Historical population connectivity and fragmentation in a tropical freshwater fish with a disjunct distribution (pennyfish, *Denarius bandata*)

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Abstract. Pennyfish, *Denarius bandata*, are small freshwater fish widely distributed in northern Australia in 4 highly disjunct regions and in the Fly River and Bensbach River drainages in southern Papua New Guinea (PNG). Alternating phases of exposure of the Australia–New Guinea Continental Shelf (ANGCS) during stands of lowered sea levels during Pleistocene glacial phases created a land bridge and fresh/brackish water habitats that intermittently connected Australia and PNG. Some biogeographic theories and empirical evidence for several freshwater crustaceans suggest that wetlands on the exposed ANGCS during the last glacial maximum were an important conduit for dispersal and gene flow in freshwater species between Australia and PNG and across northern Australia and that connectivity was severed by the most recent sea level rise in the region (~6–8 thousand years before present). We used mitochondrial deoxyribonucleic acid (mtDNA) sequence data to test phylogeographic hypotheses concerning the origin of the disjunct distribution in *D. bandata* in relation to dispersal–vicariant processes and exposure of the ANGCS during Pleistocene sea level changes. Rather than a late Pleistocene origin associated with the last glacial maximum, the coalescence of the regional populations was in the early- to mid-Pleistocene, and the molecular data indicated that the eastern regional populations split before the northern populations split (i.e., sequential vicariance), whereas the northern populations split contemporaneously (i.e., simultaneous vicariance). The complex mtDNA genealogy for *D. bandata* also indicated a phylogeographic history in which ancestral lineages were retained in the northwestern part of its distribution, and ancestral haplotype diversity was retained in the Fly River (PNG) population because several divergent clusters of PNG haplotypes were more closely related to Australian haplotypes than to each other.

Key words: statistical phylogeography, Lake Carpentaria, nested clade analysis, ancestral retention, sequential vicariance, simultaneous vicariance.

How disjunct distributions (i.e., distributions composed of multiple geographically discrete and widely spaced populations) arise is an interesting question in biogeography and has long been subject of the dispersalist–vicariance debate. Dispersalist theories emphasize the importance of species traits and dispersal in explaining the occurrence of highly isolated populations, whereas vicariant theories invoke landscape evolution and fragmentation as the principal drivers of these biotic patterns. For example, the widespread distribution of the catadromous fish *Galaxias maculatus* throughout southern hemisphere landmasses (Australia, New Zealand, South America, and the Falkland Islands) has been explained by both

dispersal (marine dispersal by euryhaline larvae) and vicariance theories (sundering of Gondwana) (see Waters et al. 2000).

Vicariance can occur simultaneously, whereby all disjunct populations form contemporaneously. Ponniah and Hughes (2004) suggested that simultaneous vicariance driven by increasing global temperatures in the Pliocene facilitated the evolution of disjunct distributions for spiny mountain crayfish (genus *Euastacus*) among high-elevation mountain tops in tropical and subtropical regions of eastern Australia because these species are cold-water specialists. Vicariance also can occur sequentially, whereby disjunct populations originate one by one from a formerly widespread population over a relatively long period of time. In the context of dispersal, disjunct distributions can arise following long-dis-

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tance colonization events, such as the recent colonization of southwestern Australia by the mud crab, *Scylla serrata*, as a consequence of the strong Leeuwin Current in 1999 and 2000 (Gopurenko et al. 2003). Colonization of landmasses throughout the South Pacific by the atyid shrimp genus *Paratya* is a more ancient example of long-range dispersal events that facilitated the evolution of a disjunct distribution in this genus (Page et al. 2005). Last, dispersal among disjunct populations can be an ongoing process among suitable disjunct habitats, such as for amphidromous species distributed throughout archipelagos and connected by interisland marine dispersal (Chubb et al. 1998, Page et al. 2008).

A series of major ice ages during the Quaternary (i.e., 2.4 million years before present [m ybp] to 10 thousand ybp [k ybp]) had an enormous influence on species distributions (Hewitt 2000), although more ancient oscillations in climate also influenced the distribution of some taxa (e.g., Ponniah and Hughes 2004). The large volume of ice held in polar ice sheets during glacial phases reduced sea levels by up to 140 m globally and produced land bridges that connected islands in several parts of the world (Hewitt 2000). Many such Pleistocene land bridges occurred in the Indo-Australian region (Hewitt 2000, Voris 2000), and oscillating exposure of the Australia–New Guinea Continental Shelf (ANGCS) putatively facilitated present-day disjunct distributions across Torres Strait for numerous terrestrial and freshwater taxa, including 33 species of freshwater fish (Allen 1991) and various freshwater invertebrates (e.g., giant river prawns [*Macrobrachium rosenbergii*], de Bruyn et al. 2004, de Bruyn and Mather 2007; redclaw crayfish [*Cherax quadricarinatus*], Baker et al. 2008). The ANGCS also connected many freshwater habitats (both lotic and lentic) across northern Australia during the Pleistocene, although the precise timing of when fish faunas between Australia and Papua New Guinea (PNG) and across northern Australia were connected is unclear (Unmack 2001). During the last glacial maximum (10–14 k ybp), a large fresh-to-brackish water lake, Lake Carpentaria (Fig. 1), existed in the present day Gulf of Carpentaria (Jones and Torgersen 1988, Torgersen et al. 1998, Reeves et al. 2007, see Collier 2009 for visualization of temporal fluctuations of Lake Carpentaria). Lake Carpentaria facilitated hydrological connectivity between rivers and other wetlands in northern Australia and southern PNG, and some biogeographers suggest that the severing of the ANGCS land bridge and inundation of Lake Carpentaria during the last sea level rise (5.5–8 k ybp) represents the most recent initiation of discontinuity among freshwater faunas in

