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DNA barcoding of Nereididae polychaetes based on COI gene barcoding in intertidal shores of Bushehr and Bandar Abbas provinces, Iran

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Abstract

Although polychaetes are the most abundant organisms in marine ecosystems, still their genetic diversity is understood inadequately. In this study, molecular identification of Nereididae polychaetes was performed by sequencing a segment of mitochondrial COI gene, isolated from mitochondrial DNA, and comparing nucleotide divergence, Molecular taxonomy, interspecific and intraspecific relations of Nereid species among 4 intertidal stations assigned in Persian Gulf of Iran. Analysis of 109 identified specimens, revealed 78 provisional gene sequences, related to 9 species and 6 genera, in which interspecific divergence was 1.4 times higher than intraspecific divergence (2.82% versus 1.95%). The average pairwise sequence divergence for all sequences was estimated at 1.37%. In three cases maximum divergence within a lineage exceeded the minimum nearest-neighbor distance: *Perinereis* sp., *Platynereis* sp. and *Platynereis bicanaliculata* .Maximum species similarity was observed amongst 3 sampling sites assigned in Bushehr Province whilst Bandar Abbas`s specimens showed less similarity to Bushehr station. Out of the 109 COI gene sequences of Nereididae polychaetes in this study, 34 contained multiple lineages .These results support the assertion that many Nereid populations in the Persian Gulf previously thought of as a single species, actually consist of two or more divergent lineages.

Keywords: Polychaetes, Nereididae, COI, Barcoding, Persian Gulf

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Introduction

Although polychaetes are the most abundant marine organisms, however, their taxonomy based on molecular data is poorly studied, in contrast to other marine species with high ecological importance (Quijo´n and Snelgrove, 2005). Nereididae (Blainville, 1818) is a family of class Polychaeta (Annelida). Most members of this group are widely distributed in global marine systems ranging from the intertidal to abyssal depths (Dean, 2001), while a few of them also settle down in brackish or swim upstream to rivers even to land (Rouse and Pleijel, 2001). They are commercially and ecologically important because they can contribute as abundant food sources in the marine communities and improve the quality of benthic sediment as well as monitor pollution (Jiang and Liu, 2008).

Current taxonomy in Nereididae principally depends on morphological characters such as prostomium, palps, parapodium, jaws and so on (Rouse and Pleijel, 2001). The types and numbers of morphologic characters used and hypotheses on origination, vertical or horizontal evolutions both may generate different taxonomic results (Fauchald, 1977). In addition, color polymorphisms in polychaete species also make confusion in traditional taxonomy (Nygren *et al.,* 2011). Polychaetes like many other marine invertebrates include polymorphic planktonic larvae in early stages of their life cycle. Although polychaete larval dispersal is a passive kind in which the larvae are unable to control their horizontal movement (Chia *et al.,* 1984; Scheltema, 1986), however their ability to control vertical movement has a significant impact on polychaetes' distribution pattern because the water current speed varies with depth (Young, 1995; Metaxas, 2001). It is now recognized that traditional taxonomic approaches often overlook polychaete species (Westheide and Schmidt, 2003) or are confused by color polymorphisms (Nygren and Pleijel, 2010; Nygren *et al.,* 2011).

Genetic methods have revolutionized the systematics field, especially in organisms for which morphological taxonomy is tough. Molecular tools are increasingly recognized as necessary for delineating species boundaries, quantifying diversity, and clarifying distributions in understudied groups (Westheide and Schmidt, 2003; Worheide *et al.,* 2005; Witt *et al.,* 2006). DNA barcoding can be of significant help for taxonomical, ecological and biological studies, mainly for species identification in research on biological communities and biodiversity in general (Valentini *et al*., 2009). The relatively recent detection of sibling species with restricted ranges, detectable only with molecular tools, supports the use of an integrative taxonomic approach to species delineation and range determination in ocean environments (Knowlton, 1993, 2000).

To address this issue, many recent studies have examined variation in mitochondrial DNA (mtDNA) sequences and demonstrated that such analysis is valuable for the discrimination of closely related

polychaete (Glover *et al.,* 2005; Bastrop and Blank, 2006; Bleidorn *et al.,* 2006; Rice *et al.,* 2008; Barroso *et al.,* 2009; Nygren and Pleijel, 2010). mtDNA has been applied widely in the studies of phylogenetics and evolution as well as an effective marker to assist taxonomy because of its uniparental inheritance (in a majority of animal phyla), high evolutionary rate, lack of introns, large copy numbers in every cell, and limited recombination.

While other molecular markers such as single nucleotide polymorphisms (SNPs) and microsatellites (SSRs) have shown promise in polychaete genomics, for instance transcriptome sequencing (Mehr *et al.,* 2015), patterns of nuclear genetic variation (Rockman, 2012), or genomic analysis in relation to ocean acidification (Valvassori, 2017), the mitochondrial cytochrome c oxidase subunit I (COI) gene has been proposed as a DNA barcode and frequently used to recognize provisional species in groups with an incomplete taxonomy, and morphological, ecological, and behavioral differences are regularly detected upon further examination of divergent taxa (Carr *et al.,* 2011). The COI gene is a suitable barcoding gene and exhibits a greater degree of genetic distance between than within species (Glover *et al.,* 2005). Although several recent studies have focused on the discrimination of closely related polychaete species (Dahlgren *et al.,* 2000; Santos *et al.,* 2005; Bakken and Wilson, 2005), the systematics of Nereididae species by employing molecular data still has received few attentions.

It is necessary to distinguish the purposes of DNA barcoding from DNA taxonomy. DNA taxonomy includes the circumscription and delineation of species based on evolution and utilizes DNA sequences to identify and classify species (Vogler and Monaghan 2006), while DNA barcoding is a method of distinguishing known species by DNA sequence similarity and does not consider species identity.

