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Population structure in Chilean hake *Merluccius gayi* as revealed by mitochondrial DNA sequences

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Genetic variation and divergence among samples of Chilean hake *Merluccius gayi*, from three localities off the coast of Chile and one locality off the coast of northern Peru, were assessed using sequences from the control region of mitochondrial DNA. Homogeneity tests revealed occurrence of at least three distinct genetic stocks of *M. gayi* within the region sampled. Factors potentially contributing to genetic divergence among *M. gayi* probably include hydrodynamics and behaviour.

Key words: Chile; genetic variation; Pacific Ocean; Peru; stock structure; South America.

The Chilean hake *Merluccius gayi* (Guichenot 1848) is a demersal marine fish found in cold, poorly oxygenated, coastal upwelling ecosystems along the Pacific Ocean coast of South America from southern Chile to northern Peru. The species is mainly concentrated between 100 and 200 m depth (Guevara-Carrasco & Lleonart, 2008) and supports an extremely important bottom-trawl fishery in both countries, especially in Chile where there are both domestic and export markets. Annual landings of *M. gayi* in Chile historically exceeded 100 000 t (Giddings, 1980). Since 2000, however, there have been declines in length at catch, proportion of mature females, catch per unit effort and adult biomass (Arancibia & Neira, 2008). The species is fully exploited in Chile (Payá & Ehrhardt, 2005), but remains an important low-cost, fresh-market commodity throughout most of the country.

The *M. gayi* resource is managed in Chilean waters as a single stock (Aguayo-Hernández, 1995), consistent with a study by Galleguillos *et al*. (2000) who found no differences in allele frequencies at six allozyme loci among geographic samples spread across a distance of *c*.1335 km. Galleguillos *et al*. (2000) did report heterozygote deficiencies at *idh*-1 between samples from one locality and at *aat*-1 between samples from another locality, suggesting possibly a mixture of different subpopulations (Wahlund, 1928). George-Nascimiento (1996) and Oliva & Ballón (2002) suggested that there were two ecological stocks of *M. gayi* along the Chilean

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coast, based on consistent geographic differences in abundance of metazoan parasite assemblages. A tag-and-release experiment of *M. gayi* in Chilean waters (Villegas & Saetersdal, 1968) indicated that adult *M. gayi* can move considerable distances.

In this study, sequences of mitochondrial (mt)DNA acquired in an earlier, unpublished study, undertaken in 1998–1999, involving four geographic samples of *M. gayi* (three from Chile) taken along the central coast of South America were analysed. Because mtDNA can be more sensitive to demographic disruptions than nuclear-encoded genes (Birky *et al*., 1983), the current stock-structure model for *M. gayi* in Chilean waters was re-examined and the null hypothesis that *M. gayi* along the Chilean coast are a single genetic stock was tested. The reasons were two-fold: (1) population structure is a central issue for management of fishery resources (Begg *et al*., 1999; Hilborn *et al*., 2003), especially if a species is fully exploited and there is a possibility of overfishing a non-identified stock (Ruzzante *et al*., 1999) and (2) there are simply too few resources available at present to undertake a more comprehensive study.

The fourth sample of *M. gayi* was from Peruvian waters. This was included in part because *M. gayi* in Peruvian waters is considered a different subspecies: *Merluccius gayi peruanus* Ginsburg 1954 (Peruvian waters) and *Merluccius gayi gayi* (Guichenot 1848) (Chilean waters). The two subspecies differ in morphology (Ginsburg, 1954; Inada, 1981) and allozyme alleles at three monomorphic loci (Hernandez *et al*., 2000), and are separated physically by a narrow continental shelf and concentrations of hydrogen sulphide in bottom layers in the region between their non-overlapping ranges (Inada, 1981). The two subspecies are managed essentially as two distinct stocks (Aguayo-Hernández, 1995; Espino *et al*., 1995).

