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Limited ecological population connectivity suggests low demands on self-recruitment in a tropical inshore marine fish (*Eleutheronema tetradactylum*: Polynemidae)

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Abstract

The diversity of geographic scales at which marine organisms display genetic variation mirrors the biophysical and ecological complexity of dispersal by pelagic larvae. Yet little is known about the effect of larval ecology on genetic population patterns, partly because detailed data of larval ecology do not yet exist for most taxa. One species for which this data is available is *Eleutheronema tetradactylum*, a tropical Indo-West Pacific shorefish. Here, we use a partial sequence mitochondrial cytochrome oxidase subunit 1 (COI) marker and five microsatellite loci to survey the genetic structure of *E. tetradactylum* across northern Australia. Structure was found throughout the range and isolation by distance was strong, explaining approximately 87 and 64% of the genetic variation in microsatellites and mtDNA, respectively. Populations separated by as little as 15 km also showed significant genetic structure, implying that local populations are mainly insular and self-seeding on an ecological time frame. Because the larvae of *E. tetradactylum* have lower swimming performance and poor orientation compared with other tropical fishes, even modest larval abilities may permit self-recruitment rather than passive dispersal.

Keywords: Australia, hermaphroditism, Indo-Pacfic, pelagic larvae, Polynemidae

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Introduction

Populations of most marine organisms are often described as discrete adult subpopulations that exchange migrants through a dispersive pelagic larval phase. The degree to which these populations mix through larval migration is referred to as population connectivity and is a rigorously studied topic in marine biology. There is a mounting effort to study marine connectivity because an awareness of the way marine populations exchange migrants is essential for the proper management of fisheries and the conservation of marine ecosystems (Botsford *et al.* 2001; Palumbi 2003; Rocha *et al.* 2007; Jones *et al.* 2009).

In recent years, efforts to study marine population connectivity have relied heavily on molecular approaches (Jones *et al.* 2009). Inferring population connectivity indirectly from molecular markers (genetic connectivity) has been widely used in many terrestrial and aquatic taxa but is particularly advantageous for marine organisms because: (i) the amplitude and scale of dispersal in marine organisms are potentially great (Waples 1998), (ii) the pelagic propagules of most marine organisms are too small and numerous to directly track from spawning to settlement in the wild (Hellberg 2007; Selkoe *et al.* 2008), (iii) the barriers that spatially limit the connectivity of marine populations are often

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not obvious to human observers (Palumbi 1994; Rocha *et al.* 2007; Selkoe *et al.* 2008).

Molecular studies have revealed a diversity of geographic scales of genetic connectivity among different marine taxa. Some species appear to be effectively panmictic throughout considerably large geographic ranges (Lessios *et al.* 2003; Klanten *et al.* 2007; Horne *et al.* 2008; Reece *et al.* 2010), while others exhibit genetic heterogeneity at small scales (Barber *et al.* 2006; Gerlach *et al.* 2007) and at a variety of intermediate scales (Drew & Barber 2009; van Herwerden *et al.* 2009a). Additionally, similar species may have dispersal regimes that are quite different (Horne *et al.* 2008; Eble *et al.* 2009; Crandall *et al.* 2010; Gaither *et al.* 2010), and both dispersal and self-recruitment seem demographically significant for some species (Jones *et al.* 2009; Planes *et al.* 2009).

Notwithstanding the utility of molecular markers for investigating marine population dynamics, there are several instances where the information provided by genetic patterns is limited. For example, when it comes to understanding patterns of migration in ecological time, many molecular inferences are unable to distinguish recent migration from historical connectivity (Palumbi 2004; Hellberg 2007; Hedgecock *et al.* 2007; Hellberg 2009). One exception to this is when migration between populations is extremely low. In this case, strong geographic variation, such as in mtDNA, may indicate a lack of recent migration (Avise 2000), and certain multilocus analyses are able to quantify migration within the time frame of a few generations (Wilson & Rannala 2003; Faubet *et al.* 2007). Parentage analysis is another way around this problem (Jones *et al.* 2009) but requires large numbers of unlinked loci and extensive sampling and may not be feasible for many marine species.

Molecular data are also less informative if it is unable to be interpreted in an ecological or demographic context supplied from other types of research (Waples 1998; Hedgecock *et al.* 2007; Selkoe *et al.* 2008; Lowe & Allendorf 2010). Even when migration is low, failure of marine populations to exchange genes may be attributed to several unknown factors (Swearer *et al.* 2002; Palumbi 2004; Choat 2006; Connover *et al.* 2006; Gerlach *et al.* 2007; Marshall *et al.* 2010). Yet, relatively few marine species have been sufficiently studied in demographically or ecologically meaningful ways that can be used to provide context for genetic connectivity studies.

Here, we present a genetic survey of the blue threadfin, *Eleutheronema tetradactylum* (Polynemidae), an inshore marine fish, across its north Australian distribution using a mitochondrial marker and five microsatellite loci developed for this study. *E. tetradactylum* is a widespread tropical Indo-West Pacific shorefish that prefers shallow turbid water and soft substrates and is found in a variety of near-shore habitats along the coast of tropical Australia. Most of what we know about the biology of *E. tetradactylum* is summarized by Motomura (2004). Like most polynemids, *E. tetradactylum* is a protandrous hermaphrodite, becoming female generally after 2 years, with a maximum lifespan of about 7 years and attain lengths of >1 m. The location of spawning is unknown in this species but both eggs and larvae are pelagic, suggesting a high dispersal potential. There are no data on the pelagic larval duration (PLD) of *E. tetradactylum* in the wild, where the larvae reach a maximum length of 30 mm (Motomura 2004). Based on unsubstantiated information provided to JM Leis (personal communication) by aquaculture farmers in Taiwan, in culture this species can reach 20–21 mm within 16–27 days. Yet, how well this approximates natural PLD is uncertain. This species is also a commercially important food fish that is harvested in high numbers between Kuwait and northern Australia (Motomura 2004) but knowledge about the stock structure is needed for proper management of this fishery (Welch *et al.* 2002). Our results show that dispersal is sufficiently low in this species that some inferences may be made about levels of ecological connectivity, which are most relevant to management concerns (Palumbi 2003; Jones *et al.* 2009).

Apart from other motivations for studying *E. tetradactylum*, this is also one of the few marine species for which detailed studies of ontogenetic larval swimming performance and larval behaviour are available (Leis *et al.* 2007, 2009), which are pertinent to genetic patterns of connectivity because they are directly related to dispersal. Marine dispersal is a complex biophysical process, and the consequences of larval abilities and behaviour on dispersal outcomes are mostly unknown (Leis 2006). Generally, however, larvae with lesser swimming and sensory abilities are thought to be less likely to self-recruit to their natal populations than larvae with acute directional senses and superior swimming performance because passivity in pelagic larval phase would lead to diffusion by oceanographic forces (Leis 2006; Cowen & Sponaugle 2009). Specifically, larval dispersal in *E. tetradactylum*, which are low- to average-performance swimmers and have poor orientation compared with the larvae of other tropical species, specifically those of coral reef fishes, was predicted not to depart from passive drift expectations (Leis *et al.* 2009). To increase the probability of successful settlement, enhanced larval abilities may be more necessary for fishes of coral reefs with patchily distributed adult habitat than those of continental coastlines.