Although a number of researches have stated that mitochondrial DNA is unsuitable to test for isolation by distance (Teske *et al*., 2018), several studies have suggested the efficacy of COI to discriminate polychaete species (e.g., Bleidorn *et al.,* 2006; Rice *et al*., 2008; Barroso *et al.,* 2009; Olson *et al*., 2009; Pleijel *et al.,* 2009; Brasier *et al*., 2017; Qian *et al*., 2018), no broad genetic investigation, geographic or taxonomic, has been undertaken in Persian Gulf region.

Currently there is no evidence regarding the amount of genetic similarity among Persian Gulf polychaetes, for there is a huge gap in molecular studies of marine organisms in this region. The aim of this study was to distinguish polychaete species by DNA barcoding, examine the genetic structure and connectivity of local Nereid polychaetes in Persian Gulf, in order to have a better understanding of interspecific - intraspecific relationships in morphologically identified Nereididae species.

Materials and methods

Specimens

A total of 56 specimens were collected in the intertidal zone or from nearshore coastal habitats using a quadrat of 50×50cm and a box corer of 0.025 m^2. Sediments were sieved under running tap water using a 0.5mm mesh-size sieve.

The polychaetes were obtained on May 2016 in four stations: Deylam Port (30°04'17.6"N, 50°08'35.7"E); Bushehr Port (28°56'25.3"N, 50°48'25.9"E); Dayyer Port (27°50'12.1"N, 51°57'04.6"E); and Bandar Abbas (27°11'04.8"N, 56°21'57.1"E) (Fig. 1).

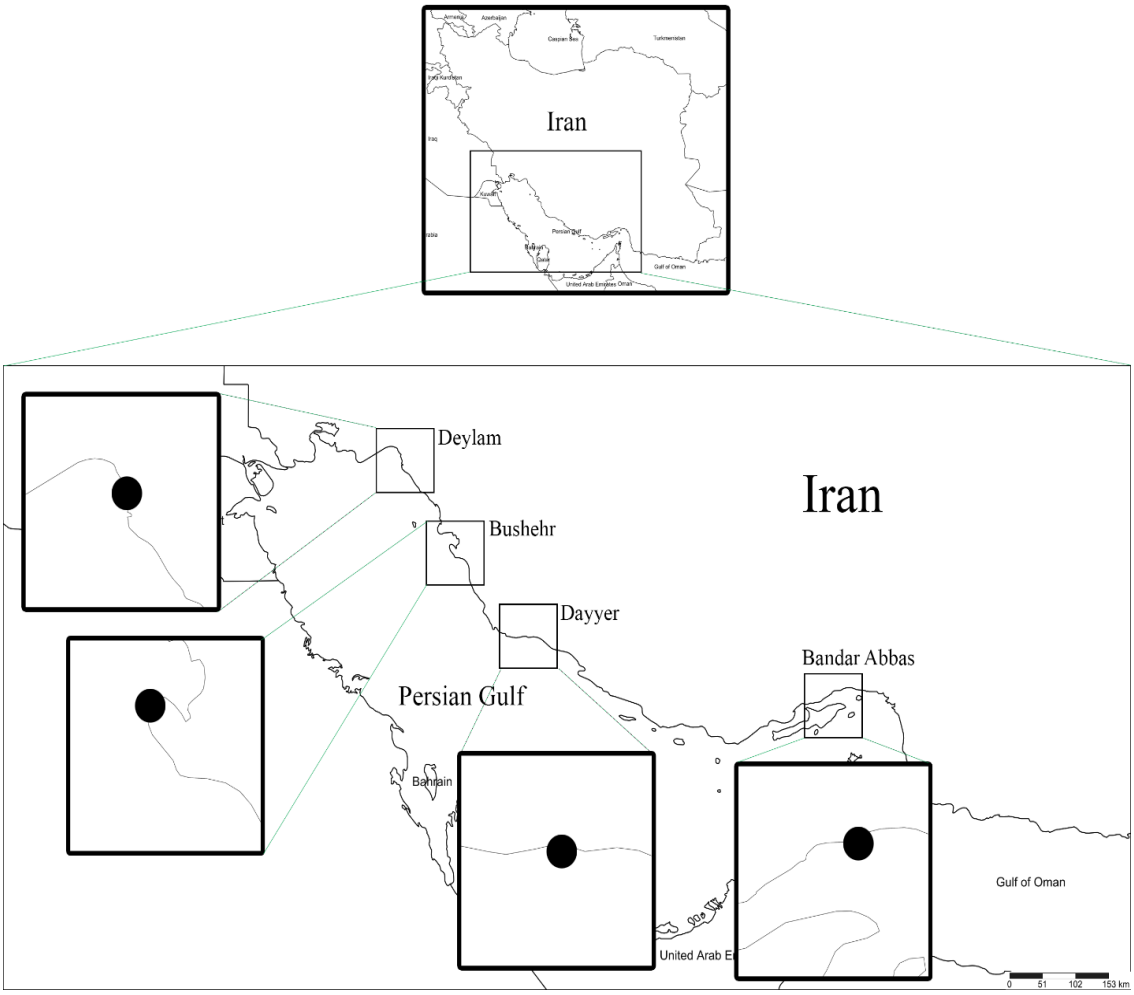

Figure 1: Sampling sites along intertidal shores of Persian Gulf.

Magnesium chloride diluted in 8% of seawater was added to specimens and fixed in 70% ethanol, which was replaced at least three times to prevent dilution. Samples were preserved in 95% ethanol and stored at 4°C (recent collections) or -8°C (older collections) until processed for DNA extraction (Brasier *et al.*, 2017).

In laboratory, after washing of specimens they were identified to the lowest practical taxonomic level (mostly genus) according to available references (Fauchald, 1977; Jones,

1986; Rouse and Fauchald, 1997; Rouse and Pleijel, 2001; Rozbaczylo *et al.*, 2005) and Nereididae polychaetes were picked out from other families. Specimens were morphologically analyzed using TUCSEN Dhyana 400DC CCD camera, attached to Olympus SZ6045 (Olympus, Japan) compound microscope and CETI stereo microscope.