Muscle tissues from fish sampled at four offshore localities (Fig. 1) were obtained from the commercial fishery. Latitude and longitude for each sample are as follows: Peru 7° S; 79° W, Coquimbo 29° S; 71° W, Valparaíso 33° S; 71° W and Corral 39° S; 73° W. Tissues were fixed in non-denatured ethanol and stored at room temperature. Samples were obtained during the commercial fishing season: the Austral spring in Chile and the Austral winter in Peru. Extraction and storage of genomic DNA followed Quintero *et al*. (2000). Polymerase chain reaction (PCR) primers L15998 (Alvarado-Bremer, 1994) and heavy-strand primer B (Lee *et al*., 1995) were used to amplify a 395 base pair (bp) fragment of the mitochondrial control region. Amplified mtDNA fragments were sequenced using an ABI PRISM 377 (Applied Biosystems; www.appliedbiosystems.com). Sequences were compiled and checked manually for accuracy. CLUSTAL-W (Thompson *et al*., 1994) was used for multiple sequence alignments; mtDNA sequences were deposited in GenBank.

Summary statistics were computed using DnaSP, v5.10.01 (Rozas *et al*., 2003). Homogeneity in haplotype number and haplotype diversity was tested using a bootstrap re-sampling approach. PopTools (www.poptools.org) was used to generate random samples of 20 haplotypes from the overall dataset of 80 individuals; the expected average number of haplotypes, average haplotype diversity and their upper (0·975) and lower (0·025) percentiles were recorded based on random sampling (1000 iterations) under Monte-Carlo simulation. Observed values were considered to differ significantly from expected values under the null hypothesis if they fell outside the obtained confidence intervals. Selective neutrality of mtDNA was tested *via* Fu's (1997) F_S statistic and Fu & Li's (1993) D^* and F^* statistics, with significance of F_S,