Because adult *E. tetradactylum* are shown to be largely sedentary using methods such as mark–recapture techniques, parasite loading and otolith microchemistry

(Zischke *et al.* 2009; Newman *et al.* 2011; Moore *et al.* 2011), any genetic population patterns would likely be primarily attributable to the movements of pelagic eggs and larvae. It should therefore be possible to interpret genetic patterns of this species in a context of the known larval ecology. Also, because the adult habitat of *E. tetradactylum* is more or less continuous across northern Australia, if the larvae of this species are passively diffused by oceanographic forces, the potential for genetic homogeneity among populations is high, particularly in the Gulf of Carpentaria, which is surrounded on three sides by coastal marine habitat. Hereafter, we conclude that the genetic patterns of *E. tetradactylum* do not conform to the assumption of passive larval diffusion. Therefore, high-performance motility and directional senses may not be necessary for marine larvae to recruit back to their natal populations.

Methods

Sampling

Six hundred *Eleutheronema tetradactylum* adults were sampled in two phases between 2007 and 2009 from eleven locations within four regions across the northern coast of Australia: northwest Western Australia (NWWA), western Gulf of Carpentaria (WGoC), eastern Gulf of Carpentaria (EGoC) and the east Queensland Coast (EQldC). Samples were obtained from commercial fishing activities and collected by fishermen using monofilament gillnets. Fin clips were taken from individuals at each location and stored in a 20% dimethyl sulphoxide buffer with 0.25 M EDTA or 80% ethanol. One location (Roebuck Bay, NWWA) was resampled in consecutive years to assess temporal stability in genetic structure (Fig. 1).

Laboratory procedures

DNA was extracted from fin clips using a chelex extraction protocol (Walsh *et al.* 1991). We amplified ~650 bp of the mitochondrial gene cytochrome oxidase subunit 1 (COI) of *E. tetradactylum* using universal primers (FishF1; 5′-TCAACCAACCACAAAGACATTGGCAC3′ and FishR1; 5′-TAGACTTCTGGGTGGCCAAAGAATCA3′) furnished by Ward *et al.* (2005). PCR was conducted as follows: 20-μL PCR containing 2.5 mM Tris–Cl (pH 8.7), 5 mM KCL, 5 mM $(NH_4)2SO_4$, 200 μM each dNTP, 2.5–3.5 mM $MgCl_2$, 10 μM each primer and 1 U of Taq Polymerase (Qiagen Ltd.). Thermocycling was carried out with an initial denaturation of 94 °C for 2 min, 35 cycles of denaturation, annealing and extension (94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s) and a final extension of 72 °C for 10 min. PCR products were confirmed by gel electrophoresis and purified using a standard ammonium acetate ethanol clean-up. PCR products were sequenced with the FishF1 primer using ABI (Applied Biosystems Incorporated) technologies at

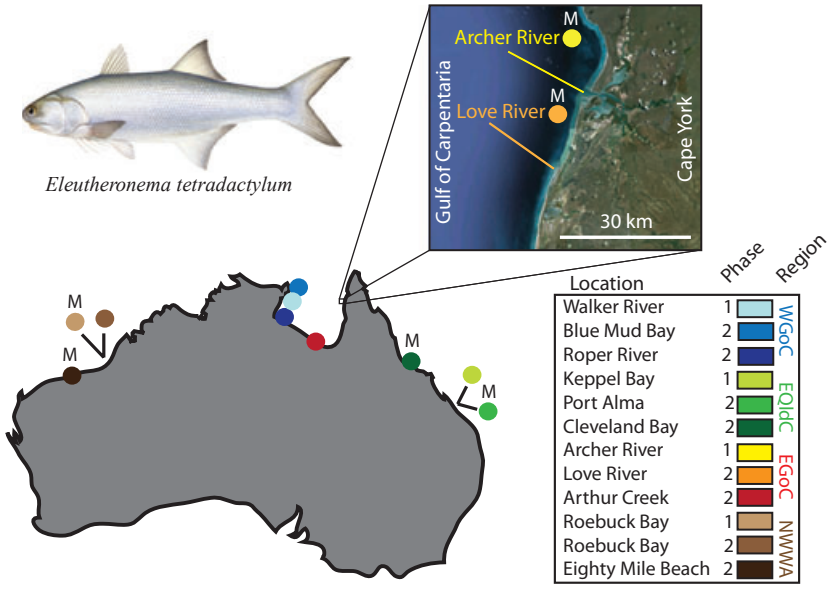

Fig. 1 Sample collections of *Eleutheronema tetradactylum* between 2007 and 2009 across four regions of northern Australia: NWWA (North West Western Australia), WGoC (Western Gulf of Carpentaria), EGoC (Eastern Gulf of Carpentaria) and EQldC (East Queensland Coast). Roebuck Bay was sampled consecutively in phase 1 and 2. Populations marked with 'M' were also analysed for five microsatellite loci. Illustration © R. Swainston http://anima.net.au.

Macrogen sequencing service Seoul, South Korea. GenBank accession numbers for all sequences are JF513489-JF513973.

During the course of this research, microsatellite loci were also developed. Genomic DNA was microsatellite enriched using the magnetic bead capture protocol of Glenn & Schable (2005). Genomic fragments were cloned into a plasmid vector (Invitrogen, Carlsbad, California), transformed and sequenced using universal M13 primers. Sequences were screened, microsatellite loci identified and primers designed using the program MSATCOMMANDER (Faircloth 2008). A total of 20 loci were detected, and the nine best (those with the longest number of repeats) were chosen for further screening. Sample DNA was PCR amplified using a tailed-primer approach as described by Boutin-Ganache *et al.* (2001). Forward or reverse primers were designed with tails in MSATCOMMANDER, and primers of the same tail sequence were fluorolabelled with either HEX, TET or FAM. PCR amplifications of microsatellite loci were carried out in 10-μL reactions with the following reagents: 1 μL 10× *Pfu* buffer, 200 μM of each dNTP, 10 μM each of forward and reverse primers and 20 μM of fluorolabelled primer (10 μM), 0.1 U *Pfu* DNA polymerase (Promega, Madison, Wisconsin) and 1 μL of chelex-extracted DNA template. PCR products were purified using an ethanol and ammonium acetate precipitation and read using Amersham MegaBACE instrumentation at the James Cook University Genetics Analysis Facility.

The five most polymorphic microsatellite loci were chosen for the final analysis (Table 1), and the sequences for each may be found in Genbank, accession numbers JF513974-JF513978. Owing to limited resources, samples from only six locations (total $n = 288$) were genotyped for each locus, representing two sites per region: in NWWA—Roebuck 1, Eighty-mile beach 2; in EGoC—Archer River 1, Love River 2; in EQldC—Cleveland Bay 2 and Port Alma 2. Forty samples were genotyped twice for quality control and returned consistent results.

Data analysis

COI sequences were initially aligned using a Clustal W alignment (Higgins *et al.* 1994) implemented in BIOEDIT 7.0 (Hall 1999). All sequences aligned easily. Microsatellite peaks were scored using the program FRAGMENT PROFILER 1.2 (©Amersham Biosciences, 2003). Microsatellite scores were organized and explored initially using GENALEX 6.1 (Peakall & Smouse 2006).