Morphological traits of Family Nereididae include large elongate body. prostomium usually with 2 pairs of antennae and always with a pair of bi-articulate palps. Peristome with usually 4 but sometimes 3 pairs of tentacular cirri , eversible pharynx with a pair of jaws, some genera are armed with many chitinous paragnaths or papillae, while in several genera the pharynx is unarmed (Day, 1967). Parapodia uniramous for first two setigers then usually biramous but some genera are uniramous throughout. Most genera usually without branchiae/gills; where branchiae occur, they are usually branched and arise on the mid anterior segments of the body. Setae mainly compound, with both falcigers and spinigers (Ahmad Al-Omari, 2011).

DNA extraction and amplification

The extraction of genomic DNA was carried out using standard CTAB 2X protocol by means of digestion with proteinase K (Sinaclon Ltd.), extraction with chloroform and precipitation with absolute ethanol described by Chen *et al*. (1995). A ~815bp fragment of the COI gene was amplified using the universal primers LCO1490 and HCO2198 from Folmer *et al*. (1994) (Table 1). All polymerase chain reactions (PCR) were carried out in a 12.5 µl volume, containing 6.25 µl 10% trehalose, 2 µl ddH20, 1.25 µl 10× PCR buffer, 0.625 µl $MgCl_2$ (50 mM), 0.25 µl of each primer (10 µM), 0.0625 µl dNTPs (10 mM), 0.06 µl Platinum Taq polymerase, and 2 µl of DNA template (10–50 ng). The amplification was carried out in a PCT- 200 thermal cycler (MJ-Research® GMI Inc.) with the following cycling conditions: 94°C for 1 min, five cycles at 94 ^{o}C for 40 s, 45°C for 40s, 72 ^{o}C for 1 min, and 35 cycles at 94 ^{o}C for 40 s, 51 ^{o}C for 40 s, 72 ^{o}C for 1 min, and final extension for 5 min at 72 ^{o}C.

A total of 131 PCR products were visualized in 1–1.5% agarose gels and vacuum purified using 96-well Millipore Multiscreen® plates and sequenced at the Macrogen® Company (Korea) on an ABI 3730XL DNA Analyser (Applied Biosystems). Chromatograms were visualized, edited, and assembled using ChromasPro v4.7 (Technelysium) and aligned by Clustal W method in MEGA 7.0. (www.megasoftware.net) Sequences that were obvious pseudogenes (i.e. frameshifts, not in full-length) or contaminants (following BLAST-type searches) were removed from the collection.

Table 1: Primers applied in this study.

Primer Name	Sequence (5'-3')	Annealing Temperature
polyLCO (F)	GAYTATWTTCAACAAATCATAAAGATATTGG	48-55 ^{o}C
polyHCO (R)	TAMACTTCWGGGTGACCAAARAATCA	48-55 ^{o}C

Phylogenetic analysis

The dataset was analyzed by parsimony, using PAUP, version 4.0b8 (Swofford, 2001). The outgroup was selected from the same order. Bootstrap values (Felsenstein, 1985) were determined from 1000 replicates subject to full heuristic searches with random taxon addition, holding one tree per step, and keeping all most-parsimonious trees to provide measures of relative clade support. Tree searches were followed by subtree pruning and regrafting (SPR) and tree bisection and reconnection (TBR) branch swapping and continuing with multiples rounds of tree fusing (Goloboff, 1999). Our sequences were compared to a database of 75 selected species from GenBank (www.ncbi.nlm.nih.gov/genbank). Taxa related to specimens collected were marked alphabetically (i.e. *Perinereis nuntia* A) and taxa from GenBank were included with accession number (i.e. JX392055.1 *Nereis denhamensis*).

The Maximum Parsimony phylogenetic tree was drawn utilizing the Subtree Pruning Regrafting (SPR) algorithm with search level 1 in which the initial trees were chosen by the random joining of sequences with 1000 replicates. Transition-transversion ratios were weighted in accordance with the model of sequence evolution supported by the result of the model test. *Sigambra magnuncus* (Pilargidae) was used as the outgroup. Bootstrap values (Felsenstein, 1985) were determined from 1,000 replicates subject to full heuristic searches with random taxon addition to providing measures of relative clade support. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated.

Statistical analysis

Bray–Curtis similarity indices were used to provide an overview of the similarity in species composition between collection sites with a dendrogram. The K2P (Kimura two-parameter) distance metric was chosen for consistency and comparability with other barcode studies and for its suitability when sequence divergences are low, such as for the construction of intra- and interspecies phylogenies (Nei and Kumar, 2000; Hebert *et al.,* 2003). To determine whether the number of individuals analyzed affected the level of variation within genetic clusters, a linear regression was performed. To examine differences in provisional and identified species, individual-based rarefaction curves were generated separately for identified species and for provisional species using the software Estimate S v.8 (Colwell, 2009) with 100 randomizations and sampling without replacement. To provide an overview of the relativity in species

composition between collection sites, Chao's Abundance-based Sørensen similarity index (Sørensen, 1948; Chao *et al.*, 2005) was calculated.

Results

Altogether 256 forms of polychaetes were sampled during the study. In total 212 specimens belonging to Family Nereididae were identified morphologically and separated for further analysis. Polychaetes belonging to genus *Perinereis* were the most dominant species (n=50) while other species belonging to 6 genera *Platynereis* sp. (n=29), *Nereis* sp. (n=16), *Pseudonereis* sp. (n=9), *Laeonereis* sp. (n=3) and *Neanthes* sp. (n=2) have also been identified.