the region (Allen and Hoese 1980, McGuigan et al. 2000). In contrast, Unmack (2001) suggests that aridity during Pleistocene glacial cycles coupled with the brackish water quality of Lake Carpentaria might have limited opportunities for faunal exchanges by freshwater fish between northern Australia and southern PNG and across northern Australia. Instead, Unmack (2001) suggests that many present-day disjunct distributions of freshwater fauna in the region might have originated in the late Miocene (5.3–23 m ybp), a time when sea levels were lower and global climate was wetter than at present.

The pennyfish *Denariusa bandata* Whitley 1948 (Ambassidae) is predominantly a wetland (i.e., lentic habitat) species with strong habitat associations with macrophytes and is commonly found in oxbow lakes, lagoons, and other floodplain wetlands that are permanently, seasonally, or sporadically connected to lotic systems within a drainage basin (Pusey et al. 2004). Populations of *D. bandata* at Cape Flattery (southeastern Cape York Peninsula region) also occur in coastal sand dune lakes that are not connected to lotic systems. The species is widely distributed in northern Australia within 4 highly disjunct regional populations and in the Fly River and Bensbach River drainages in southern PNG (Allen 1991, Pusey et al. 2004; Fig. 1). We used mitochondrial deoxyribonucleic acid (mtDNA) sequence data to test phylogeographic hypotheses concerning the origin of the disjunct distribution in *D. bandata* in relation to dispersal–vicariant processes and exposure of the ANGCS during Pleistocene sea level changes. We tested 2 alternative hypotheses. First, if all 5 regional populations of *D. bandata* comprised a single, reasonably cohesive population at the time of the last glacial maximum when Lake Carpentaria was at its maximum extent in accordance with some biogeographic theories (e.g., Allen and Hoese 1980, McGuigan et al. 2000), we would expect the present disjunct regional populations to have formed via a single vicariant event during the last sea level rise ~6 to 8 k ybp (simultaneous vicariance hypotheses). Second, the eastern populations (Wet Tropics and southeastern Cape York Peninsula regions) might represent long-isolated populations of *D. bandata* because it is unlikely that fluctuating sea levels on ANGCS during the Pleistocene facilitated connectivity of these populations with the northern populations (Cape York, Top End, and PNG). Thus, we would expect that the eastern populations diverged from the 3 northern ones earlier than the late Pleistocene, although we would still expect the 3 northern populations to arise contemporaneously in the late Pleistocene (sequential vicariance hypothesis).

FIG. 1. Northern Australia and southern Papua New Guinea (PNG) showing regions and drainage basins sampled for *Denariusa bandata*. Drainage basins are numbered as in Table 1, and the 3 main unsampled populations are indicated with the letters A (Cape Flattery dune lakes), B (Archer and Wenlock River drainages), and C (Bensbach River drainage in southern PNG). The exposed Australia–New Guinea Continental Shelf is shown by the broken black lines, and putative Pleistocene hydrological connections, including Lake Carpentaria, between Australia and PNG and across northern Australia, are indicated by thin black lines. Map redrawn from Voris (2000).

Methods

A total of 163 individuals from 18 drainage basins across 5 disjunct regional distributions, 4 in Australia and 1 (the Fly River drainage) in southern PNG (Table 1), were sequenced for a fragment of the ATPase 6 and 8 mtDNA genes using the LYS.22F (5'-AAAGCGTTAGCCTTTTAAGC-3') and CO3.62R (5'-TTATTAGAAGGGCGGCAACTG-3') primers (P. J. Unmack, Brigham Young University, unpublished data; Genbank Accession Numbers GU474206–GU474370). Polymerase chain reaction (PCR) was used to amplify an 835-base pair (bp) segment of the ATPase mtDNA gene. The PCR thermal cycling profile was: 5 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 45°C, and 90 s at 72°C, an extension step of 7 min at 72°C, and a final hold period at 4°C. PCR product was purified and sequenced as described in Cook et al. (2007).

