

Origin of a Spanish population of *Fundulus heteroclitus* inferred by cytochrome *b* sequence analysis

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Fundulus heteroclitus in the estuary of the Guadalquivir River in southern Spain competes with a native killifish, *Aphanius iberus*. Cytochrome *b* sequence analysis shows that the Spanish individuals are derived from the northern of two possible American populations of *F. heteroclitus*. © 1995 The Fisheries Society of the British Isles

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In the last 50 years, interest in the phenomenon of colonization of new habitats by immigrating organisms has increased considerably. Advances in transportation, construction and industrial technologies have enabled the transfer of plants and animals to areas not occupied previously. Despite efforts by ecologists and geneticists, no universal criteria for predicting successful potential colonizers have been formulated (Ehrlich, 1986). Moreover, factors that determine colonization success appear to be so diverse that the formulation of universal criteria is impossible (Safriel & Ritte, 1980; Golani, 1993). A unique opportunity for the study of colonization events was the involuntary introduction of *Fundulus heteroclitus* L. in southern Spain. The history of *F. heteroclitus* introduction in Spain is difficult to retrace. Its introduction might be fairly ancient. Machado-Nunez as early as 1857 finds a Mediterranean coast species *Valencia hispanica* in the south of the Iberian Peninsula. Later, Buen (1930, 1935) renames it *Fundulus hispanicus*. More recently, possibly the same species from southern Spain was described as a new species, *Valencia lozanoi* (Gomez-Caruana *et al.*, 1984). *Valencia lozanoi* was finally synonymized with *F. heteroclitus* (Fernandez-Delgado *et al.*, 1986). Fernandez-Delgado (1989) proposes a later date of introduction, around 1970, probably when two other American species were voluntarily introduced in this region: the largemouth bass (*Micropterus salmoides* Lacépède) and the crayfish (*Procambarus clarki*).

F. heteroclitus is a killifish that inhabits the estuary waters of the East Coast of the United States and Canada. Populations living from the cold waters of Nova Scotia, to the warmer waters of Florida have been shown to differ at the behavioural, fitness, physiological, and DNA level (Powers *et al.*, 1993). In this study we have tried to determine from which of the American populations the Spanish individuals are derived. Since the American populations were shown to be adaptationally different, our long-term goal will be to investigate the fitness of the Spanish population relative to its new environment, and its efficiency to compete with a local killifish *Aphanius iberus* (Valenciennes).

Cytochrome *b* (Cyt *b*) sequences for North American *F. heteroclitus* and for its close relative *F. grandis* Baird & Girard were taken from the literature (Bernardi *et al.*, 1993; Bernardi & Powers, 1994). We will refer in this paper to northern population for

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individuals from Nova Scotia and Maine, and southern population for individuals from Georgia and Florida. Spanish specimens of *F. heteroclitus* were collected from the estuary of the Guadalquivir River (Lebrija, Sevilla) in southern Spain. DNA was extracted from liver tissue of ethanol preserved samples following Bernardi & Bernardi (1990). The polymerase chain reaction (Saiki *et al.*, 1988) was used to amplify a region of the Cyt *b* gene starting at amino acid 34 (human sequence used as a reference). Primers and protocols were from Kocher *et al.* (1989) and Palumbi *et al.* (1991). Approximately 100 ng of DNA were used as template for 100 μ l polymerase chain reactions (PCR) that contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin, 200 mM of each dNTP, 2.5 units of *Taq* DNA polymerase (Perkin-Elmer, Cetus) and 1 μ M of each amplification primer. PCR products were sequenced directly using the femtomole sequencing kit (Promega) and dATP³³ (NEN) as the radiolabelled nucleotide. Sequencing primers used were CB2-H, CB1-L, and GLUDG-L (Palumbi *et al.*, 1991). The sequences presented in this manuscript were deposited in GenBank with the following accession numbers: L31593 to L31599, and L34411. Sequences were aligned using the software Geneworks (IntelliGenetics, Mountain View, CA).