Population genetic analysis

Molecular diversity indices, haplotype diversity (h) and nucleotide diversity (π) of COI haplotypes were calculated in DNAsp 5.10 (Rozas *et al.* 2003). A minimum-spanning network of COI haplotypes was constructed in TCS 1.21 (Clement *et al.* 2000). An assessment of spatial heterogeneity for COI—analysis of molecular variance (AMOVA) and pairwise F_{st} values—was performed in Arlequin 3.1 (Excoffier *et al.* 2005) with 10 000 permutations.

Genetic diversity metrics for the microsatellite markers—number of alleles, private alleles, observed and expected heterozygosities and the average inbreeding coefficient (F_{IS}) —were calculated in GENALEX and FSTAT 2.9 (Goudet 2001). Significance testing of overall regional F_{IS} was performed in the 'compare-groups' function in FSTAT using 10 000 permutations. Exact tests for departures from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium (LD) were performed in GENEPOP 4.0 (Rousset 2008) using the

Table 1 Description of five novel microsatellite loci for (*Eleutheronema tetradactylum)*

Locus	Repeat motif	Primer sequences (5′-3′)	T_a (°C)	N_a	Size range (bp)
Etet1	$(AAAAC)_{32}$	F 5′-CCCAGAAGGCAGCGTGAG R 5′-TCCCATGAGTGATAGCTTTTGC	58°	33	128–264
Etet2	$(GT)_{15}$	F 5′-TTGGGCATGGTGGCTTTTG R 5′-GTCAGATGGACCAGATTAACTTCC	51–58°	19	206–241
Etet3	$(GTTT)_6$	F 5′-GTGCAACGAGGTCATCAGC R 5′-TGCACACCTTCTCCAGCTC	51–58°	8	194–215
Etet4	$(GT)_{16}$	F 5′-GCACAGCTCGGTTTTCTGG R 5′-TACAACCTCCCTGCTGGAC	51–58°	19	155–191
Etet5	$(GT)_{14}(GAGT)_4$	F 5′-CACCGTGTGCTTGTGCAG R 5′-TAGTCCAGGTTCGCTCCAG	51–58°	25	202–260

Data generated from the analysis of 288 individuals from six putative populations: name of locus, repetitive sequence motif, forward and reverse primer sequences, annealing temperature (T_a °C), number of alleles (N_a) and size range of resulting fragment in units of base pairs.

Markov chain algorithm, a dememorization of 10 000 iterations, 20 batches and 5000 iterations per batch. When departure was observed, the program MICRO-CHECKER 2.2.3 (van Oosterhout *et al.* 2004) was used to detect the presence of null alleles, large allele drop-out and other scoring errors.

Raw estimates of population differentiation in the microsatellite loci were estimated locus by locus and as the average over five loci using AMOVA performed in ARLEQUIN with 10 000 permutations (Φ_{st}; Excoffier *et al.* 1992). For raw estimates of population differentiation, 42 samples with missing data at more than one locus were excluded (leaving $n = 246$) so that each individual had a minimum of four loci and each locus had <5% missing data. Estimates of population differentiation corrected for null allele frequencies were performed in FREENA (Chapuis & Estoup 2007) using the excluding null alleles (ENA) correction method and 1000 bootstrap replicates. No missing data were excluded when estimating population differentiation with null allele frequencies. Rather, all missing data were treated as null homozygotes. Because microsatellite data sets have maximum fixation index values of less than 1 (Hedrick 1999), values of population subdivision were standardized to a scale of 0–1 (Φ'_{st} and F'_{st}), (Hedrick 2005; Meirmans 2006) in the program RECODEDATA 0.1 (Meirmans 2006).

Isolation-by-distance (IBD) analyses were conducted for both COI and microsatellite data independently. Shoreline distances between sampled populations were estimated in km using Google Earth version 4.3 and plotted against genetic distance, pairwise $F_{st}/(1 - F_{st})$ and $F'_{st}/(1 - F'_{st})$ for COI and microsatellites, respectively (Rousset 1997). IBD regression analysis was performed online using the IBD web service (Jensen *et al.* 2005) with 10 000 randomizations of the data.

Discriminant analysis of principal components

Discriminant analysis of principal components (DAPC) is a multivariate analysis that may be used to extract information from large genetic data sets and assign individual genotypes to predefined groups (Jombart *et al.* 2010). First, data are transformed into uncorrelated components, containing most of the genetic information, using principal component analysis (PCA). These components are then subjected to a linear discriminant analysis (DA) that minimizes the genetic variance within populations while maximizing among-population variation, thus providing the best discrimination of predefined genetic groups. This method has been shown to be as sensitive as Bayesian clustering programs (Jombart *et al.* 2010) but does not require large amounts of computational time. Furthermore, DAPC does not assume HWE or LD and has very few assumptions making it an extremely versatile methodology.

PCA was performed on the microsatellite data in R 2.12 (R development core team 2009; http://www.r-project.org) using the dudi.pca function (written by Daniel Chessel & Anne B Dufour) in the R package ade4. Data were scaled and all missing data were replaced with the mean (the origin of the X and Y axes). A number of principal components (PCs) were retained as predictors for DA. There are no strict guidelines for determining how many PCs should be retained during this dimensions-reduction step but it is a compromise between the statistical power of more PCs and the stability of assignments (Jombart *et al.* 2010 & references therein). For the purposes of this study, 40 PCs were kept comprising 92% of the total genetic information (see Supporting information). PCs were discriminated and genotypes assigned to populations using the lda function from the R package MASS (Venables & Ripley 2002). For this analysis, the six genotyped populations were used as a priori population criteria for genotype assignment. Discriminant functions and microsatellite genotypes were visualized as scatterplots created in the R package adegenet (Jombart 2008; Jombart *et al.* 2010).

Results

Six hundred and twenty-eight base pairs of the mitochondrial COI region were resolved for 485 *Eleutheronema tetradactylum* individuals. There were a total of 39 polymorphic sites, of which 15 were parsimony informative (24 singletons), and 47 individual haplotypes were identified (Fig. 2). Molecular diversity statistics of COI for the entire data set were 0.79 and 0.003 for h and π, respectively. COI diversity statistics for each region and population are reported in Table 2. Regionally, EQldC had the highest haplotype and nucleotide diversity while NWWA populations had the least.