Patterns of genetic variation

Where sample sizes permitted ($n > 5$), measures of molecular diversity (nucleotide diversity [π], haplotype diversity [h], number of pairwise bp differences [k]) were calculated for populations at the drainage-basin and regional scales (ARLEQUIN, version 2; Schneider et al. 2000). The demographic parameters F_s (Fu 1997) and D (Tajima 1989) were calculated using 10,000 coalescent simulations given the observed number of segregating sites (DnaSP version 5; Librado and Rozas 2009). These parameters belong to different classes of demographic statistics, each with particular approaches and assumptions (see Finn et al. 2009), but both indicate demographic stability when nonsignificant, population growth when significantly negative, and demographic bottlenecks when significantly positive. Partitioning of mtDNA genetic variation was assessed with hierarchical Φ indices in the

TABLE 1. Regions, drainage basins, and localities sampled for *Denariusa bandata*. Drainages are listed from west to east following coastline distance in Australia. Numbers next to drainage basin names correspond to numbers in Fig. 1. *n* indicates the number of individuals sampled in each drainage. Lowest-level and highest-level clades numbers correspond with labels in the haplotype network (Fig. 4). Clades A and B represent single haplotypes within Clades 1-5 and 1-20, respectively (Fig. 4). CYP = Cape York Peninsula, PNG = Papua New Guinea.

Region	Drainage	Localities	<i>n</i>	Lowest-level clades	Highest-level clades
Top End	1. Moyle	Woody Billabong	2	2-10	4-2
	2. Daly ^a	Milkwood Billabong, Mission Waterhole	25	2-1	4-1
	3. Finniss	Unnamed billabong on east branch	2	1-19	4-2
	4. Howard	Giraween Lagoon	10	B	4-2
	5. Adelaide ^a	Manton River Crossing, Marakai Crossing	21	1-22, 1-20 excluding B, 2-8	4-1, 4-2
	6. South Alligator	Sandy Billabong	1	1-9	4-1
	7. Murgarella	Murrkan Lagoon	1	1-8	4-1
	8. Liverpool ^a	Liverpool River, Mann River	9	1-10	4-1
	9. Blyth ^a	Blyth River, Caddell River	14	1-11	4-1
	10. Cato	Unnamed billabong	4	1-17	4-1
Northwestern CYP	11. Koolatong	Unnamed billabong	1	1-16	4-1
	12. Mitchell	Ten Mile Lagoon	11	1-2, 1-4	4-1
	13. Edward	Eight Mile Yard Lagoon	10	1-3	4-1
	14. Jardine ^a	Jardine Creek, Burster Creek	17	1-5, 1-7	4-1
Southeastern CYP	15. Normanby	Low Lake	3	1-14	4-1
Wet Tropics	16. Russell	Eubanangee Swamp	9	2-7	4-1
	17. Tully	Tully-Murray Swamp	11	1-21, 1-15	4-1
PNG	18. Fly	Suki	11	A, 1-6, 1-13, 1-12	4-1

^a Drainages in which 2 subcatchments were sampled

analysis of molecular variance (AMOVA; Excoffier et al. 1992) framework as implemented by ARLEQUIN. Regions were used as groups, and drainages within regions were used as populations within groups. Statistical significance was assessed based on 10,000 nonparametric permutations of the observed genotypes. Pairwise Φ_{ST} values were calculated among populations from all drainages and analysed with 10,000 multidimensional scaling replicates in PRIMER (Clarke and Gorley 2001). Isolation-by-distance (IBD) in the genetic data was tested with 5000 permutations of a Mantel test (Mantel 1967) in PRIMER with pairwise Φ_{ST} among populations as the measure of genetic distance and coastline distances among river mouths as the measure of geographic distance. Geographic distances between Australian drainages and the Fly River in PNG also incorporated straight line distances across Torres Strait between the northern tip of Cape York Peninsula and the estuary of the Fly River.

Phylogeographic approaches

Times of population divergence.—Several phylogeographic approaches were used to test the simulta-

neous vicariance and sequential vicariance hypotheses. First, the program MDIV (Nielsen and Wakeley 2001) was used to estimate divergence times for pairwise population comparisons among regions. Coalescent parameters were estimated using the finite (Hasegawa, Kishino, Yano [HKY]) sites model and 5,000,000 Markov chain iterations with a 20% burn-in. M_{MAX} (the maximum value for the scaled migration rate considered in the likelihood analyses) and T_{MAX} (the maximum value for the scaled divergence time considered in the likelihood analyses) were both set to 10, and 3 replicate runs, each with a different starting seed number, were made for each pairwise comparison. The highest likelihood values for the parameters T (divergence time in mutational time) and the mutation parameter θ ($\theta = 2Ne\mu$, where Ne is the effective female population size and μ is the substitution rate/nucleotide) were converted to time in y (t) using the formula, $t = T(\theta/2\mu)$. The mtDNA ATPase sequence divergence rate used was 1.3% per million y (Bermingham et al. 1997), which is the divergence rate of this gene estimated for various species of tropical freshwater fish from several orders. This divergence rate was converted to μ with the formula,

$$\mu = \frac{\% \text{ sequence divergence per million y/200}}{10^6} \times \text{number of bp in fragment.}$$

