The Southern Hemisphere ascidian *Asterocarpa humilis* is unrecognised but widely established in NW France and Great Britain

Biological Invasions

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Online Resource 1 (text)

Molecular barcoding methodology

Two overlapping fragments of the 18S gene were amplified over a total length of ca. 1640 base pairs with the following primers: (1) 18S1 (Fwd) 5'-CCTGGTTGATCCTGCCAG-3’ and 18S4 (Rev) 5'-GATTAAAGAAAACATTCTTGGC-3' (Tsagkogeorga et al. 2009) and (2) 18SA (Fwd) 5'-CAGCAGCCGCGGTAATTCCAGCTC-3' and 18SB (Rev) 5'-AAAGGGCAGGGACGTAATCAACG-3' (Wada et al. 1992). For COI we first used the universal primers LCO1491 and HCO2198 of Folmer et al. (1994) from which a ca. 650 bp fragment was obtained. However, because of PCR failures with the samples from New Zealand, we designed specific primers for COI based on preliminary sequences to obtain more reliable amplification; these are detailed in the published paper.

Specimens were preserved in ethanol after being relaxed using Propylene phenoxetol (1-Phenoxy-2-propanol) and were dissected under the microscope to obtain branchial basket tissue. Less than 15 mg of tissue was used per specimen. Total DNA was extracted using Nucleospin 96 Tissue Kit (Macherey-Nagel) following the manufacturer’s protocol but with a final elution of DNA in only 80 µl of elution buffer (two elution steps of 40 µL each). PCR reactions were undertaken in 30 µl volumes which contained 3 µL of 10x reaction buffer (100 mM Tris-HCl pH 8.3 and 500 mM KCl) (Thermoprime AbGene), 0.6 µL of each 2.5 mM dNTP (Promega), 2.4 µL of 25 mM $MgCl_2$, 1 unit of *Thermus aquaticus* DNA polymerase (Thermoprime AbGene), 4µL of template DNA (1:50 stock solution) and 1.2 µL of each primer (10 mM), the balance being Milli-Q filtered and autoclaved water. Touchdown PCR was used for 18S. This included initial denaturation for 4 min at 94°C, then 9 cycles of 40s at 94°C, 40s at 62°C initially but decreasing by 1°C per cycle, 1 min at 72°C, then 30 cycles of 40s at 94°C, 40s at 57°C, 1 min at 72°C, with a final elongation step of 10 min at 72°C. For COI, PCR cycling conditions were as described in Lejeusne et al. (2010). Double stranded PCR products were purified using ExoSAP-IT® before being sequenced at the BioGenouest sequencing platform (Roscoff) or at LGC Genomics (Berlin Germany). Sequences were checked with CodonCode Aligner (CodonCode Corporation) and aligned using BioEdit (Hall 1999).

Neighbour-joining trees and bootstrap tests were computed with the software MEGA v. 5.03 (Kumar et al. 2001) following a Kimura 2-parameter model with all sites (excluding gaps and missing data in pairwise comparisons) and uniform rates. Maximum Parsimony methods were also used and delivered the same topology. The tree of the nuclear 18S gene (Online Resource 3) was based on a 589 base-pair fragment to allow comparison with a large number of sequences available in the GenBank dataset.

Internal anatomy (see Online Resource 2)

The branchial basket, with four distinct folds on each side, has the following complement of longitudinal vessels (figures in brackets are for each fold; E = endostyle; DL = dorsal lamina):

Holyhead, N. Wales, 23/11/2011, specimen (in tunic) 24 mm long–

(Right) E 1 (9) 3 (10) 3 (11) 3 (10) 0 **DL** 0 (10) 3 (11) 3 (10) 3 (9) 2 **E (Left)** (total 101)

Plymouth, England, 18/11/2011, specimen (in tunic) 29 mm long–

(Right) E 2 (9) 3 (10) 3 (10) 3 (10) 0 **DL** 0 (9) 3 (10) 3 (10) 3 (10) 2 **E (Left)** (total 100).

About 15 stigmata separate successive longitudinal vessels in each mesh of the branchial basket (counted between the branchial folds approximately mid-way along the branchial basket). The dorsal lamina (just visible in Online Resource 2e) is smooth-edged. The opening of the large dorsal tubercle is variable: it is commonly U- or C-shaped with its horns turned either inwards or outwards, but is occasionally S-shaped (Online Resource 2e, f, and h). The anus has two smooth, outwardly curled lips (Online Resource 2g), as variously noted by Millar (1982), Kott (1985) and Clarke and Castilla (2000), although Van Name (1945) appears to illustrate a series of small lobes. Relatively large, flat-topped endocarps are present on the left side of the body adjacent to the gut, particularly within the gut loop and alongside the descending (dorsal) arm of the loop (Online Resource 2d, right inset).

Comments on generic assignment

The anus of *A. humilis* has two lips (Online Resource 2g). This is distinct from the multi-lobed anus commonly seen in *Cnemidocarpa*. A similar absence of anus lobes is noted by Brewin (1948) in the other *Asterocarpa* species, *A. coerulea*.

In the DNA-based phylogenetic analysis by Tsagkogeorga et al. (2009), *Asterocarpa humilis* (as *Cnemidocarpa humilis*) and *Cnemidocarpa finmarkiensis* failed to cluster closely within the Styelidae. Sanamyan (2000) noted that *C. finmarkiensis* is very similar to, and possibly conspecific with, the type species of *Cnemidocarpa, C. joannae* (Herdman, 1898). Our own trees (Online Resource 3) similarly failed to group *Asterocarpa humilis* with *Cnemidocarpa* species. The limited molecular data thus support the view that *Asterocarpa* and *Cnemidocarpa* should be maintained as separate genera.

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