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Is the subspecies classification of the freshwater eels *Anguilla australis australis* Richardson and *A. a. schmidtii* Phillipps still valid?

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DNA extraction

Tissue was placed in 500 µl of extraction buffer (1% SDS, 1 × STE (150mM NaCl, 20 mM Tris, 1mM EDTA pH 8.0), 0.25 mg/ml Proteinase K (Boehringer Mannheim)) and incubated at 50°C for 3 hours. Tubes were cooled to room temperature before the addition of 5 M NaCl to a final concentration of 1.6 M. Centrifugation at 8000 G for 10 min at room temperature removed excess protein and the supernatant was transferred to a fresh tube. Total nucleic acids were recovered using an equal volume of isopropyl alcohol (BDH), followed by incubation at –20°C for 1 hour and centrifugation at 12 000 G for 10 minutes. The pellet was washed using a 1 ml volume of 70% ethanol (BDH). After air-drying for 30 min, the pellet was resuspended in 50 µl sterile deionised water. Yield was assessed by UV spectrophotometry at 260 nm.

PCR amplification and DNA sequencing

No reliable amplification was achieved using the primers of Sang et al. (1994). Instead the full control region (1.2 kb) was amplified for three eels using primers P3 (5'-AACTTC-CATCCTCAACTCCCAAAGC-3' (Sang et al. 1994)) and S-Phe (5'-GCTTTAGTTAAGCTACG-3' (Nielsen et al. 1994)). Primers were synthesised by Custom Primers, BRL Life Technologies, Auckland, New Zealand. Reactions (50 µl per fish) consisted of 100 total nucleic acids, 100 µM dNTPs, 1 µM each primer, 1 × Boehringer Mannheim Taq reaction buffer and 2U Boehringer Mannheim Expand. The PCR machine used was a Stratagene Robocycler programmed to cycle at 94°C 4 min; 94°C 1 min, 50°C 1 m 30 sec, 72°C 1 min 30 sec (35 cycles); 72°C 5 min.

PCR products were purified prior to sequencing by ethanol precipitation using 1/10th volume of 10 M ammonium acetate and 2.5 volumes of absolute ethanol. Pellets were washed in 70% ethanol, dried, and resuspended in 20 µl of sterile deionised water. Templates were quantified by running a 3 ml aliquot on a 1.0% agarose gel adjacent to a GibcoBRL low DNA mass ladder. PCR products were sequenced from the 5' terminal end by using primer P3 and a Terminator Ready Reaction Mix (TRRM) with Amplitaq FS supplied by the University of Waikato, New Zealand DNA Sequencing Facility on an automated DNA sequencer (ABI prism). Data for bases 20-633 (A. australis) and bases 20-645 (A. dieffenbachii) were reliable and were used in the analyses.

Data analysis

Sequence ambiguities were checked and resolved by visual examination of the electropherogram. Edited sequence data were aligned using DNAMAN (Lynnon BioSoft, Quebec) according to the methods of Feng and Doolittle (1987) and Thompson et al. (1994). DNA divergence and gene flow between populations was estimated using DNASP (Rozas and Rozas 1997). Haplotypic correlations in the form of Φ statistics were calculated by AMOVA according to the method of Excoffier et al. (1992) with indels omitted. These statistics are equivalent to F-statistics (Cockerham 1969, 1973) and were developed for the haploid mitochondrial genome: Φ_{ST} is the correlation of random haplotypes within populations relative to that of random pairs of haplotypes drawn from the total species, Φ_{CT} is the correlation of random haplotypes within a group of populations (e.g. a region) relative to that of random pairs of haplotypes drawn from the total species, and Φ_{SC} is the correlation of random haplotypes within populations relative to that of random pairs of haplotypes drawn from a group of populations (e.g. a region).

Population structure was further assessed using bootstrapped neighbour joining trees derived from the sequence data. One hundred resampled sequences were produced using SEQBOOT of the PHYLIP 3.5 package (Felsenstein 1985, 1995). Genetic distances (Kimura 1980) were calculated by DNADIST using the multiple data set option, and a transition/transversion weighting of 20:1. From the distance matrices, neighbour joining trees were produced using NEIGHBOUR and the CONSENSE algorithm. New Zealand eel sequences were compared to those deposited in GenBank via the BLAST email server and to those of Sang et al.(1994).

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