Statistical phylogeography.—The statistical phylogeographic approach of Knowles (2001) and Knowles and Maddison (2002) was used. The mtDNA gene tree for *D. bandata* was reconstructed using maximum likelihood (ML) analysis as implemented in PHYML (Guindon and Gascuel 2003) and incorporated 1000 bootstrap replications and input parameters as determined in MODELTEST (Posada and Crandall 1998). This gene tree was compared to a hypothetical phylogenetic tree in which the regional populations were monophyletic using MESQUITE (Maddison and Maddison 2000). The degree of discord between the observed gene tree and the hypothetical gene tree (monophyletic regional populations in this case) was assessed using the *s* statistic (Slatkin and Maddison 1989) in MESQUITE, with *s* being a measure of the observed number of interpopulation coalescent events. Batches of 100 sequences each were simulated for both of our hypothesized biogeographic models with an empirically built model of sequence evolution (i.e., parameters determined by MODELTEST from the empirical sequence data). *Ne* and the scaling factor were varied among the batches. For the simultaneous vicariance hypothesis, the interpopulation branch lengths all were set to 10,000 generations. For the sequential vicariance hypothesis, branch lengths between the northern and eastern populations were set to 200,000 generations. The *Ne* values used were the ancestral population size (APS) and APS/10. APS was determined by rearranging the formula $\theta_0 = 2Ne\mu$, and the 95% probability value for θ_0 was calculated using mismatch distribution analysis (Rogers and Harpending 1992) in ARLEQUIN. The 3 values used for the scaling factor were 1.0, 0.001, 0.0000001. Each batch of simulated sequences was converted into a consensus tree file with PAUP* (Swofford 2002), and the distribution of *s* values was calculated in MESQUITE. If the observed value of *s* fell within the 95% confidence interval (CI) of the distribution, the hypothesis could not be rejected.

Nested clade phylogeographic analysis.—Last, nested clade phylogeographic analysis (NCPA; Templeton et al. 1995) was used to identify fragmentation among the regional populations. For the simultaneous vicariance hypothesis, signatures of allopatric fragmentation were expected to be at equivalent clade levels among all populations. For the sequential vicariance hypothesis, signatures of allopatric fragmentation were expected to occur at a higher clade level between

the eastern and northern regions than among the 3 northern populations. TCS (Clement et al. 2000) was used to build a parsimony haplotype network, and haplotypes within the genealogy were nested according to the rules of Templeton et al. (1995). Significant associations between genealogy and geography were assessed with 10,000 χ^2 permutations in the NCPA framework as implemented in GEODIS (version 2.6; Posada et al. 2000). Putative biological inferences of statistically significant clades within the genealogy were made with the December 2008 inference key (available from: <http://darwin.uvigo.es/software/geodis.html>).

Results

Patterns of genetic variation

Measures of molecular diversity were highest for the Adelaide River drainage and Top End region, respectively, and the population demographic parameters *F_s* and *D* were not significant for any analysis (Table 2). AMOVA indicated that a relatively small and nonsignificant portion (7.27%; $\Phi_{CT} = 0.073$, $p = 0.260$) of mtDNA genetic variation in *D. bandata* was partitioned among the regions and that most (70.91%; $\Phi_{SC} = 0.765$, $p < 0.001$) of the genetic variation was partitioned among drainages within regions. The global Φ_{ST} was 0.782 ($p < 0.001$), with pairwise Φ_{ST} values among drainages ranging from 0.112 to 1.000 (mean = 0.811 ± 0.197 , $n = 153$). The multidimensional scaling (MDS) plot of pairwise Φ_{ST} values among all drainages (Fig. 2) also showed greater variation within regions than among regions. Genetic and geographic distances were not correlated (Mantel test, $\rho = 0.106$, $p = 0.139$).

Times of population divergence

Replicate coalescence simulations gave identical results for each pairwise interpopulation comparison; thus, the predefined values for *M_{MAX}* and *T_{MAX}* and the length of the Markov chains used allowed exhaustive likelihood analyses in each case. Estimates of time (t) of divergence between pairs of regional populations indicated consistently older population splits when one member of the pair was the Wet Tropics region (comparisons B, D, F in Fig. 3), regardless of whether the divergent haplotypes from the western drainage basins of the Top End region (Clade 4-2; see Fig. 4) were included or excluded from analyses. The comparison of populations in the eastern and western parts of the Top End region (Clades 4-1 and 4-2) yielded the oldest estimated phylogeographic split for *D. bandata* (early Pleistocene, ~1.5 m ybp; comparison G in Fig. 3). Thus, the

TABLE 2. Mean (± 1 SD) measures of molecular diversity (nucleotide diversity [π], haplotype diversity [h], number of pairwise base pair differences [k]) and results of population demographic analyses (F_s [Fu 1997], D [Tajima 1989]) (p values in parentheses) for populations of *Denariusus bandata* at drainage-basin and regional scales. Populations where the number of individuals sampled was <5 or where the number of haplotypes = 1 were not analyzed at the drainage scale, but all populations were included in regional-scale analyses (see Table 1). All haplotypes from Clades 4-1 and 4-2 were included in analyses. CYP = Cape York Peninsula, PNG = Papua New Guinea.