Phylogenetic analyses employed maximum parsimony (MP) and neighbour-joining (NJ). The most parsimonious trees were obtained using the Branch and Bound option of PAUP (Swofford, 1993). The NJ tree (Saitou & Nei, 1987) was generated using PHYLIP (Felsenstein, 1989). The degree of confidence assigned to nodes in trees obtained by MP or NJ was determined by bootstrapping (Felsenstein, 1985) with 2000 replicates (Hedges, 1992).

Nucleotide sequences for ingroup and outgroup taxa for a 268 bp segment of the Cyt *b* gene were aligned. Of 268 aligned positions, 27 were variable, and five were phylogenetically informative. Transitions were more frequent than transversions with an average transition/transversion ratio of 4.4. This ratio indicates that the sequences compared are not deeply within the multiple-hit zone (Brown *et al.*, 1982; Meyer & Wilson, 1990) and can be used for phylogenetic analysis. Cyt *b* sequences from Spanish individuals had an average similarity of 97.5% with the *F. heteroclitus* sequences and were found within the *F. heteroclitus* clade (Fig. 1). Thus confirming the renaming of *V. lozanoi* into *F. heteroclitus* (Fernandez-Delgado *et al.*, 1986). No variable sites were found between the three sequences from the Spanish individuals. A contamination during the amplification procedure is unlikely since negative and positive controls were always used, and that the three individuals were amplified at intervals of several weeks.

A single most parsimonious tree was found by MP and was identical to the tree obtained using NJ (Fig. 1). The MP tree had a length of 27 steps and a consistency index of 1.0. Both methods of phylogenetic reconstruction (MP and NJ) grouped the Spanish individuals with the northern population (Fig. 1) with a high statistical support. This grouping was found in 90 and 100% of bootstrap replicates for the MP and NJ methods, respectively.

The introduction of exotic species is usually associated with risk of environmental disturbance. Such was the case for the introduction of the red swamp crayfish in the estuary of the Guadalquivir River, possibly concomitantly with *F. heteroclitus* (Delibes & Adrian, 1987). Three small fish species coexist with *F. heteroclitus* in the Guadalquivir estuary, *Aphanius iberus*, *Gambusia affinis* (Baird & Girard), and *Atherina boyeri* Risso (Fernandez-Delgado, 1989). *F. heteroclitus* is now thriving in the hypersaline waters of the estuary, and is likely to compete only with the native *A. iberus*. Whether the alien species affects the *A. iberus* population is not known at present.

Several features of the northern population might have been conserved or lost after their introduction in Spain, including the following:

(i) *Genetic variability*. The northern population has a lower genetic diversity than the southern population (Gonzales-Villasenor & Powers, 1990; Bernardi *et al.*, 1993). Our results seem to indicate a low genetic diversity for the Spanish population as well. However, the reduced sample size, the type of sequence analysed, the possible founder effect, might be evoked to explain this result. Further analysis with a larger sample size will determine the degree of homogeneity of the Spanish population.