Molecular identification

This study involved the analysis of 131 COI sequences, which 22 sequences were related to polychaetes and 109 sequences were related to polychaetes and 22 sequences were related to organisms unrelated to this study and were removed from sample set. 78 sequences were identified to 9 described species and 31 to genus level (*Perinereis* sp., *Perinereis aibuhitensis*, *P. brevicirris, P. cultrifera, P. nuntia, Platynereis* sp., *P. bicanaliculata, P. dumerilii, Nereis* sp., *N. denhamensis, N. zonata, Laeonereis culveri, Neanthes* sp. and *Pseudonereis* sp.). In total 78 provisional Nereid species, representing 23 taxa at genera level and 55 taxa at species level resulted from this analysis. The average COI sequence size was 800 bp, with no stop codons, deletions or insertions.

Phylogenetic analysis

The evolutionary history was inferred using the Maximum Parsimony method (Swofford, 2001). Tree #1 out of 10 most parsimonious trees (length=3047) is shown (Fig. 2). The consistency index was 0.581542, the retention index was 0.944360, and the composite index was 0.561762 for all sites. Phylogeny was tested by Bootstrap method and each node's bootstrap value is placed adjacent to it.

The parsimony tree showed paraphyly of *Perinereis* sp., as *Perinereis* sp. D, E from Bushehr and F from Deylam formed a clade together with strong bootstrap support (100%) while *Perinereis* sp. A from Bushehr and B, C from Deylam formed a sister clade (98%). Both clades above were monophyletic to a clade from the Indian Ocean with accession numbers KX525498.1, KX525499.1, and KX525500.1 (100%). A polyphyletic third clade included *Perinereis* sp. G from Bushehr, H from Bandar Abbas and I from Dayyer (100%) together.

A monophyletic *P. nuntia* including *P. nuntia* A-M from all sampling stations in one clade and *P. nuntia* C, E from Dayyer in a sister clade yielded high support (98%). Both clades showed monophyletic relationship with *P. nuntia* from Australia by accession number JX420257.1 (100%).

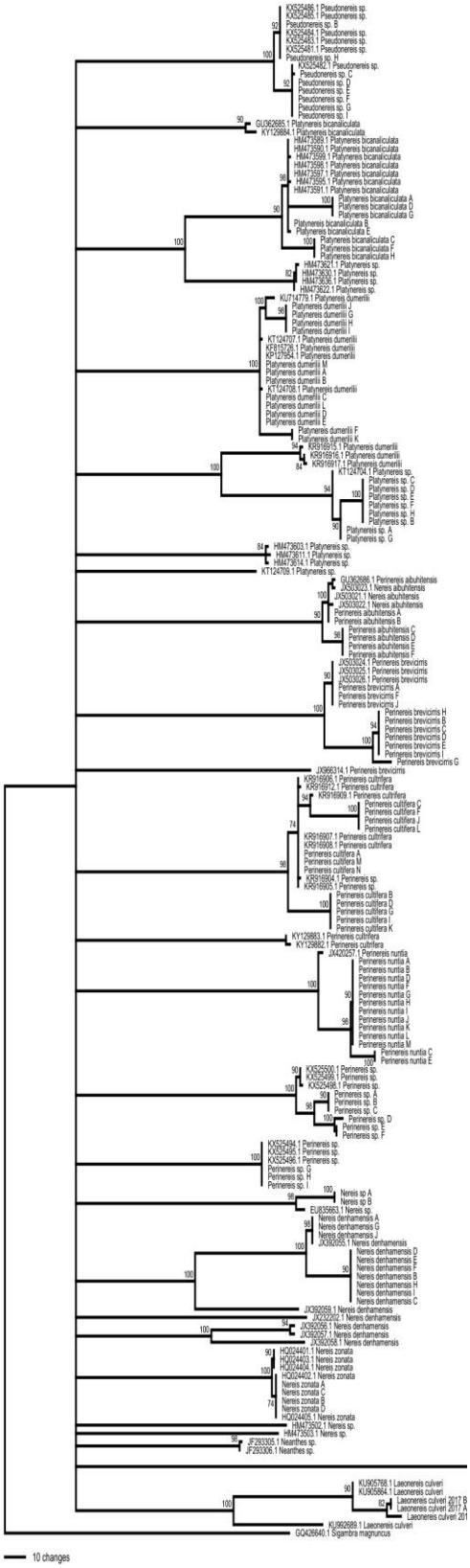

Figure 2: Maximum parsimony phylogenetic tree reconstruction based on Subtree-Pruning-Regrafting (SPR) method. Phylogenetic relationship for 109 Nereididae specimens in this study together with GenBank taxa has shown.

All *P. cultrifera* B, D, G, I, K collected from Dayyer station formed a clade with significant support (100%). Also, *P. cultrifera* C, L from Deylam site showed sister relationship with *P. cultrifera* F, J from Bushehr station with strong bootstrap support (100%). *Perinereis cultrifera* A, M, N from Bushehr joined samples from European waters by accession numbers KR916904.1, KR916905.1, KR916907.1 and KR916908.1 (74%). No evidence has been found for the monophyly of our samples and specimens from China by accession numbers KY129882.1 and KY129883.1. All clades including sampled *P. cultrifera* clustered together with significant support (98%).

The inclusion of *P.aibuhitensis* in a cluster is also well supported (90%), as *P. aibuhitensis* A, B from Deylam and Bushehr sites formed a clade (100%) while *P. aibuhitensis* C, D, E, F from Bushehr, Dayyer and Deylam stations formed a sister clade (98%). All specimens were clustered together with GenBank taxa from South Korea by accession numbers JX503021.1, JX503022.1, JX503023.1 and GU362686.1 from China (90%).

The cluster of *P. brevicirris* clades yielded high support (100%) as *P. brevicirris* A, F, J from Bushehr station together with GenBank taxa from South Korea by accession numbers JX503024.1, JX503025.1 and JX503026.1 formed a clade (90%) and *P. brevicirris* B, C, D, E, H, I from Dayyer and Bandar Abbas stations in a sister clade (94%). However, *P.*

brevicirris G from Deylam formed a single sister clade (100%).