Region	Drainage	π	h	k	No. of haplotypes	F_s	D
Top End	Daly	0.003 $\pm$ 0.002	0.490 $\pm$ 0.123	2.480 $\pm$ 1.385	7	-0.226 (0.373)	-0.098 (0.565)
	Adelaide	0.010 $\pm$ 0.006	0.876 $\pm$ 0.037	8.733 $\pm$ 4.199	8	-0.095 (0.907)	-0.114 (0.569)
	Liverpool	0.001 $\pm$ 0.001	0.500 $\pm$ 0.128	0.500 $\pm$ 0.467	2	0.311 (0.664)	-0.052 (0.436)
	Blyth	0.004 $\pm$ 0.002	0.582 $\pm$ 0.092	3.209 $\pm$ 1.762	3	0.033 (0.961)	-0.094 (0.553)
	Region	0.015 $\pm$ 0.008	0.917 $\pm$ 0.015	12.661 $\pm$ 5.762	27	-0.525 (0.288)	-0.095 (0.570)
Northwestern CYP	Mitchell	0.001 $\pm$ 0.001	0.691 $\pm$ 0.128	1.164 $\pm$ 0.810	4	0.030 (0.398)	0.069 (0.531)
	Jardine	0.001 $\pm$ 0.001	0.772 $\pm$ 0.057	1.103 $\pm$ 0.759	5	-0.027 (0.266)	-0.079 (0.544)
	Region	0.002 $\pm$ 0.002	0.868 $\pm$ 0.028	2.236 $\pm$ 1.260	10	-0.267 (0.215)	-0.093 (0.568)
Wet Tropics	Russell	0.001 $\pm$ 0.001	0.389 $\pm$ 0.164	0.778 $\pm$ 0.622	2	0.157 (0.887)	-0.050 (0.525)
	Tully	0.001 $\pm$ 0.001	0.346 $\pm$ 0.172	0.364 $\pm$ 0.378	3	0.148 (0.125)	-0.055 (0.513)
	Region	0.003 $\pm$ 0.002	0.695 $\pm$ 0.070	2.663 $\pm$ 1.481	5	0.082 (0.794)	-0.091 (0.558)
PNG	Fly	0.006 $\pm$ 0.003	0.764 $\pm$ 0.107	4.655 $\pm$ 2.471	5	0.065 (0.801)	-0.061 (0.532)

phylogeographic break between the eastern and western parts of the Top End region is older than the among-region breaks, which date to the mid-Pleistocene (~ 300 – 500 k ybp; Fig. 3).

Statistical phylogeography

The ML tree indicated a number of well supported ($>70\%$ bootstrap support) clades related mainly to

populations at the river-basin scale (Supplemental file; available online from: <http://dx.doi.org/10.1899/09-087.1.s1>). The regional populations were not monophyletic, and haplotypes from PNG were polyphyletic with respect to Australian haplotypes. Nevertheless, haplotypes were not shared among regions or among drainages within regions, a result indicating significant phylogeographic structuring in the species. The 2 most divergent clades (Clades 4-1 and 4-2; Fig. 4) did

FIG. 2. Two-dimensional multidimensional scaling (MDS) plot of pairwise Φ_{ST} among drainage basins showing patterns of genetic differentiation among populations of *Denariusus bandata* from drainage basins and regions. Labels refer to drainage basins; regions are indicated by the fills in the diamonds. CYP = Cape York Peninsula, PNG = Papua New Guinea.

FIG. 3. Plot of estimated coalescent times for pairwise comparisons of regional populations. A = Top End vs northwestern Cape York Peninsula, B = Top End vs Wet Tropics, C = Top End vs Papua New Guinea, D = northwestern Cape York Peninsula vs Wet Tropics, E = northwestern Cape York Peninsula vs Papua New Guinea, F = Wet Tropics vs Papua New Guinea, G = Clade 4-1 vs Clade 4-2.

not receive high bootstrap support, but their distributions in the Top End region were interesting. Clade 4-2 was restricted to the western ½ of the region, and Clade 4-1 was present mainly in the eastern ½ of the region, except that Clade 4-1 also occurred in the Daly and Adelaide Rivers in the western ½ of the region (Table 1). Individuals from Clades 4-1 and 4-2 were sympatric at only 1 site, Marakai Crossing, in the Adelaide River drainage. The observed value of s for the ML tree was 6, indicating reasonable discordance between the ML genealogy and the regional population trees. However, this value is considerably smaller than values within the 95% CIs of s , given our 2 hypotheses (mean s values of 62–67 for scaling factors of 1.0 and 0.001, >26 for a scaling factor of 0.0000001). Thus, the ML genealogy and regional population trees align to a greater degree than would be expected under either hypothesis (simultaneous vicariance 10 k ybp, or sequential vicariance [eastern populations maintained in a refugium since the early Pleistocene and then the northern populations split 10 k ybp]).