Summary statistics for microsatellite loci are reported in Table 2. Allelic diversity was lowest in NWWA but highest in the EGoC. F_{IS} did not differ significantly across the three regions surveyed ($F_{IS} = 0.122$, 0.054, 0.042, $P = 0.53$). Significant single-locus departures from HWE were detected in five of 30 tests at the population level before Bonferroni adjustment and four afterwards ($\alpha = 0.0016$). Likewise, three single-locus HWE departures were detected at the regional level before Bonferroni adjustment and one afterwards ($\alpha = 0.003$), and all regional departures from HWE were in the EGoC (Appendix). Gulf populations also had the most private alleles, 19 across five loci, while

Table 2 Sample sizes and diversity indices

	n (COI)	n_h	h	π	n (msat)	N_a	P_a	H_O	H_E	F_{IS}
East Queensland Coast	117	19	0.83	0.0024	96	15.2	11	0.785	0.831	0.042
Keppel Bay 1	41	10	0.84	0.0021	–	–	–	–	–	–
Cleveland Bay 2	30	7	0.77	0.0021	48	13.2	5	0.795	0.829	0.054
Port Alma 2	46	7	0.62	0.0011	48	10.4	2	0.766	0.781	0.032
Eastern Gulf of Carpentaria	124	14	0.59	0.0012	96	17.8	19	0.773	0.866	0.112
Archer River 1	39	11	0.74	0.0018	48	15.6	10	0.759	0.859	0.128
Arthur River 2	45	5	0.53	0.0010	–	–	–	–	–	–
Love River 2	40	5	0.45	0.0007	48	14.2	4	0.787	0.860	0.097
Western Gulf of Carpentaria	112	19	0.52	0.0011	–	–	–	–	–	–
Walker River 1	27	9	0.51	0.0012	–	–	–	–	–	–
Blue Mud Bay 2	39	6	0.53	0.0009	–	–	–	–	–	–
Roper River 2	46	9	0.50	0.0011	–	–	–	–	–	–
North West Western Australia	132	8	0.18	0.0003	96	10.6	3	0.704	0.740	0.054
Roebuck 1	38	1	0.00	0.0000	48	9.0	2	0.661	0.735	0.112
Eighty-mile Beach 2	47	7	0.43	0.0007	48	8.6	1	0.746	0.735	−0.002
Roebuck 2	47	2	0.04	0.0001	–	–	–	–	–	–

Number of haplotypes (n_h), haplotype diversity (h), nucleotide diversities (π) of COI for all regions and populations of *Eleutheronema tetradactylum* (total n = 485). Average number of alleles per locus (N_a), observed number of private alleles (P_a), observed heterozygosity (H_O) and expected heterozygosity (H_E) and the inbreeding coefficient (F_{IS}) averaged over five microsatellite loci for six populations in three regions (total n = 288).

NWWA had only three private alleles across all loci. Null alleles identified in some populations may contribute to departures from HWE in loci Etet1, Etet2 and Etet5 as indicated in MICROCHECKER. Of 60 locus x locus exact tests of linkage disequilibrium (ten per population), only three were significant before Bonferroni adjustment and all significant values were for different locus pairs. Only one test was significant after Bonferroni (α = 0.00083).

Population structure

The level of genetic population differentiation between sampled populations was high for all comparisons (Tables 3–5). AMOVA fixation indices for the COI marker were Φ_{st} = 0.62, $P < 0.0001$. Pairwise F_{st} comparisons were nearly all significant for COI, indicating that population connectivity is low in this species. Also, population structure was significant between the Archer and

Table 3 Pairwise population structures (F_{st}) generated from 628 bp of the mitochondrial COI region, for 12 sampled *Eleutheronema tetradactylum* populations (n = 485) and corresponding P values (upper diagonal)

	Arch	Arth	Love	Kepp	Clev	Alma	Walk	Blue	Rope	Roe1	Eighty	Roe2
Archer		**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Arthur	**0.107**		0.495	**0.000**	**0.008†**	**0.000**	**0.012†**	**0.000**	0.366	**0.000**	**0.000**	**0.000**
Love	**0.111**	−0.005		**0.000**	**0.001†**	**0.000**	0.104	**0.005†**	0.701	**0.000**	**0.000**	**0.000**
Keppel	**0.298**	**0.460**	**0.471**		**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Cleveland	**0.134**	**0.074†**	**0.110†**	**0.385**		**0.000**	**0.000**	**0.000**	**0.001†**	**0.000**	**0.000**	**0.000**
Alma	**0.469**	**0.509**	**0.532**	**0.250**	**0.420**		**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Walker	**0.104**	**0.055†**	0.021	**0.416**	**0.116**	**0.475**		0.230	0.055	**0.000**	**0.000**	**0.000**
Blue Mud	**0.135**	**0.092**	**0.054†**	**0.455**	**0.162**	**0.508**	0.007		**0.000**	**0.000**	**0.000**	**0.000**
Roper	**0.106**	−0.000	−0.008	**0.451**	**0.093†**	**0.496**	0.023	**0.057**		**0.000**	**0.000**	**0.000**
Roebuck1	**0.782**	**0.840**	**0.888**	**0.797**	**0.724**	**0.863**	**0.858**	**0.865**	**0.838**		**0.013†**	0.999
Eighty Mile	**0.724**	**0.769**	**0.804**	**0.754**	**0.651**	**0.807**	**0.768**	**0.785**	**0.768**	**0.068†**		**0.012†**
Roebuck2	**0.792**	**0.845**	**0.889**	**0.807**	**0.737**	**0.867**	**0.862**	**0.868**	**0.842**	−0.004	**0.071†**	

Significant values are highlighted in bold. Comparisons with (†) were not significant after Bonferroni correction, experiment-wide α = 0.00083 (Rice 1989).

Table 4 Pairwise population structures (F'_{st}) generated from five microsatellite loci for six sampled *Eleutheronema tetradactylum* populations ($n = 288$) after correction for null allele frequencies

	Archer	Love	Cleveland	Alma	Roebuck	Eighty mile
Archer		0.477	**0.000**	**0.000**	**0.000**	**0.000**
Love	0.006		**0.000**	**0.000**	**0.000**	**0.000**
Cleveland	**0.088**	**0.115**		**0.000**	**0.000**	**0.000**
Alma	**0.193**	**0.140**	**0.149**		**0.000**	**0.000**
Roebuck	**0.376**	**0.382**	**0.448**	**0.483**		0.425
Eighty mile	**0.448**	**0.481**	**0.521**	**0.581**	0.013	

Significant values are highlighted in bold. All bold values were significant after Bonferroni correction, experiment-wide $\alpha = 0.00416$ (Rice 1989)

Table 5 AMOVA fixation indices (Φ_{st}) for *Eleutheronema tetradactylum* across all populations surveyed

Marker class and analysis	Average	Etet1	Etet2	Etet3	Etet4	Etet5
COI	0.620					
Raw msat	0.073	0.013	0.083	0.023	0.086	0.159
Msat corrected for null allele freq.	0.062	0.010	0.079	0.023	0.069	0.127
Standardized msat (Φ'_{st})	0.322	0.136	0.424	0.080	0.380	0.558

Average population differentiation for mitochondrial COI, raw population differentiation from microsatellite allele frequencies for each individual loci and the average across all loci, population differentiation corrected for null allele frequencies using the ENA correction of Chapuis & Estoup (2007) and standardized population differentiation for and across all loci (Φ'_{st}). All values are significant to the 95% confidence interval.

