occurred in one, possibly two other species, indicates that such is a viable possibility.

The genetic results we have presented here are preliminary. They involve only one type of genetic marker and do leave many questions unresolved about the sequence(s) of events leading to the two isolated populations of non-native regal demoiselles becoming established in the NWA. However, those data do show that those populations, in the only two areas in the NWA with offshore oilfields, came from the same general source in the native range, and could be the products of a single introduction of fish likely carried there from the native range by oilfield infrastructure. Further genetic studies using a broader range of genetic markers and analytical techniques could help resolve some of the remaining issues. More comprehensive, quantitative sampling of populations of this species across the areas of the native range occupied by haplogroups 1 and 4 also is essential for answering many questions.

## Acknowledgements

We thank Solomon Baksh and Jonah McComie for diving support at Trinidad, and Kristin Saltonstall for comments on a draft of the ms. Thanks to two reviewers whose comments improved the ms.

## Ethics and Permits

Permission to collect and export specimens was provided by the Fisheries Division, Ministry of Agricultures, Lands and Fisheries, Government of the Republic of Trinidad and Tobago. Methods of capture and handling of live fish were approved by the Smithsonian Tropical Research Institute IACUC committee (2017-1107-2020-A1).

## Funding

Personal funds of the authors were used to conduct the fieldwork. Laboratory work at Universidad Michoacana de San Nicolás de Hidalgo was funded by the project CIC-UMSNH-2019.

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