The haplotype network indicated 2 divergent haplotype clusters (Clades 4-1 and 4-2; ~1.5% divergent) that were connected with 90% probability; all connections up to 12 steps were inferred with >95% confidence (Fig. 4). Within Clade 4-1, the most widespread haplotype cluster, the number of bp differences was often greater within than among regions, as shown by the large number of missing haplotypes between numerous haplotypes within regions, especially the Top End region (Fig. 4). Within Clade 4-2, the most distantly related haplotypes

(Clade 1-20 excluding Haplotype B and Clade 2-8; Fig. 4) were sympatric within the Adelaide River drainage (Table 1). Both the Top End and PNG regions had >1 grouping of haplotypes within Clade 4-1. The Daly River (Clade 2-1) was an allopatric subset of haplotypes distinct from all other rivers in the Top End region. In contrast, the divergent clusters of haplotypes from the PNG region were sympatric in the Fly River drainage, with each PNG cluster being more closely related to Australian haplotypes than to each other.

Nested clade phylogeographic analysis

Two reticulations that each linked only missing haplotypes were observed in the network. These reticulations were resolved using the method of Templeton et al. (1992) prior to NCPA. The geographic association of most 1st-step clades was not significant. Many clades at this level tended to have only a single extant haplotype or representation from a single drainage. The few 1st-step clades that were significantly associated with geography lead to inconclusive biological inferences caused by insufficient genetic resolution (Table 3). Results for 2nd- and 3rd-step clades mainly indicated past fragmentation or long-distance colonization as the inferred biological process giving rise to patterns at this level in the genealogy, although the analysis suggested that Clade 3-4 had molecular signatures of allopatric fragmentation (Table 3). The biological inference for Clade 4-1 was restricted gene flow/dispersal with some long distance dispersal over intermediate areas not occupied by the species, or past gene flow followed by extinction of intermediate populations. Conclusive biological inferences could not be made for Clades 4-2 or the total cladogram.

Discussion

We predicted that inundation of the land bridge on the ANGCS and associated wetland habitats (e.g., Lake Carpentaria and connected palaeo-river systems) by rising sea levels at the end of the last glacial maximum (~10 k ybp) would have had an important role in the formation of the present-day disjunct distribution of *D. bandata*. We hypothesized that fragmentation of all regional populations could have occurred contemporaneously in response to this marine incursion (simultaneous vicariance hypothesis) or that the eastern populations might have been isolated from the northern populations before the last glacial maximum and that only the northern populations underwent late Pleistocene simultaneous vicar-

FIG. 4. Haplotype network for *Denariusus bandata* indicating evolutionary relationships among haplotypes, nesting levels, and clades used in Nested Clade Phylogeographic Analysis (NCPA) (see Table 3). Small open circles between colored circles represent missing haplotypes. Numbers indicate clade names. Clades A and B represent single haplotypes within Clades 1-5 and 1-20, respectively. Each dark circle represents a haplotype; the size of each circle indicates the number of individuals with that haplotype, as shown in the legend, and the fill pattern of each circle indicates the region from which it was sampled as follows: black circles = Top End, dark grey circles = northwestern Cape York Peninsula, light blue circle = southeastern Cape York Peninsula, open circles = Wet Tropics, grey cross-hatched circles = Papua New Guinea. (See Table 1 for drainages from which each haplotype was sampled.)

TABLE 3. Results of nested clade phylogeographic analysis (NCPA) of *Denariusa bandata*. Dc = clade distance, Dn = nested clade distance, ^L = significantly large, ^S = significantly small, I-T = interior clade to tip clade. The 2nd column shows the nested clades within the significant clade (1st column) that are statistically associated with geography and whether the nature of the geographic association is the result of small or large distributions of the nested clade (2nd column) with respect to the distribution of significant clade (1st column). The inference chain numbers (3rd column) indicate the sequence of steps followed when Templeton's (2008) inference key was applied in NCPA of the *D. bandata* genealogy; the biological interpretation (4th column) presents the biological interpretation as presented in the inference key (available from: <http://darwin.uvigo.es/software/geodis.html>).