Love Rivers in the EGoC ($F_{st} = 0.111$, $P < 0.0001$), which is unexpected because the estuaries of these rivers are only about 15 km apart and are expected to exchange migrants easily. A similar situation is found between Keppel Bay and Port Alma, like the Love and Archer Rivers, these two sites share a close proximity, were sampled in 2008 and 2009, respectively, and are significantly differentiated ($F_{st} = 0.250$, $P < 0.0001$). Consecutive 2008–2009 samples from Roebuck Bay were genetically homogenous. These unusual results prompted the development of microsatellite loci for this species. The microsatellite loci Etet1-5 also revealed a high degree of population structuring, raw $\Phi_{st} = 0.073$, $P < 0.001$; $\Phi'_{st} = 0.322$, $P < 0.001$. Locus-by-locus AMOVA yielded concurrent results (Table 5), and raw values were comparable to values corrected for null alleles. The structuring between the Archer and Love Rivers that was unexpectedly deep in the mtDNA was not observed in the microsatellite data.

Discriminant analysis of principal components

The strong separation between eastern and western populations was visible in DAPC (Fig. 3) and is represented primarily on the *X*-axis. Eastern populations overlap but are differentiated on the *Y*-axis, which probably represents coastline distances. The Port Alma population largely occupies a separate quadrant from the other eastern populations and might be interpreted as a distinct genetic cluster. Using the six sampled populations as a priori population criteria, DAPC was able to assign 70.83% of all individuals to the population where they were sampled, and none of the eastern populations had a proportion of miss-assigned individuals greater than 32% (see Supporting information).

Isolation by distance

Plots of geographic vs. genetic distance are shown in Fig. 4. For both sets of data, distance appears to be a significant driver of the observed genetic patterns. For microsatellite data, the relationship between geographic and genetic distance was positive and strong ($r^2 = 0.868$, $P < 0.01$) with a regression line slope of 1.308e–04. In the mitochondrial COI data, the relationship between geographic and genetic distance was also strong ($r^2 = 0.635$, $P < 0.0001$, slope = 2.4e–04). The IBD result for COI was not substantially altered when comparisons between Love River, Archer River, Keppel Bay and Port Alma were excluded or when only the six populations used for microsatellite analysis were compared.

Discussion

A low-dispersal species

The deepest genetic structuring observed in *Eleutheronema tetradactylum* was at a large spatial scale, between populations in NWWA and the rest of the sampled range. A population break between eastern and western coasts is exhibited by many marine fishes across northern Australia (van Herwerden *et al.* 2009a,b; Edmunds *et al.* 2010; Veilleux *et al.* 2010), perhaps in relation to an emergent land barrier in the Torres strait (Chenoweth *et al.* 1998; but see Chenoweth & Hughes 2003). At a larger spatial scale, many marine fishes and invertebrates also show genetic differentiation between Indian and Pacific Ocean basins (Benzie 1999; Rocha *et al.* 2007). While an east–west segregation in the Indo-Australian Archipelago is common for marine taxa,

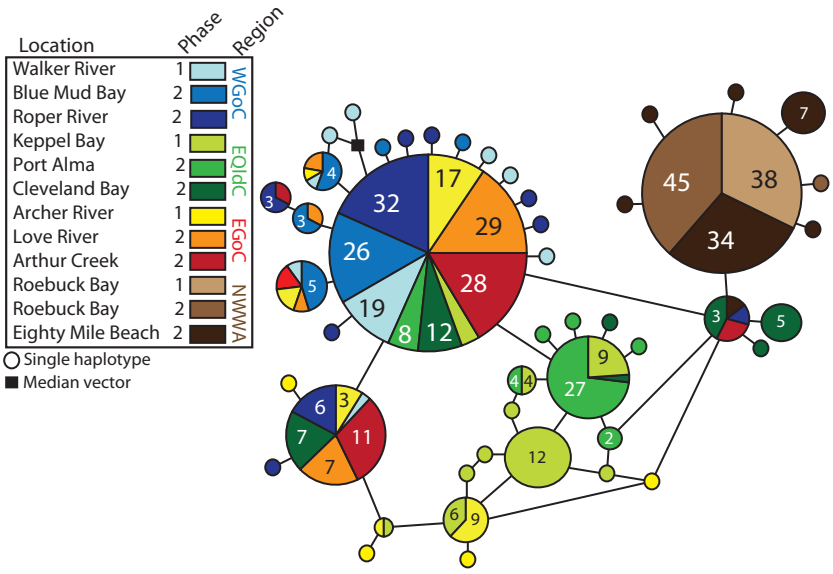

Fig. 2 Minimum-spanning network of 485 *Eleutheronema tetradactylum* cytochrome oxidase subunit 1 (COI) sequences comprising 47 mtDNA haplotypes.

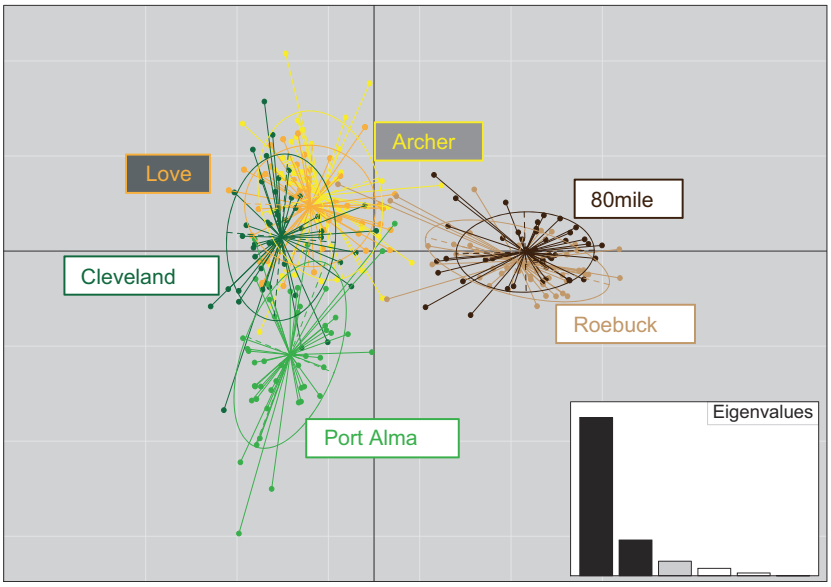

Fig. 3 Scatterplots of the discriminant analysis of principal components of the microsatellite data for six *Eleutheronema tetradactylum* populations using geographic sample site as priors for genetic clusters. Individual genotypes appear as dots, and populations are depicted by colours and 95% inertia ellipses. Eigenvalues show the amount of genetic information contained in each successive principal component. X and Y axes are the first two principle components, respectively.

genetic structuring within regions of Australia, such as the EQldC, is found in only a small number of fishes, and in several cases, low dispersal can probably be attributed to demersal spawning, lack of a pelagic larval stage, diadromy or other life history characteristics (Chenoweth *et al.* 1998; Bay *et al.* 2006; van Herwerden & Doherty 2006). With a pelagic spawning mode, a PLD that probably lasts for weeks, seemingly passive larvae and a largely continuous coastal adult habitat, *E. tetradactylum* might be expected to have highly connected populations. Nevertheless, all indications suggest that this is a low-dispersal species.