A monophyletic *Platynereis* sp. including two clades was significantly supported (100%), as *Platynereis* sp. A, G from Bandar Abbas and Bushehr were in a single clade (90%) while other *Platynereis* sp. samples from Bushehr and Deylam stations formed a sister clade (100%). Both clades showed monophyly with GenBank's *Platynereis* sp. from Italy by accession number KT124704.1 (94%). However, none of the samples had a monophyletic or polyphyletic relationship with other GenBank taxa from Italy and Canada.

The inclusion of *P. dumerilii* in a cluster was highly supported as three clades clustered together (100%). *P. dumerilii* G-J from Dayyer station formed a single clade (98%), while *P. dumerilii* A-E, L, M from Deylam and Bushehr were in a sister clade (100%). *P. dumerilii* F, K from Bandar Abbas formed a single sister clade (100%). All three clades were monophyletic to *P. dumerilii* from Spain by accession number KU714779.1 (100%).

The polyphyly of *P. bicanaliculata* yielded high support (100%), as *P. bicanaliculata* A, D, G from Bushehr site formed a single clade (100%) which was monophyletic to the second clade including *P. bicanaliculata* B, E from Bushehr and Bandar Abbas (98%) and GenBank taxa from Canada by accession numbers HM473589.1, HM473590.1, HM473591.1, HM473595.1, HM473597.1, HM473598.1, HM473599.1. Also, *P. bicanaliculata* C, F, H from Dayyer were in a single clade polyphyletic to other clades (100%). However, None of samples showed a monophyletic or polyphyletic relationship with other GenBank taxa from China by accession numbers GU362685.1 and KY129884.1.

The phylogenetic tree indicated polyphyly of *Nereis* sp., as *Nereis* sp. A, B from Dayyer and Deylam stations formed a clade together with strong support (100%) which was monophyletic with GenBank's *Nereis* sp. from Australia by accession number EU835663.1 (98%). However, none of the specimens formed a polyphyletic or monophyletic relationship with those from Canada by accession numbers HM473502.1 and HM473503.1.

A monophyletic *Nereis denhamensis* including two clades yielded high support (100%), as *Nereis denhamensis* A, G, J from Bandar Abbas, Bushehr and Dayyer formed a clade together with GenBank taxon from Australia by accession number JX392055.1 (98%). Other *N. denhamensis* samples from Bushehr, Deylam and Bandar Abbas were in a sister clade (90%). However, none of the samples had a monophyletic or polyphyletic relationship with other GenBank taxa from Australia.

The inclusion of *Nereis zonata* in a cluster was highly supported (100%) as all specimens from Deylam and Bandar Abbas together with taxa from Canada by accession numbers HQ024402.1 and HQ024405.1 formed a single clade (74%). However, other Canadian taxa by accession numbers HQ024401.1, HQ024403.1 and HQ024404.1 were placed in a sister clade with high support (90%).

The cluster of *Pseudonereis* sp. yielded high support (100%), as *Pseudonereis* sp. C, D, E, F, G, I from Deylam, Dayyer and Bandar Abbas sites together with GenBank taxon from India by accession number KX525482.1 formed a single clade (92%) which was monophyletic to the second clade including *Pseudonereis* sp. B, H from Bushehr and other taxa from India by accession numbers KX525481.1, KX525483.1, KX525484.1, KX525485.1 and KX525486.1 (92%).

Monophyly of *Neanthes* sp. was highly supported (100%), as *Neanthes* sp. A, B from Bandar Abbas station formed a clade together with GenBank taxon from India by accession number JX866607.1 (100%).

A monophyletic *Laeonereis culveri* including a single clade significantly supported (100%), as *Laeonereis culveri* A, B from Deylam were in a single clade while *L. culveri* C from Bandar Abbas station formed a sister clade (82%). Both clades showed monophyly with GenBank's *Laeonereis culveri* from the USA by accession numbers KU905768.1 and KU905768.1 (90%). Also, the cluster including clades above was monophyletic to a clade with single GenBank taxon from the USA by accession number KU992689.1 (100%).

COI gene divergence

Mean COI Kimura two-parameter (K2P) sequence divergence between congeneric clusters was 1.4 times higher than within-cluster variation (2.82% and 1.95%, respectively) (Figs. 3 and 4). The average pairwise sequence divergence for all sequences was estimated 1.37%. In total, 78 provisional species representing 4 genera and 8 species resulted from analysis of 109 specimens. Among sampling stations, the highest number of provisional species were discovered in Dayyer, Deylam, Bushehr and Bandar Abbas (29.48%, 26.92%, 24.35% and 19.23%, respectively). The ratio of provisional species to species with 100% likelihood to GenBank database (homologue species hereafter) was 1:27.

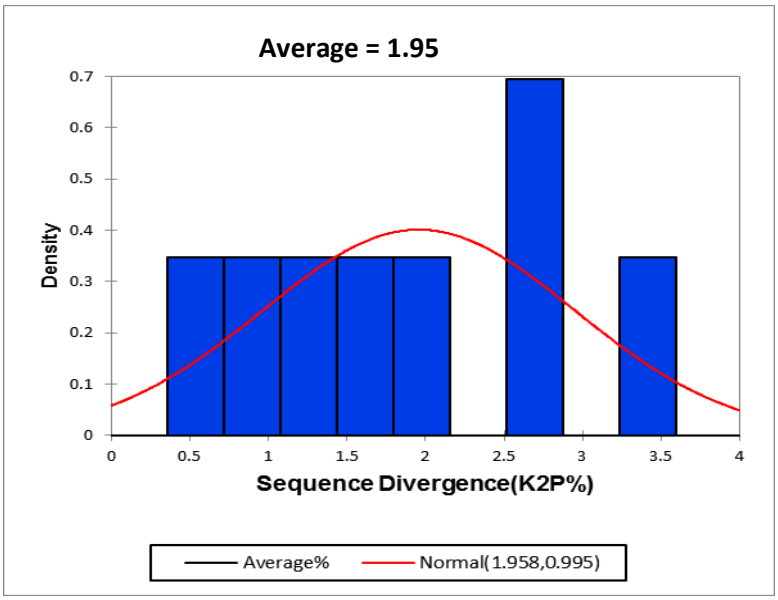

Figure 3: K2P distances for the barcode region within species.