Significant clades	Nature of significant result	Inference chain	Biological Interpretation
Clade 1-5	Tip (Clade A) Dn ^L I-T Dn ^S	1-2-11-17: No	Inconclusive outcome
Clade 1-20	Tip (Clade B) Dn ^L I-T Dc ^L	1-2-3-5-6: Too few clades	Insufficient genetic resolution
Clade 2-2	Tip (Clade 1-3) Dc ^S , Dn ^L Interior (Clade 1-2) Dc ^S , Dn ^L I-T Dn ^S	1-2-3-5-15: No	Past fragmentation or long-distance colonization
Clade 2-4	Tip (Clade 1-10) Dc ^S Interior (Clade 1-11) Dc ^S , Dn ^S I-T Dn ^S	1-19-20-2-3-5-15: No	Past fragmentation or long-distance colonization
Clade 3-1	Tip (Clade 2-1) Dc ^S , Dn ^L Interior (Clade 2-2) Dc ^S , Dn ^S I-T Dn ^S	1-19-20-2-3-5-15: No	Past fragmentation or long-distance colonization
Clade 3-2	Tip (Clade 2-11) Dc ^S Tip (Clade 2-12) Dn ^L Interior (Clade 2-4) Dc ^S , Dn ^S I-T Dn ^S	1-2-3-5-15: No	Past fragmentation or long-distance colonization
Clade 3-4	Tip (Clade 2-7) Dc ^S , Dn ^L Interior (Clade 2-6) Dc ^S , Dn ^L I-T Dn ^S	1-19: No	Allopatric fragmentation
Clade 3-6	Tip (Clade 2-9) Dc ^S , Dn ^S Tip (Clade 2-10) Dn ^L	1-19-20-2-3-5-15: No	Past fragmentation or long-distance colonisation
Clade 4-1	Tip (Clade 3-3) Dc ^S Tip (Clade 3-4) Dc ^S , Dn ^L Interior (Clade 3-2) Dc ^S I-T Dn ^S	1-2-3-5-6-7-8: Yes	Restricted gene flow with some long distance dispersal or past gene flow and extinction of intermediate populations
Clade 4-2	Tip (Clade 3-5) Dc ^S Tip (Clade 3-6) Dn ^S	1-2-11-17: No	Inconclusive outcome
Total cladogram	Tip (Clade 4-1) Dn ^L Tip (Clade 4-2) Dc ^S	1-2-11-17: No	Inconclusive outcome

iance (sequential vicariance hypothesis). However, population splits of both mid- and early-Pleistocene origin were revealed, and statistical phylogeographic analysis indicated that the discord between the genealogy and the regional population tree should have been much greater than was observed had the regional populations been formed by inundation of the ANGCS 10 k ybp. Moreover, results of the demographic analyses were all nonsignificant, so the populations did not undergo bottlenecks in the recent past, as would have been expected if the populations were fragmented in the late Pleistocene. Thus, oscillating low sea level stands in the mid- to late-Pleistocene do not appear to have influenced the extant disjunct distribution or phylogeographic history of *D. bandata*. This result lends support to the suggestion by Unmack (2001) that aridity during late

Pleistocene glacial phases might not have facilitated formation of extensive freshwater habitats for dispersal by freshwater fish on the exposed ANGCS.

Recent phylogeographic studies have indicated that population splits in other freshwater fish species in northern Australia are older than the late Pleistocene. These studies have focused on sooty grunter, *Hephaestus fuliginosus*, between Australia and PNG (B. J. Pusey, A. Bently, D. Burrows, C. Perna, M. J. Kennard, C. Mills, and JMH, Australian Rivers Institute, Griffith University, unpublished data) and glass perchlet, *Ambassis* sp. (Huey et al. 2010), and eel-tailed catfish, *Neosilurus hyrtlui* (Huey et al. 2008), among Australian rivers draining to the Gulf of Carpentaria. Repeated genetic connectivity across the ANGCS in 3 genera of elapid (terrestrial) snakes is thought to have occurred in the late Miocene and early Pleistocene, rather than

in the late Pleistocene (Wüster et al. 2005). Populations of giant river prawn, *Macrobrachium rosenbergii*, from Australian rivers draining to the Gulf of Carpentaria (the intermediate geographical area where *D. bandata* does not occur) underwent a range expansion in the late Pleistocene, putatively associated with the timing of Lake Carpentaria (de Bruyn et al. 2004). However, even this species, which is catadromous and tolerant of brackish water, does not have a late Pleistocene association with southern PNG (Fly River) or more distant regions in Australia (de Bruyn et al. 2004). In contrast, populations from a genetic lineage of the redclaw crayfish, *Cherax quadricarinatus*, species complex from Australian rivers draining to the Gulf of Carpentaria have close genetic affinities with populations from the Bensback River in southern PNG (Baker et al. 2008).

The temporal expectations of our hypotheses were rejected, but the MDIV analyses clearly showed that the eastern populations diverged from the northern populations earlier than the northern populations diverged from one another, as expected under the sequential vicariance hypotheses. NCPA indicated that most haplotypes of the Wet Tropics region (Clade 3-2) arose by allopatric fragmentation, also in accordance with our expectations for the sequential vicariance hypothesis, although not all eastern haplotypes (including one from the Wet Tropics region) were contained within this clade. NCPA has recently received considerable criticism in the literature (Knowles 2008, Petit 2008; but see Templeton 2008) because it falsely fails to identify phylogeographic events (false negative rate for fragmentation is ~12%). Our identification of allopatric fragmentation for most haplotypes from the eastern region almost corresponds to a biologically realistic scenario, but the fact that NCPA identified this phylogeographic process within a regional population highlights limitations of the method. We suggest that nesting of haplotypes within a relatively complex and shallow genealogy might force creation of clade levels that do not necessarily reflect most recent historical genetic associations among haplotypes, and thus, might create error in interpretation of phylogeographic events. Sequence data from a more rapidly evolving gene (e.g., mitochondrial control region) might have enabled the eastern populations to be contained within a significant 3rd-step clade level. The sequential vicariance hypothesis was rejected under the statistical phylogeographic approach (Knowles 2001, Knowles and Maddison 2002) because the approach uses very explicit temporal bounds for inferring phylogeographic processes based on the a priori hypothesis (in our study, the end of the last glacial

maximum). Templeton (2008) suggested that the very explicit and, thus, limited number of hypotheses that statistical phylogeography can assess is a limitation of the method. Our analyses suggest that coupled testing of processes and time is restrictive in phylogeographic analyses. Post hoc implementation of this method with revised temporal expectations based on the genetic data (e.g., MDIV analyses) might facilitate discrimination between competing phylogeographic processes.