Several lines of evidence support low dispersal in *E. tetradactylum*. (i) Each population surveyed possessed at least one but as many as ten private alleles (Table 2), which indicates long-term isolation and low

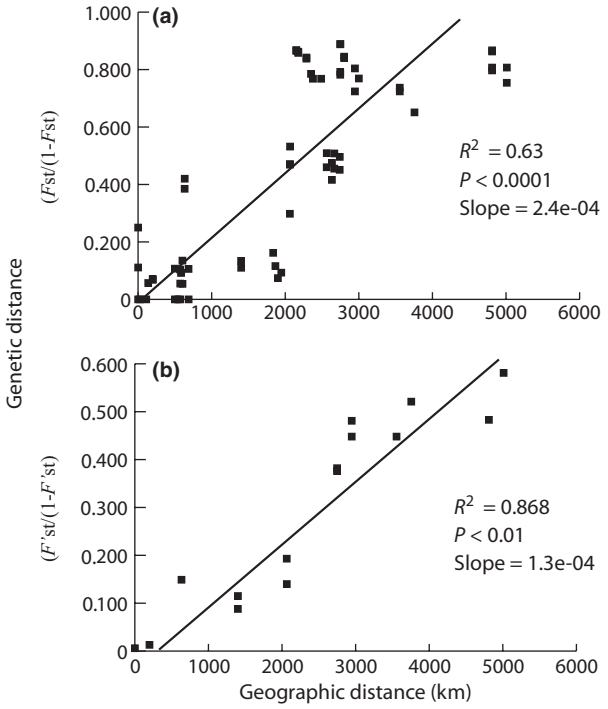

Fig. 4 Isolation-by-distance analysis generated from 10 000 Mantel test randomizations. (a) Genetic distance [mitochondrial cytochrome oxidase subunit 1 (COI)] $F_{st}/(1 - F_{st})$ against geographic distance and corresponding coefficient of determination (r^2), P value and slope of the regression line. (b) Genetic distance (microsatellite loci) $F'_{st}/(1 - F'_{st})$ against geographic distance (km) and corresponding values.

migration (Slatkin 1985; Lowe & Allendorf 2010). (ii) A strong signal of IBD, particularly in the microsatellite loci, indicates that gene flow is primarily restricted to neighbouring populations in a linear stepping stone fashion. Such a distinct pattern of IBD is expected under an assumption of selective neutrality (Slatkin 1993); therefore, departures from HWE found in some populations may partly be attributed to inbreeding (and partly to null alleles). (iii) The genotypes of most individuals in the data were assigned to the populations where they were collected. (iv) Pairwise fixation indices often resolved a substantial amount of genetic structure between neighbouring populations, even at very small spatial scales, most notably in the mtDNA, suggesting that even short-distance dispersal is limited. One example of this is between the adjacent estuaries of the Love and Archer Rivers in the EGoC, which are separated by only 15 km of coastline. No environmental obstruction to gene flow is apparent between these populations, and ocean circulation in the Gulf of Carpentaria is shore-parallel (Wolanski 1993), which would intuitively promote the diffusion of propagules. The data from the gulf does not appear to be anomalous because a similar situation is observed between Port Alma and Keppel Bay on the EQldC.

Differences were observed in the degree of genetic differentiation between mtDNA and microsatellite loci. In *E. tetradactylum*, depth of divergence in the overall Φ'_{st} values for microsatellites is about 52% of that seen in the mtDNA. This same relationship of ~54% between the two classes of markers was also seen in the steepness of the IBD regression slope (Fig. 4), which is another indicator of the diffusion rate of genes (Slatkin 1993; Palumbi 2003). These results suggest that the mitochondrial genes are diffusing at approximately half the rate of the nuclear genome, rather than the expected one-fourth, under the expectation of neutrality, in the absence of sex-biased dispersal and given the fourfold smaller effective population size of the mitochondrial genome (Birky *et al.* 1989; see also Buonaccorsi *et al.* 2001; Lukoschek *et al.* 2008). Higher than expected diffusion of mitochondrial genes, relative to nuclear markers, can probably be reconciled by the fact that *E. tetradactylum* are protandrous hermaphrodites, in which all males become female upon reaching 240–470 mm in length (Motomura 2004). If most individuals that survive to reproductive maturity have the opportunity to spawn as both male and female during their lifetime, then the observed estimates of population differentiation across all populations between the two classes of markers are probably consistent.

Congruence between classes of markers dissolved at the finest spatial scale, between the adjacent Archer and Love Rivers. No differentiation was detected in the microsatellite F'_{st} values but mtDNA structure was significant and comparably strong (Table 3). At first glance, this small-scale discrepancy appears to be some type of bias against maternally inherited mitochondrial genes but cannot be sex-biased dispersal because of hermaphroditism. Another explanation is that there has not been sufficient time for polymorphisms in the nuclear genome to accumulate between these neighbouring, albeit insular, populations. The present-day Gulf of Carpentaria is a very young body of salt water that has only become marine as recently as 9 kya (Harris *et al.* 2008). Therefore, gulf populations of *E. tetradactylum*, as presently constituted, have had limited time to become differentiated. Further, an assessment of stock structure from stable isotopes from sagittal otoliths between the Love and Archer Rivers (on the same samples used in this genetic study) showed significant differentiation, congruent with the mtDNA structure (Newman *et al.* 2011).

Lukoschek *et al.* (2008) also report nonequilibrium in microsatellite loci at a small scale but not a regional one (relative to mtDNA) for a sea snake (*Aipysurus laevis*) across northern Australia. In fact, there are several

parallels between the patterns of *E. tetradactlyum* and *A. laevis* in spite of drastically different life history characteristics. Lukoschek *et al.* (2008) posit that time since recent range expansions into shallow water habitat following the flooding of the Australian continental shelves less than 10 kya may have been insufficient for nuclear genes to reach equilibrium but not for mtDNA, given a large effective population size. Nonequilibrium is hard to reconcile in the face of a strong IBD signal (Hellberg 1995), as seen in the present study, but with more observations, a deterioration of this signal at finer scales might be predicted.

Overall, the genetic patterns exhibited by *E. tetradactylum* are probably best explained by a combination of larval retention and sporadic short-distance dispersal, with longer dispersal occurring infrequently. On an evolutionary timescale, migration is sufficient to connect populations through stepping stone dispersal. Ecologically, migration, even between neighbouring populations separated by a few km, is small and restricted. Because breeding populations appear to be highly localized, at least in ecological time frames, this implies a large degree of spawning site fidelity in adults and a high level of self-recruitment by pelagic larvae. In this regard, populations in NWWA differed, exhibiting high genetic homogeneity. The reason for this difference is unknown but may be attributed to habitat. *E. tetradactylum* was collected from open beaches in NWWA but from estuaries in EGoC. It is also possible that occasional migrants are able to make large genetic contributions to recipient populations even after one or two generations.

Comparisons to pelagic larval ecology

The larvae of *E. tetradactylum* are low-performance swimmers compared with many other fishes, in terms of speed and endurance (Leis *et al.* 2007, 2009; see also Stobutzki & Bellwood 1997). Even so, through much of the larval phase, larvae should still be able to swim against ambient ocean currents in most places (Leis *et al.* 2007), and with a maximum unfed endurance of about 40 km (Leis *et al.* 2009), this appears to be enough for most larvae to recruit back to natal populations. *E. tetradactylum* larvae have an overall significant sense of direction but not to the same extent of accuracy as the larvae of coral reef fishes. For this reason, Leis *et al.* (2009) believed that *E. tetradactylum* would have little control over dispersal outcomes and that dispersal would not depart significantly from passive diffusion. In contrast, the results of this study indicate that active self-recruitment is highly likely. Ergo, high-performance larval swimming and acute orientation may not be required for marine populations to be self-seeding. However, much more research will be required before the linkage between larval ecology and genetic patterns in benthic marine organisms can be fully appreciated. As detailed data on the larval ecology of more taxa become available, multi-species comparisons will be particularly desirable.