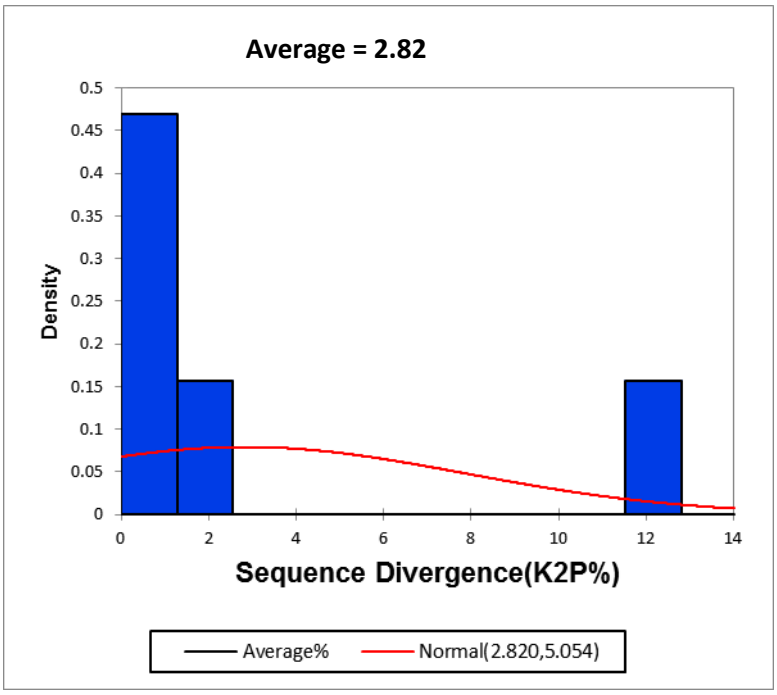

Figure 4: K2P distances for the barcode region within genera.

Rarefaction curves were generated to compare species richness of provisional and homologue species (Figure 5). The provisional barcode curve showed a higher slope than the curve for homologue species, with significant divergence apparent after 15 specimens were sampled.

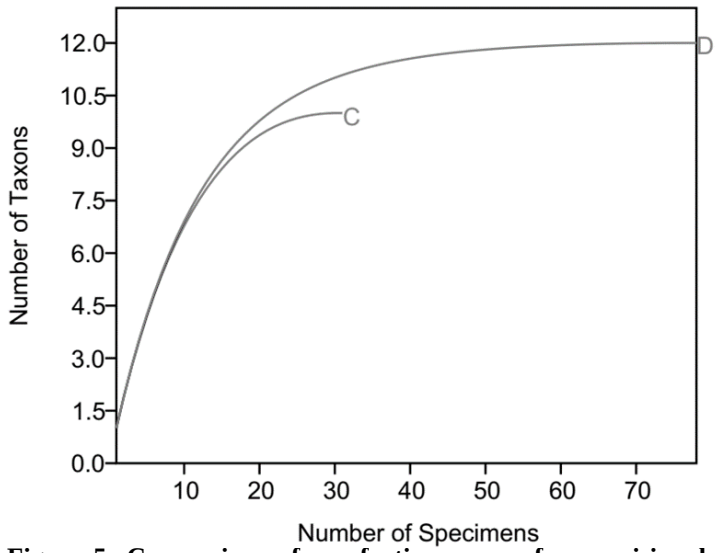

Figure 5: Comparison of rarefaction curves for provisional species (D) and homologue species (C).

The average number of individuals analyzed per cluster was 3.4 (range of 1–11), but 2 out of 31 were represented by a single specimen. The number of individuals analyzed per cluster affected the average within-cluster variation (Fig. 6A); Also, maximum within-cluster variation increased with the number of individuals analyzed per cluster, though most divergences were under 4% (Fig. 6B). The divergence within homologue species was between 15-30%. Most specimens formed two or more clades, in which the maximum K2P distance within clusters ranged between 0-3.6%. None of the specimens shared a barcode cluster with different species.

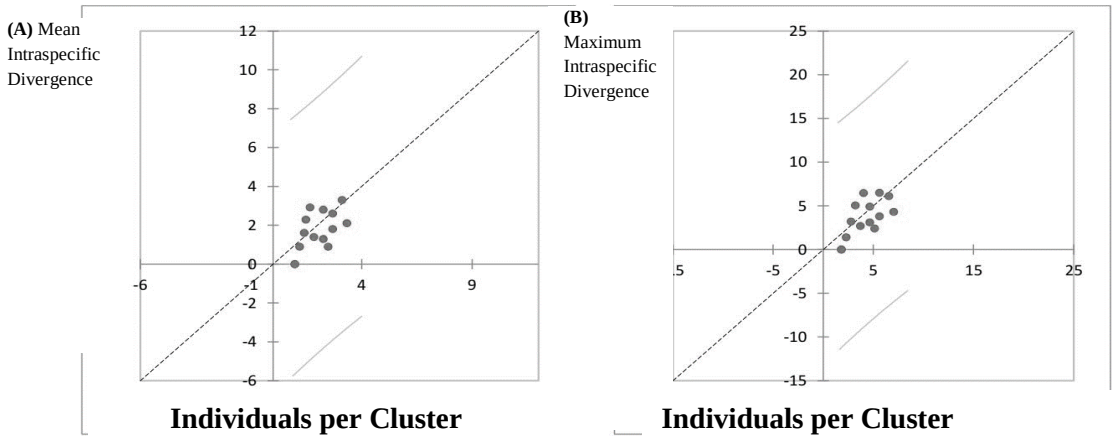

Figure 6: The effect of sample size on genetic distances. Linear regression of the number of individuals in each barcode cluster versus maximum intraspecific distance (A) and mean intraspecific distance (B).