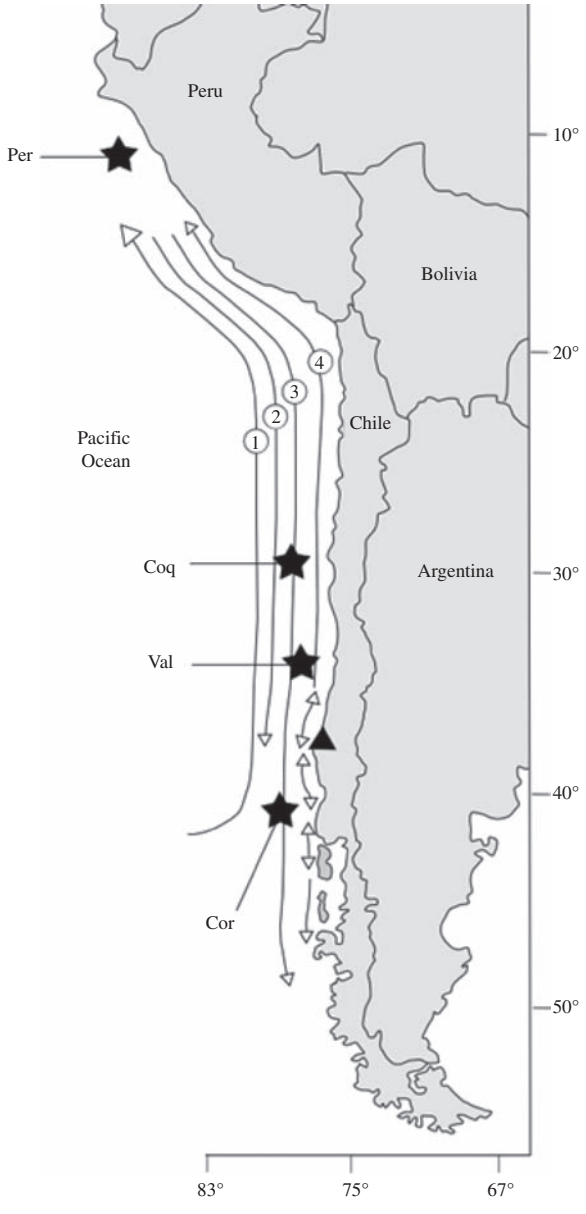

FIG. 1. Approximate collection localities (★) for samples of *Merluccius gayi*. Per, Peru; Coq, Coquimbo; Val, Valparaíso; Cor, Corral. 1, Peru–Chile Current; 2, Peru–Chile Counter Current; 3, Peru–Chile Undercurrent; 4, Chile Coastal Current. Information on currents was obtained from Payá & Ehrhardt (2005), based on a study by Leth (2000). ▲, the town of Talcahuano.

*D** and *F** assessed from 10 000 coalescent simulations (Rozas *et al*., 2003). A minimum spanning network of mtDNA haplotypes was constructed in Fluxus Network v4.6 (http://fluxus-engineering.com), using the full median-joining algorithm (Bandelt *et al*., 1999). Epsilon and character weights for the median-joining algorithm

were set to the default value (= 0), with the exception of character weights at indel sites, which were double weighted (= 20), as recommended in the user manual; additional testing of higher and lower weights did not change network structure.

Homogeneity of mtDNA haplotype distribution was tested *via* exact tests and analysis of molecular variance (AMOVA) as implemented in Arlequin (Excoffier & Lischer, 2010). Pair-wise estimates of ϕ_{ST}, generated using Arlequin, were tested for homogeneity, using exact tests. Isolation by distance was assessed *via* spatial autocorrelation analysis (Smouse & Peakall, 1999) as implemented in GenAlEx v. 6.0 (Peakall & Smouse, 2006). Geographic distance between individual localities was determined from longitude and latitude. Multiple distance-class sizes (0–500, 0–1000 and 0–3000 km) were determined based on the distribution of geographic distances between localities. Significance of spatial autocorrelation (r) was determined *via* random permutations of genotypes of individuals sampled in each locality, following (Saillant *et al*., 2010). Significance of r also was tested by generating 95% C.I. for r, using 1000 bootstrap values obtained by sampling pairs of localities within a given distance class. Significance of r was inferred when the 95% C.I. did not overlap zero.

A total of 20 mtDNA haplotypes was detected among the four localities (Appendix). Variable positions within the 395 bp fragment included 23 base substitutions (18 transitions and five transversions) and three one-base deletions. Haplotypes that contained one-base deletions are designated with a subscript. Haplotypes B_1 and B_2 contained deletions at nucleotide sites 79 and 150, respectively, but were otherwise identical to haplotype B; haplotype H_1 contained a deletion at nucleotide site 3 and remained unique even if the deletion was not considered.

Summary statistics for the four localities are presented in Table I. Bootstrap resampling indicated that the observed number of haplotypes in the samples from Peru, Coquimbo and Valparaíso deviated significantly from the lower (0·025) percentile of 8, generated from random subsamples of the overall dataset; observed haplotype diversity in the sample from Valparaíso was marginally (but significantly) less than the lower (0·025) percentile of 0·832. The reduced haplotype variability observed could in theory reflect reduced female effective size. Tests of selective neutrality were non-significant for all four localities. The minimum spanning network of mtDNA haplotypes (Fig. 2) consisted of two Steiner trees that were identical in topology except for the relationship of haplotype L to haplotype C, where two alternative connections had 50% support each. Haplotypes found in samples from Chilean waters were included in the three principle clusters; except for divergent haplotype L, haplotypes in the sample from Peru were related to haplotype A. Haplotype L differed from haplotype A by either six or seven base substitutions.