The larval behaviour of *E. tetradactylum* is also suggestive of sensory-guided behaviour (olfaction) and may indicate natal homing. When released in open water, *E. tetradactylum* larvae initially swam in circles. The reason for this may be an attempt to detect olfactory cues from settlement habitat (Leis *et al.* 2009). Unlike the larvae of many marine fishes that undergo vertical movements (Leis 2006), the larvae of *E. tetradactylum* stayed primarily within the top 7 m of the water column throughout the larval stage (Leis *et al.* 2009), which may be optimal for the olfactory detection of chemical cues from mangroves, estuaries or wetlands. Nevertheless, vertical movement of larvae between strata in the water column is often thought to be a measure that prevents advection (Leis 2006; Cowen & Sponaugle 2009). The absence of this behaviour in *E. tetradactylum* is unusual, as is swimming in circles, but Leis *et al.* (2009) recognized that the environment in which their study was conducted may have been strange for the larvae of turbid water species and might have influenced their observations.

Selection pressures on dispersal?

Many shallow water fishes of oceanic environments, namely coral reef fishes, often show little population structure at spatial scales of thousands or even tens of thousands of kilometres (Bay *et al.* 2004; Craig *et al.* 2007; Shultz *et al.* 2007; Horne *et al.* 2008; Gaither *et al.* 2010; Leray *et al.* 2010; Reece *et al.* 2010), while structuring in coastal inshore marine fishes is generally stronger and most commonly observed at a scale of hundreds of kilometres or less (Gold & Turner 2002; Chenoweth & Hughes 2003; Durand *et al.* 2005; Hickerson & Cunningham 2005; Bradbury *et al.* 2008; Hua *et al.* 2009). Stepping stone dispersal across a one-dimensional coastline may have contributed to the high level of structuring in coastal habitats (Wright 1943; Kimura & Weiss 1964; de Aguiar *et al.* 2009), and physical oceanography may favour larval retention along some coastlines (Cowen & Sponaugle 2009). Nonetheless, the population genetic patterns of *E. tetradactylum* imply biologically restrained gene flow.

Habitat density and stability may be related to population structure. Crandall *et al.* (2010) observed that population structure is weak or absent in some amphidromous gastropods of oceanic islands, even when islands are separated by thousands of kilometres. The

same authors noticed that pelagic larval dispersal is often lost altogether in species of otherwise amphidromous genera found in continental watersheds, which are closer together and less-volatile habitats. Crandall *et al.* (2010) reason that organisms of isolated and volatile environments must deal with the dual pressures of staying close to suitable habitat and at the same time safeguard against local extinction through long-distance dispersal. Contrastingly, in dense, stable habitats, the pressure to safeguard against extinction by dispersing is less but the need for suitable habitat remains the same. Jones *et al.* (2009) also contribute to this argument by pointing out that self-recruitment may be greater in Great Barrier Reef populations, where coral reef habitat is dense, than on Lord Howe island, which, being the most southerly reef in the world, is isolated, even by coral reef standards. Whether shallow inshore marine habitats are stable is questionable, but compared with coral reef habitat, continental coastline habitat is much more dense. Therefore, in coastal marine fishes, such as *E. tetradactylum*, selection pressures on dispersal may be satisfied by occasional, short-distance dispersal.

If the physical swimming requirements of self-recruitment in marine larvae lie well within the levels exhibited by *E. tetradactylum*, the evolutionary significance of superior larval swimming ability, such as seen in many reef fishes, may most likely be an adaptation for dispersal, not self-recruitment. This is not to suggest that these species cannot also show high levels of self-recruitment (Almany *et al.* 2007), rather it suggests that these species are under selective pressure to maintain ecologically significant dispersal as well as self-recruitment (Jones *et al.* 2009; Planes *et al.* 2009). As a case in point, in a study by Gerlach *et al.* (2007), the study species with the highest swimming performance showed the least degree of olfactory natal homing.

Conclusions

Fine-scale spatial population structuring and a strong pattern of IBD imply that *Eleutheronema tetradactylum* has an aversion to dispersal at all phases of its life cycle. Because *E. tetradactylum* larvae are not high-performance swimmers compared with other species (Leis *et al.* 2007), it may be concluded that high-performance swimming is probably not an absolute requirement of populations of marine organisms to be self-seeding. Furthermore, observations of swimming patterns of *E. tetradactylum* larvae may indicate olfactory-guided behaviour and possibly natal homing. These larval attributes may, in part, be a consequence of the coastal marine habitat of *E. tetradactylum*, in which selection pressures on dispersal may not be great. If so, then population connectivity and dispersal processes of inshore marine habitats could differ from those of other marine systems. Optimal conservation and fisheries management strategies regarding inshore marine species, including mangrove and brackish water specialists, may therefore need to be distinct from management of other marine biota, such as that of coral reefs. In regard to *E. tetradactylum* populations, fisheries managers need to consider the fine-scale spatial population structuring and self-recruitment of this species in determining the appropriate spatial scale for management and monitoring purposes.

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Data accessibility

mtDNA COI sequences: Genbank accessions JF513489-JF513973; Microsatellite loci sequences: Genbank accessions JF513974-JF513978; Microsatellite data deposited at Dryad: doi:10.5061/dryad.8852.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Proportion of the genetic information contained by each additional principal component. The red line indicates the fortieth component, at which 92% of the genetic information is available.

Fig. S2 Posterior probability of assignment of each individual genotype to six different populations. Along the *x*-axis names of the possible assignment populations are given. Along the *y*-axis 288 genotypes are listed, along with the population from which they were sampled. Darkness of the shaded bars corresponds proportionally to the probability of assignment to a given population.

Fig. S3 Proportion of miss-assigned individuals belonging to each of six populations.