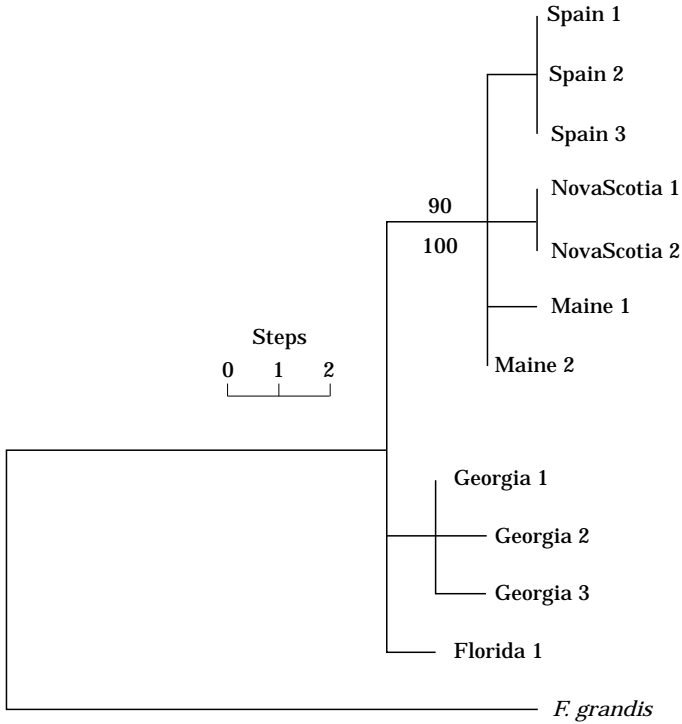


FIG. 1. Tree derived from 268 bp of the cytochrome *b* gene from *Fundulus heteroclitus*. Five localities were studied: northern population: Nova Scotia and Maine; southern population: Georgia and Florida; Spain: estuary of the Guadalquivir River. The most parsimonious tree is shown (length=27 steps, C.I.=1.0). Numbers on branches indicate results of bootstrapping (2000 replicates) using maximum parsimony (above) or neighbour-joining (below). Only values grouping Spanish and American populations are shown. Bar corresponds to two steps in maximum parsimony analysis.

(ii) *Meristics, morphometrics, and egg size.* Northern and southern populations show differences at the morphological, meristic, and egg size level (Able & Felley, 1986). Morphological and meristic descriptions of *F. heteroclitus* from Spain (Gomez-Caruaña *et al.*, 1984) are unfortunately not comparable with the results obtained from American individuals (Able & Felley, 1986). Egg sizes have been scored from both Spanish and American populations. Average egg sizes from northern and southern populations were 1.89 and 2.18 mm, respectively (Marteinsdottir & Able, 1988). The average egg size from the Spanish population was 1.90 mm (Drake *et al.*, 1987). The Spanish fish seem to have retained the egg size characteristics from their original population.

(iii) *Physiological and molecular adaptation.* Northern and southern populations were physiologically different (Powers *et al.*, 1993). Their swimming abilities at cold temperatures, egg development timing, and metabolic enzyme levels were shown to be genetically determined. The study of these characteristics in the newly introduced Spanish population will be the subject of our future investigations.

References

- Able, K. W. & Felley, J. D. (1986). Geographical variation in *Fundulus heteroclitus*. Test for concordance between egg and adult morphologies. *American Zoologist* **26**, 145–157.
- Bernardi, G. & Bernardi, G. (1990). Compositional patterns in the nuclear genome of cold-blooded vertebrates. *Journal of Molecular Evolution* **31**, 265–281.