Significant heterogeneity among the four localities in mtDNA haplotype distribution was indicated by a global exact test ($P < 0{\cdot}001$) and AMOVA ($F_{ST} = 0{\cdot}093$, $P < 0{\cdot}001$). AMOVA also revealed significant heterogeneity ($F_{ST} = 0{\cdot}083$, $P < 0{\cdot}001$) among the three localities in Chile. Pair-wise ϕ_{ST} values (Table II) ranged between 0·070 (Coquimbo *v*. Valparaíso) and 0·123 (Peru *v*. Corral). The comparison of Coquimbo *v*. Valparaíso was significant before but not after sequential Bonferroni correction; the remaining comparisons were significant before and after correction. Significant, positive autocorrelation (r), based on both bootstrapping and random permutation (Table III), was observed for distance classes 0–500 and 0–1000 km;

TABLE I. Summary statistics for the 395 base pair sequence of the mitochondrial control region among four geographic samples of *Merluccius gayi* (see Fig. 1)

Variable	Peru	Coquimbo	Valparaíso	Corral
Sample size	20	20	20	20
Haplotype number	6*	7*	6*	9
Haplotype diversity	0·832	0·889	0·826*	0·905

*Values significantly lower than expectation based on random subsampling 20 mtDNA haplotypes from the overall dataset of 80 individuals.

the *r* value for the distance class 0–3000 km was significant for confidence intervals generated by random permutation but not for intervals generated by bootstrapping.

Genetic separation of *M. gayi* in Peruvian waters (recognized as *M. g. peruanus*) from those in Chilean waters (recognized as *M. g. gayi*) is consistent with the prior study by Hernandez *et al*. (2000), who found fixed differences at three monomorphic allozyme loci between *M. g. peruanus* and *M. g. gayi*, and with the long-held assumption that the two constitute distinct stocks (Aguayo-Hernández, 1995; Espino *et al*., 1995). Reduced gene flow between Peruvian and Chilean *M. gayi* also is consistent with the narrow continental shelf and presence of hydrogen sulphide in bottom layers in the region between the non-overlapping ranges of the two subspecies (Inada, 1981). The minimum spanning network of mtDNA haplotypes did not reveal monophyletic clades containing only haplotypes from any of the localities, suggesting that mtDNA lineage sorting (Avise *et al*., 1984) in *M. gayi* from Peru may be incomplete. All but one of the haplotypes found in the sample from Peru were related to haplotype A. The exception was haplotype L, which differed from

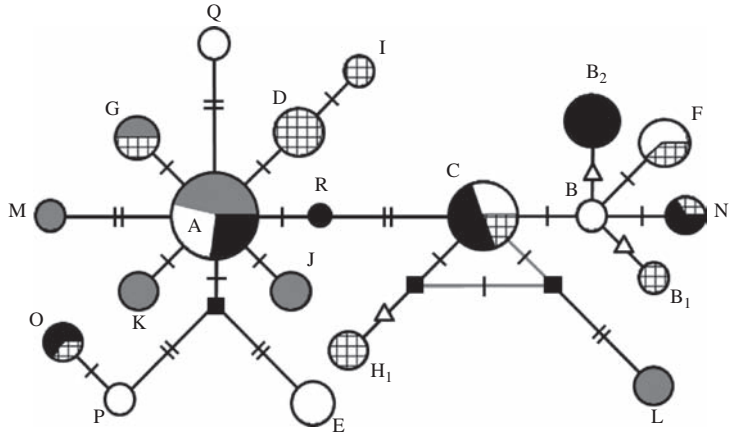

FIG. 2. Minimum spanning network of 20 mitochondrial (mt)DNA haplotypes of *Merluccius gayi*. Haplotypes sampled from Peru (●) and from localities along the coast of Chile [Coquimbo (○), Valparaíso (●) and Corral (⊕)]; haplotypes that differ in base substitutions are identified by an upper-case letter and haplotypes that differ by a one-base deletion are identified by a letter with a numeric subscript. Circle size is scaled to haplotype frequency. ▮, single-base substitutions; △, single-base deletions; ■, undetected mtDNA haplotypes.