NCPA could not discriminate between past fragmentation or long-distance colonization for 2nd- and most 3rd-step clades. This problem reduces the method's usefulness for answering fundamental phylogeographic questions, such as discriminating dispersal and vicariance, when phylogeographic patterns are complex. We suggest that a large population of *D. bandata* existed on the ANGCS during a historical period of lower sea levels, perhaps in the Miocene or Pliocene. This population became fragmented by rising sea levels, which occurred repeatedly in the region (Hewitt 2000, Voris 2000). Evidence for the existence of this large historical population includes the retention of genetic diversity by the Fly River population and the polyphyletic relationship of Fly River haplotypes with the Australian haplotypes. MDIV analyses suggest that the northern populations might have become fragmented contemporaneously from one another, although molecular signatures also indicated substantial phylogeographic structuring within the Top End region (see below). Thus, we suggest that past fragmentation rather than long-distance colonization explains phylogeographic patterns of the 2nd- and most 3rd-step clades. NCPA suggested 2 biological interpretations for Clade 4-1 (past gene flow with extinction of intermediate populations and restricted gene flow with some long-distance colonization). However, we invoke the former interpretation because the large historical population probably extended over geographic areas that are intermediate to the present-day disjunct regional populations. This suggested extinction of intermediate historical populations of *D. bandata* accords with the global pattern of late Pleistocene extinctions of biota (Hewitt 2000).

The phylogeographic patterns in *D. bandata* in the western part of the Top End region are interesting and indicate 2 temporal episodes of ancestral lineage retention in this area. First, within Clade 4-1, haplotypes from the Daly River drainage (Clade 2-1, Top End region) were more closely related to haplotypes from the Jardine River drainage (north-western Cape York Peninsula region) than to other haplotypes from the Top End region. This pattern

suggests the possibility that the Daly River population has been isolated from the more easterly populations (from both the Top End and northwestern Cape York Peninsula regions) for a greater length of time than the regional populations have been isolated from one another and that stochastic lineage sorting processes have facilitated the unexpected phylogeographic pattern. A second pattern of retention of divergent haplotypes is shown by the restriction of Clade 4-2 to this geographic area. This pattern suggests that 2 highly isolated populations of *D. bandata* have persisted throughout most of the Pleistocene (~1.5 m ybp), and that only 1 clade (Clade 4-1) expanded its range to all 5 regions, while a cryptic barrier to range expansion restricted Clade 4-2 to the western Top End region. Similar old and cryptic barriers to range expansion have facilitated the retention of ancestral lineages within Melanotaeniid fishes in northern PNG (McGuigan et al. 2000) and in some terrestrial fauna in southern PNG, such as palm cockatoos, *Probosciger aterrimus* (Murphy et al. 2007). Retention of ancestral lineages in the Top End region might be one explanation for the absence of an isolation-by-distance pattern in the genetic data. Clades 4-1 and 4-2 are sympatric only in the Adelaide River, probably because of secondary contact facilitated by recolonization by Clade 4-1. Consequently, this drainage had the highest measures of molecular diversity of all drainages.

Environmental change during the Pleistocene has had a profound influence on species distributions and their phylogeographic patterns globally (Hewitt 2000). Our phylogeographic analysis of *D. bandata* shows a complex Pleistocene history of connectivity and fragmentation events at various spatial and temporal scales coupled with the geographically restricted retention of ancestral lineages (in the western part of the Top End Region) and ancestral genetic diversity (in the Fly River drainage). However, these processes did not occur in the late Pleistocene in response to sea level rise at the end of the last glacial maximum as we predicted. Instead, exposure of the ANGCS during low sea level stands in the early- to mid-Pleistocene were the important landscape changes for the biogeography and phylogeography of *D. bandata*. Sea level changes in ANGCS region have a complex and poorly understood influence on the biogeography of freshwater fauna (Unmack 2001), although the influence of the Pleistocene land bridge for phylogeographic structuring in various marine (Lavery et al. 1996, Chenoweth et al. 1998, Gopurenko and Hughes 2002) and terrestrial (Norman et al. 2002, Rawlings and Donnellan 2003, Wüster et al. 2005, Murphy et al. 2007)

species across Torres Strait is comparably well documented. Ongoing phylogeographic research on freshwater fish in the region will determine the importance of Pleistocene sea level changes for dispersal and vicariance in this biota and will identify whether the existence of Lake Carpentaria in the late Pleistocene influenced the biogeographic history of other freshwater fishes, as shown for some freshwater crustaceans (de Bruyn et al. 2004, Baker et al. 2008).

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