Based on Bray-Curtis similarity indices, the highest biological similarity occurred between Bushehr and Dayyer stations, while lowest congruence was between Dayyer and Bandar Abbas sites (Fig. 7). The sites in Deylam and

Bandar Abbas formed a separate branch, so based on species composition, stations can be divided into three categories: Bushehr + Dayyer, Deylam, and Bandar Abbas.

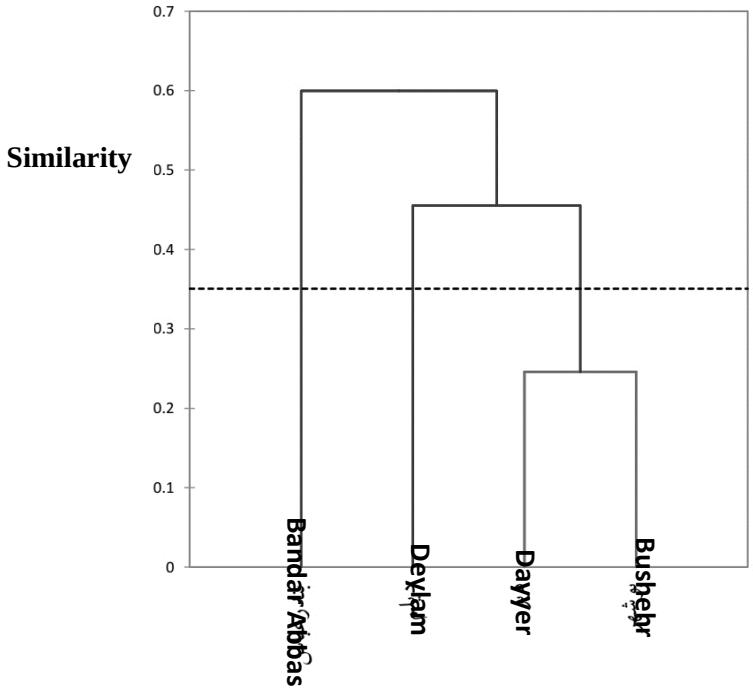

Figure 7: Hierarchical cluster analysis classification of the four sampling stations based on species composition by Bray-Curtis similarity index.

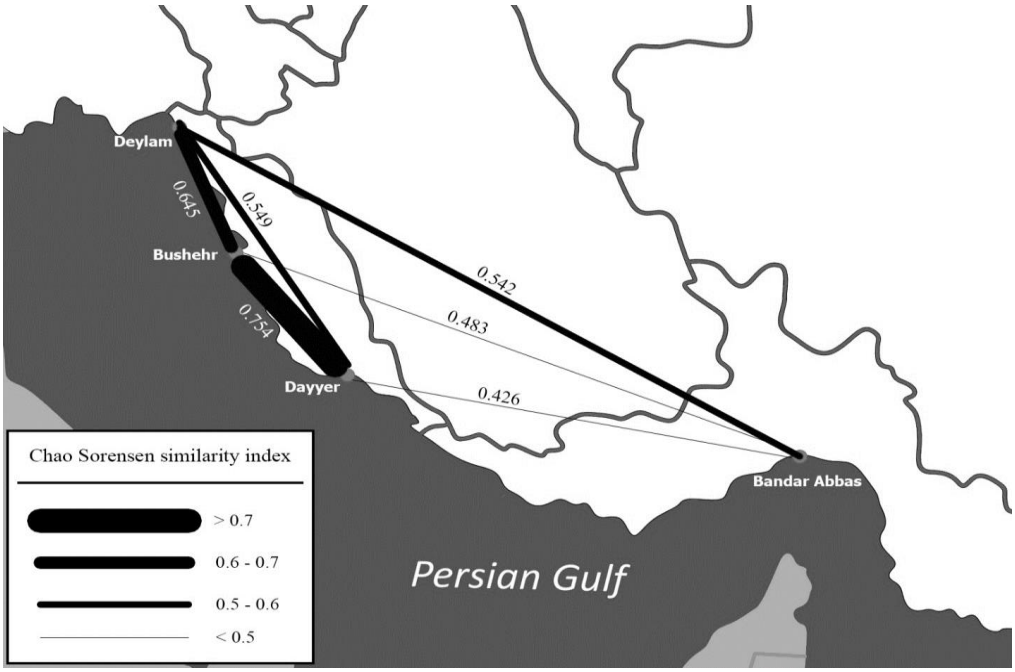

Figure 8: Faunal connectivity among sampling sites. The similarity in the species composition among seven regions measured by Chao Sørensen similarity index. Similarity values are represented by line width between two regions.

Connectivity patterns among stations in terms of species composition based on Chao-Sorensen similarity index indicated that the highest similarity in species composition was observed between Bushehr and Dayyer (0.754), followed by Bushehr and Deylam (0.645), Dayyer and Deylam (0.549), Deylam and Bandar Abbas (0.542), Bushehr and Bandar-Abbas (0.483) and Dayyer and Bandar Abbas (0.426) (Figure 8).