TABLE II. Probability values from pair-wise exact tests of homogeneity in mitochondrial (mt)DNA haplotype distributions among four geographic samples of *Merluccius gayi* (see Fig. 1). Upper diagonal, ϕ_{ST} estimates; lower diagonal, probability (P) that $\phi_{ST} = 0$. The comparison of Coquimbo *v*. Valparaíso was significant before but not after sequential Bonferroni correction; the remaining comparisons were significant before and after correction

	Peru	Coquimbo	Valparaíso	Corral
Peru	–	0·075	0·109	0·123
Coquimbo	0·009	–	0·070	0·075
Valparaíso	0·002	0·015	–	0·103
Corral	0·000	0·001	0·000	–

haplotype A by either six or seven base substitutions. Occurrence of haplotype L in Peruvian waters conceivably could stem from a sporadic migrant from Chilean waters during El Niño Southern Oscillation (ENSO) events when the southern limit of the Peruvian subspecies may be shifted to *c*. 18° S (Espino *et al*., 1995).

Occurrence of at least two (mtDNA-based) genetic units of *M. gayi* in Chilean waters, one to the north represented by the samples from Valparaíso and Coquimbo and one to the south represented by the sample from Corral, is generally consistent with studies based on geographic differences in metazoan parasites infecting *M. gayi*. George-Nascimiento (1996) found geographic differences in overall abundance of several parasites and hypothesized that one ecological stock of *M. gayi* was situated in the south near Corral, while a second stock was situated to the north in the area between Talcahuano (*c*. 430 km to the south of Valparaíso) and Coquimbo. Oliva & Ballón (2002) also found geographic differences in parasite abundance and hypothesized that one stock of *M. gayi* occurred in the area around Puerto Montt (south of Corral), while a second stock occurred between Talcahuano and Coquimbo.

TABLE III. Estimated autocorrelation (r) values and 95% C.I. from spatial autocorrelation analysis for multiple distance class sizes, based on bootstrap re-sampling and random permutation of genotypes

	Distance class size		
	0–500 km	0–1000 km	0–3000 km
Bootstrap			
r	0·028	0·020	0·006
Upper C.I.	0·046	0·034	0·018
Lower C.I.	0·012	0·005	−0·004
Permutation*			
r	0·029	0·020	0·007
Upper C.I.	0·011	0·008	0·004
Lower C.I.	−0·007	−0·004	−0·003
P	<0·001	<0·001	<0·01

*Upper and lower confidence limits in permutation bound the 95% C.I. about the null hypothesis of no spatial structure ($r = 0$). P is the probability that $r = 0$.

Taken together, the mtDNA and parasite data support the hypothesis of two stocks of *M. gayi*: one to the south of Talcahuano and one to the north of Talcahuano.

Mechanisms affecting gene flow in *M. gayi* and divergence of different genetic stocks along the Chilean coast probably include both hydrodynamic and behavioural factors. Circulation patterns off the Chilean coast (Fig. 1) include both northward and southward flowing currents; one of these, the subsurface Peru–Chile Undercurrent or Günter's Current, begins at 6° S and ends around 48° S and is primarily responsible for the major upwelling that occurs along the Chilean coast (Payá & Ehrhardt, 2005). Data on the density of eggs and larvae of *M. gayi* (Aguayo-Hernández, 1995; Bernal *et al*., 1997; Vargas & Castro, 2001) indicate that there are at least two principle spawning areas along the Chilean coast: one in the central area (32–34° S) near Valparaíso and one to the south (35–37° S) near Talcahuano. The primary spawning season is in the Austral spring and coincides with the onset of the upwelling season (Alarcón & Arancibia, 1993). Both Vargas *et al*. (1997) and Payá & Ehrhardt (2005) hypothesized that the upwelling produces eddies and filaments that could favour retention of eggs and larvae at or near the breeding grounds. There is also a secondary spawning season that occurs in the late Austral summer in association with upwelling produced by the transition from south to north winds (Aguayo-Hernández, 1995; Landaeta & Castro, 2012). Interestingly, spawning adults during the primary spawning season are older and larger (>50 cm total length, L_T) and spawn *c*.50–60 km offshore (Vargas *et al*., 1997), whereas spawning adults during the secondary spawning season are younger and smaller (<50 cm L_T) and spawning occurs in shallower waters and within gulfs and bays (Alarcón *et al*., 2004; Landaeta & Castro, 2006). Bernal *et al*. (1997) and Landaeta & Castro (2012) hypothesized that homing behaviour might be responsible for movement to either the primary or secondary (or both) spawning centres. In addition, as movement to the secondary spawning centres appears to involve individuals of different size, there also is the possibility of size-related assortative mating.