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Appendix

Summary statistics for five microsatellite loci Etet 1-5. Sample sizes (*n*), observed number of alleles (N_a), the average inbreeding coefficient (F_{IS}), observed number of private alleles (P_a), observed heterozygosity (H_O) and expected heterozygosity (H_E). Probability of departure from HWE for each locus at each population (p); significance of departure $P < 0.05$*, significance of departure after sequential Bonferroni correction $P < 0.0083$** (Rice 1989)

Population	*n*	Etet1	Etet2	Etet3	Etet4	Etet5
Eastern Gulf of Carpentaria	96	$N_a = 27$	$N_a = 16$	$N_a = 7$	$N_a = 17$	$N_a = 22$
		$P_a = 5$	$P_a = 4$	$P_a = 2$	$P_a = 5$	$P_a = 3$
		$H_O = 0.772$	$H_O = 0.787$	$H_O = 0.710$	$H_O = 0.933$	$H_O = 0.756$
		$H_E = 0.932$	$H_E = 0.882$	$H_E = 0.741$	$H_E = 0.897$	$H_E = 0.904$
		$F_{IS} = 0.179$	$F_{IS} = 0.147$	$F_{IS} = 0.124$	$F_{IS} = -0.048$	$F_{IS} = 0.160$
		$P = 0.000$**	$P = 0.023$*	$P = 0.350$	$P = 0.801$	$P = 0.039$*
Archer River	48	$N_a = 24$	$N_a = 14$	$N_a = 6$	$N_a = 16$	$N_a = 20$
		$P_a = 2$	$P_a = 3$	$P_a = 0$	$P_a = 3$	$P_a = 2$
		$H_O = 0.772$	$H_O = 0.739$	$H_O = 0.625$	$H_O = 0.975$	$H_O = 0.682$
		$H_E = 0.927$	$H_E = 0.888$	$H_E = 0.720$	$H_E = 0.911$	$H_E = 0.899$
		$F_{IS} = 0.169$	$F_{IS} = 0.170$	$F_{IS} = 0.133$	$F_{IS} = -0.072$	$F_{IS} = 0.243$
		$P = 0.030$*	$P = 0.005$**	$P = 0.486$	$P = 0.949$	$P = 0.007$**
Love River	48	$N_a = 22$	$N_a = 13$	$N_a = 7$	$N_a = 13$	$N_a = 17$
		$P_a = 0$	$P_a = 1$	$P_a = 1$	$P_a = 1$	$P_a = 1$
		$H_O = 0.760$	$H_O = 0.772$	$H_O = 0.659$	$H_O = 0.904$	$H_O = 0.837$
		$H_E = 0.9388$	$H_E = 0.886$	$H_E = 0.734$	$H_E = 0.883$	$H_E = 0.908$
		$F_{IS} = 0.191$	$F_{IS} = 0.130$	$F_{IS} = 0.103$	$F_{IS} = -0.025$	$F_{IS} = 0.079$
		$P = 0.000$**	$P = 0.199$	$P = 0.106$	$P = 0.742$	$P = 0.520$
North East Queensland Coast	96	$N_a = 24$	$N_a = 13$	$N_a = 6$	$N_a = 13$	$N_a = 20$
		$P_a = 5$	$P_a = 2$	$P_a = 1$	$P_a = 1$	$P_a = 2$
		$H_O = 0.903$	$H_O = 0.740$	$H_O = 0.722$	$H_O = 0.853$	$H_O = 0.740$
		$H_E = 0.938$	$H_E = 0.854$	$H_E = 0.705$	$H_E = 0.821$	$H_E = 0.843$
		$F_{IS} = 0.049$	$F_{IS} = 0.141$	$F_{IS} = -0.015$	$F_{IS} = -0.012$	$F_{IS} = 0.129$
		$P = 0.378$	$P = 0.511$	$P = 0.611$	$P = 0.573$	$P = 0.228$
Cleveland Bay	48	$N_a = 22$	$N_a = 13$	$N_a = 5$	$N_a = 12$	$N_a = 16$
		$P_a = 1$	$P_a = 2$	$P_a = 0$	$P_a = 1$	$P_a = 1$
		$H_O = 0.875$	$H_O = 0.717$	$H_O = 0.666$	$H_O = 0.847$	$H_O = 0.868$
		$H_E = 0.930$	$H_E = 0.867$	$H_E = 0.667$	$H_E = 0.849$	$H_E = 0.876$
		$F_{IS} = 0.060$	$F_{IS} = 0.175$	$F_{IS} = 0.015$	$F_{IS} = 0.001$	$F_{IS} = 0.007$
		$P = 0.336$	$P = 0.490$	$P = 0.468$	$P = 0.437$	$P = 0.459$
Port Alma	48	$N_a = 21$	$N_a = 7$	$N_a = 6$	$N_a = 8$	$N_a = 11$
		$P_a = 1$	$P_a = 0$	$P_a = 1$	$P_a = 0$	$P_a = 0$
		$H_O = 0.911$	$H_O = 0.756$	$H_O = 0.808$	$H_O = 0.808$	$H_O = 0.545$
		$H_E = 0.921$	$H_E = 0.824$	$H_E = 0.744$	$H_E = 0.773$	$H_E = 0.691$
		$F_{IS} = 0.012$	$F_{IS} = 0.083$	$F_{IS} = -0.087$	$F_{IS} = -0.046$	$F_{IS} = 0.214$
		$P = 0.869$	$P = 0.752$	$P = 0.740$	$P = 0.253$	$P = 0.062$
North West Western Australia	96	$N_a = 18$	$N_a = 9$	$N_a = 4$	$N_a = 10$	$N_a = 12$
		$P_a = 1$	$P_a = 1$	$P_a = 0$	$P_a = 0$	$P_a = 1$
		$H_O = 0.958$	$H_O = 0.641$	$H_O = 0.597$	$H_O = 0.750$	$H_O = 0.578$
		$H_E = 0.923$	$H_E = 0.691$	$H_E = 0.718$	$H_E = 0.733$	$H_E = 0.606$
		$F_{IS} = -0.042$	$F_{IS} = 0.059$	$F_{IS} = 0.177$	$F_{IS} = 0.016$	$F_{IS} = 0.096$
		$P = 0.175$	$P = 0.214$	$P = 0.096$	$P = 0.706$	$P = 0.298$
Roebuck Bay	48	$N_a = 17$	$N_a = 9$	$N_a = 4$	$N_a = 8$	$N_a = 8$
		$P_a = 0$	$P_a = 1$	$P_a = 0$	$P_a = 0$	$P_a = 1$
		$H_O = 0.951$	$H_O = 0.583$	$H_O = 0.600$	$H_O = 0.738$	$H_O = 0.434$
		$H_E = 0.929$	$H_E = 0.701$	$H_E = 0.715$	$H_E = 0.736$	$H_E = 0.635$
		$F_{IS} = -0.023$	$F_{IS} = 0.170$	$F_{IS} = 0.162$	$F_{IS} = -0.002$	$F_{IS} = 0.318$
		$P = 0.200$	$P = 0.115$	$P = 0.196$	$P = 0.493$	$P = 0.001$**

Appendix (*Continued*)

Population	*n*	Etet1	Etet2	Etet3	Etet4	Etet5
Eighty-Mile Beach	48	$N_a = 15$ $P_a = 1$ $H_O = 0.973$ $H_E = 0.920$ $F_{IS} = -0.059$ $P = 0.345$	$N_a = 6$ $P_a = 0$ $H_O = 0.729$ $H_E = 0.694$ $F_{IS} = -0.050$ $P = 0.467$	$N_a = 4$ $P_a = 0$ $H_O = 0.595$ $H_E = 0.736$ $F_{IS} = 0.193$ $P = 0.088$	$N_a = 10$ $P_a = 0$ $H_O = 0.720$ $H_E = 0.750$ $F_{IS} = 0.039$ $P = 0.548$	$N_a = 9$ $P_a = 0$ $H_O = 0.708$ $H_E = 0.618$ $F_{IS} = -0.147$ $P = 0.406$