- Bernardi, G., Sordino, P. & Powers, D. A. (1993). Concordant mitochondrial and nuclear DNA phylogenies for populations of the teleost fish *Fundulus heteroclitus*. *Proceedings of the National Academy of Sciences of the U.S.A.* **90**, 9271–9274.
- Bernardi, G. & Powers, D. A. (1994). Phylogenetic relationships among nine species from the genus *Fundulus* (Cyprinodontiformes, Fundulidae) inferred from sequences of the cytochrome b gene. *Copeia* **1995**(2), 469–473.
- Brown, W. M., Prager, E. M., Wang, A. & Wilson, A. C. (1982). Mitochondrial DNA sequences of primates: Tempo and mode of evolution. *Journal of Molecular Evolution* **18**, 225–239.
- Buen, F. de. (1930). Notas de sobre la fauna ictiológica de nuestras aguas dulces. *Notas y Resúmenes del Instituto Español de Oceanografía. Serie II*, **46**, 1–62.
- Buen, F. de. (1935). Fauna ictiológica. Catálogo de los peces ibéricos: de la planicie continental, aguas dulces, pelágicos y de los abismos próximos. *Notas y Resúmenes del Instituto Español de Oceanografía. Serie II*, **88/89**, 1–149.
- Delibes, M. & Adrian, I. (1987). Effects of crayfish introduction on otter *Lutra lutra* food in the Doñana National Park, SW Spain. *Biological Conservation* **42**, 153–159.
- Drake, P., Arias, A. M. & Sarasquete, M. C. (1987). Reproducción de *Fundulus heteroclitus* (Linneo, 1758) (Pisces, Cyprinodontidae) en medio hipersalino. *Investigación Pesquera* **51**, 183–197.
- Ehrlich, P. R. (1986). Which animal will invade? In *Ecology of Biological Invasion of North America and Hawaii. Ecological Studies* No. 58 (Mooney, H. A. & Drake, J. A., eds), pp. 79–95. New York, NY: Springer.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Felsenstein, J. (1989). *PHYLIP*, Manual Version 3.4, University Herbarium. Berkeley, CA: University of California.
- Fernandez-Delgado, C., Hernando, J. A., Herrera, M. & Bellido, M. (1986). Sobre el status taxonomico del genero *Valencia* Myers, 1928 en el suroeste de Iberia. *Donana. Acta Vertebrata* **13**, 161–163.
- Fernandez-Delgado, C. (1989). Life-history patterns of the salt-marsh killifish *Fundulus heteroclitus* (L.) introduced in the estuary of the Guadalquivir river (South west Spain). *Estuarine, Coastal and Shelf Science* **29**, 573–582.
- Golani, D. (1993). The sandy shores of the Red Sea—launching pad for Lessepsian (Suez Canal) migrant fish colonizers of the eastern Mediterranean. *Journal of Biogeography* **20**, 579–585.
- Gomez-Caruaña, F., Peiro, S. & Sanchez, S. (1984). Descripción de una nueva especie de pez continental ibérico, *Valencia lozanoi* n. sp. (Pisces, Cyprinodontidae). *Boletín de la Estación Central de Ecología* **13**, 67–74.
- Gonzales-Villasenor, L. I. & Powers, D. A. (1990). Mitochondrial-DNA restriction-site polymorphisms in the teleost *Fundulus heteroclitus* support secondary intergradation. *Evolution* **44**, 27–37.
- Hedges, S. B. (1992). The number of replications needed for accurate estimation of the bootstrap P value in phylogenetic studies. *Molecular Biology Evolution* **9**, 366–369.
- Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Paabo, S., Villablanca, F. X. & Wilson, A. C. (1989). Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the U.S.A.* **86**, 6196–6200.
- Machado-Núñez, A. (1857). Catálogo de los peces que habitan o frecuentan las Costas de Cadiz y Huelva y con inclusión del río Guadalquivir. *Imprenta Española y Extranjera*. Sevilla. 29 pp.
- Marteinsdóttir, G. & Able, K. W. (1988). Geographic variation in egg size among populations of the mummichog, *Fundulus heteroclitus* (Pisces, Fundulidae). *Copeia* **1988**, 471–478.
- Meyer, A. & Wilson, A. C. (1990). Origin of Tetrapods inferred from their mitochondrial DNA affiliation to Lungfish. *Journal of Molecular Evolution* **31**, 359–364.
- Palumbi, S. R., Martin, A., Romano, S., McMillan, W. O., Stice, L., Grabowski, G. (1991). *The simple fool's guide to PCR*. Honolulu, HI: University of Hawaii.
- Powers, D. A., Smith, M., Gonzales-Villasenor, I., DiMichele, L., Crawford, D., Bernardi, G. & Lauerman, T. (1993). A multidisciplinary approach to the selectionist/neutralist controversy using the model teleost *Fundulus heteroclitus*. *Oxford Surveys in Evolutionary Biology* **9**, 43–107.
- Saiki, R., Gelfand, D., Stoffel, S., Sharf, S., Higuchi, R., Horn, G., Mullis, K. and Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–491.
- Safriel, U. N. & Ritte, U. (1980). Criteria for the identification of potential colonizers. *Biological Journal of the Linnean Society, London* **13**, 287–297.
- Saitou, N. & Nei, M. (1987). The Neighbor-Joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406–425.
- Swofford, D. L. (1993). *PAUP: Phylogenetic Analysis Using Parsimony*, version 3.1. Champaign, IL: Illinois Natural History Survey.