The significant isolation-by-distance effect on divergence of mtDNA in *M. gayi* is consistent with limited north–south movement, whether due to hydrodynamic retention of eggs and larvae or homing behaviour to central spawning grounds. Generally, latitudinal movement of post-larval *M. gayi* is thought to be northwards to spawning grounds during the early Austral spring and southwards (because of prey availability) during the late Austral summer (Aguayo-Hernández, 1995). A lone tag-and-recapture experiment was carried out in the 1960s by Villegas & Saetersdal (1968). Their experiments confirmed the northward movement of adults in the spring and the southward movement in the early autumn, and their study is often cited as demonstrating that the latitudinal movement of *M. gayi* is extensive as tag recoveries occurred over 350 km from the tagging site. Inspection of the recapture data in Villegas & Saetersdal (1968), however, reveals that the distribution of recoveries in the two largest experiments was generally leptokurtic, with the majority of recoveries (*c*. 60% in one experiment and 75% in another) occurring up to 18·5 km from the release site. The tag-and-recapture data, the significant isolation-by-distance effect and the distance between the two principal spawning areas near Valparaíso and Talcahuano (>400 km) suggest there is relatively little female migration between these two spawning areas.

Results of this study indicate occurrence of discrete genetic units (stocks) of *M. gayi* along the western coast of South America. The data are sequences of mtDNA,

meaning that inferences drawn relate only to females. Nuclear sequences need to be assessed to determine if patterns of genetic divergence, including isolation by distance, are congruent for biparentally inherited genetic markers. A number of PCR primers that amplify nuclear-encoded microsatellites in related species of *Merluccius* (Machado-Schiaffino & Garcia-Vazquez, 2009; Renshaw *et al*., 2011) are available for such study. It also will be important to sample individuals from discrete primary and secondary spawning areas and in successive years, in part to examine the temporal stability of genetic divergence and the isolation-by-distance effect, and in part to assess whether the size and locality differences observed between spring and early autumn spawning fish (Alarcón *et al*., 2004; Landaeta & Castro, 2006) reflect different genetic stocks. More precise establishment of genetic boundaries and their temporal stability will be important in future management planning of *M. gayi* resources in Chilean waters.

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APPENDIX. Spatial distribution of mitochondrial (mt)DNA haplotypes and their GenBank accession number among four geographic samples of *Merluccius gayi*

Haplotype	Peru	Coquimbo	Valparaíso	Corral	Total	GenBank
A	7	4	4	–	15	JQ082371
B	–	2	–	–	2	JQ082372
B_1	–	–	–	2	2	JQ082373
B_2	–	–	6	–	6	JQ082374
C	–	3	5	2	10	JQ082375
D	–	–	–	5	5	JQ082376
E	–	4	–	–	4	JQ082377
F	–	3	–	2	5	JQ082378
G	2	–	–	2	4	JQ082379
H_1	–	–	–	3	3	JQ082380
I	–	–	–	2	2	JQ082381
J	3	–	–	–	3	JQ082382
K	3	–	–	–	3	JQ082383
L	3	–	–	–	3	JQ082384
M	2	–	–	–	2	JQ082385
N	–	–	2	1	3	JQ082386
O	–	–	2	1	3	JQ082387
P	–	2	–	–	2	JQ082388
Q	–	2	–	–	2	JQ082389
R	–	–	1	–	1	JQ082390
Total	20	20	20	20	80	