Discussion

The present study demonstrates the effectiveness of DNA barcoding as a tool for species identification in polychaetes specifically in Nereididae. Out of the 212 species morphologically identified in this study, 78 taxa have reported for the first time. Although named species often included more than one provisional species, clusters were easily identifiable and typically highly divergent from other clusters. The highest observed within-cluster divergence was 3.5% in *P. cultrifera*, indicating that barcodes naturally form tight clusters with low variation. Levels of intraspecific divergence described here are compatible with results determined in other polychaete researches (Chevaldonne *et al.*, 2002; Glover *et al.*, 2005; Bastrop and Blank, 2006; Jolly *et al.*, 2006; Rice *et al.*, 2008; Sekar *et al.*, 2016; Brasie *et al.*, 2017). Moreover, the 1.4-fold higher mean sequence divergence between than within clusters and the rarity of intermediate divergences (Figs. 3 and 4) suggest that COI barcodes have high discriminatory power for polychaetes. However, this study also revealed sibling taxa that require more detailed investigation. For instance, in three cases maximum divergence within a lineage exceeded the minimum nearest-neighbor distance: *Perinereis* sp., *Platynereis* sp. and *P. bicanaliculata*. Such a high divergence rate within a single lineage might reflect the isolated populations in the Persian Gulf. Recent origins coupled with complex historical distributions make such species particularly difficult to diagnose (Knowlton, 2000; Herbert *et al.*, 2003). Indications of ecological divergence were detected between provisional species delineated in this study. For example, habitat specialization was apparent in a number of taxa. For instance, *P. nuntia* was found in Bandar Abbas but there was no sign of *P. cultrifera* in this region. Similar results on *Eteone* and *Ophelia* (Syllidae) are reported by Carr *et al*. (2011). This divergence is the result of reproductive isolation among populations of species. For example, a research by Rice *et al*. (2008) suggests that lineages of the *Polydora cornuta* complex are reproductively isolated. Also, other studies have indicated that members of the *Hediste japonica* complex show differences in life history (Sato and Masuda, 1997), and sympatric sibling species of the *P. ciliata* complex show ecological differences (Manchenko and Radashevsky, 1993).

Divergent clusters within a species were evident in the absence of geographic barriers (e.g., *P. bicanaliculata* in Bushehr). Similarly, six lineages in the *Harmothoe*

imbricata complex have come into secondary contact in the central Canadian Arctic, but remain distinct (Hardy *et al.,* 2011). Whether these relatively young provisional species will continue on separate evolutionary trajectories or eventually merge as a result of introgressive hybridization requires further analysis of populations in zones of sympatry.

Recent studies of polychaete species recorded from the Persian Gulf have indicated that they occur in all regions of this water basin (Nikouyan and Savari, 1999; Salehi Farestani *et al.*, 2010; Ahmad Al-Omari, 2011; Soleimanirad *et al.*, 2014; Mooraki, 2014; Baghernejad *et al.*, 2015; Darya *et al.*, 2016; Bonyadi-Naeini *et al.*, 2017). However, high divergence within several of these widespread species (Figs. 3 and 4) suggests that true diversity may be much more than previously reported. Most species examined in this study contained multiple provisional species with enough genetic divergence to designate them as provisional species (Fig. 8).

The Persian Gulf region is a probable habitat for Indian Ocean taxa, for the high connectivity with oceanic waters suggests that the Indian Ocean was an important source for today's Persian Gulf species. Indeed, many species in this study have been discovered in the Persian Gulf were also reported in the Indian Ocean and other oceanic habitats. Among sampling stations, the highest overlap in species composition was observed between the Deylam, Dayyer and Bushehr sites, where Bandar Abbas site was almost separated from others. However, connectivity between Bandar Abbas and Deylam was higher than Bandar Abbas – Dayyer and Bandar Abbas – Bushehr. For instance, *Laeonereis culveri* and *Nereis zonata* were solely found in Bandar Abbas and Deylam. Although results suggest that genetic similarity of Nereididae polychaetes found in Bushehr, Deylam, and Dayyer is higher in comparison to Bandar Abbas, but in total 6 taxa of 14 identified taxa were revealed in all regions (42.8%). Such correlation might be related to the proximity of the sampling regions.

This study corroborates the divergence of closely located benthic populations, noted by Ushakov (1965) who reported a biogeographic division between the Bering Sea and British Columbia coasts. Since polychaete larvae are free swimmers and capable of migration between water basins (Chia *et al.*, 1984; Scheltema, 1986; Young, 1995; Metaxas, 2001), the anticlockwise water circulation present in the Persian Gulf may have assisted the dispersion of Nereid larvae among regions under study. Marko (2004) in a study on disparate patterns of benthic marine gastropods suggests that dispersion of larvae plays a critical role in species distribution of gastropods (Marko, 2004). In another research by Bush (2006) on identification and migration patterns of Cirratulidae polychaetes in Panama, highlights the impacts of climatic factors such as precipitation and water flow on polychaete larval dispersion. However, Bush (2006) also notes the significant impact of ecological factors (e.g. pH,

temperature and dissolved oxygen) which requires further research in Persian Gulf region.

Molecular assessment of Nereid polychaetes suggests high connectivity patterns between sampling stations (Fig. 7). Such correlation may indicate that polychaetes in these regions might have experienced relatively analogous evolutionary process.

As Nereididae larval dispersion pattern in subtidal waters is the main impacting factor on their gene flow, and because this study is focused on Nereids' molecular taxonomy in intertidal zone and thus was unable to clarify the impacts of gene flow pattern, another research on proportion of genealogies with significant or non-significant clustering under a model plotted against a scaled migration rate is strongly suggested.

Out of the 109 COI gene sequences of Nereididae polychaetes in this study, 34 contained multiple lineages representing nearly three times as many provisional species. This study's results suggest that broad application of DNA barcoding can accelerate the recognition and description process of many currently undescribed polychaete species. Results also support the assertion that many Nereid populations in the Persian Gulf previously thought of as a single species, actually consist of two or more divergent lineages, and further support that Nereis populations in Bushehr province (Bushehr, Deylam and Dayyer ports) are closely related to each other, while Bandar Abbas`s Nereids are less analogous. However, the presence of most identified polychaetes in all regions under study, emphasizes on Persian Gulf water circulation's positive impact on polychaete larval distribution and thus creating a relatively homogeneous Nereididae communities in this region.

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