SEQUENCE VARIATION OF THE INTERNAL TRANSCRIBED SPACER (ITS) REGION OF RIBOSOMAL DNA IN *CERASTODERMA* SPECIES (BIVALVIA: CARDIIDAE)

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(Received 2 February 2009; accepted 7 August 2009)

ABSTRACT

The internal transcribed spacer (ITS) region of ribosomal DNA (ITS1, 5.8S rRNA gene and ITS2) constitutes one of the most widely applied molecular markers in phylogenetic studies and species differentiation. Here, the ITS region of the cockles *Cerastoderma edule* from NW Spain and *C. glaucum* from the Mediterranean and Baltic Seas was characterized and its variation examined. The length of the ITS was 762–783 bp (ITS1, 226–251 bp; 5.8S rRNA gene, 158–161 bp; ITS2, 305–325 bp) and the GC content ranged between 57% and 60% (ITS1, 52–62%; 5.8S rRNA gene, 56–57%; ITS2, 61–63%). All individuals showed variation among ITS repeats, mostly in the spacers, with nucleotide diversity values of 0.0005–0.07. Among the *C. glaucum* sequences, two types of ITS (ITSI and ITSII) were distinguished, based on the percentage of differences observed in the spacers, sequence of the 5.8S gene, GC content of the ITS1 and clustering in phylogenetic trees. A PCR assay using specific primers for each ITS type demonstrated that both types coexist in all *C. glaucum* individuals. While type I seems to contain the *bona fide* 5.8S rRNA gene, type II could contain a pseudogene. *Cerastoderma edule* sequences grouped separately on phylogenetic trees, as expected for a differentiated species, but those of *C. glaucum* from the Mediterranean and Baltic Seas were intermixed in the two clades defining the ITS types, which supports the absence of systematic division previously inferred from other molecular markers.

INTRODUCTION

The ribosomal RNA genes (rDNA) of eukaryotes are organized in clusters of tandem repeat units, each consisting of coding regions and transcribed and nontranscribed spacers. The unit starts at the 5′ end with the external transcribed spacer (ETS), followed downstream by the 18S rRNA gene, the internal transcribed spacer 1 (ITS1), the 5.8S rRNA gene, the internal transcribed spacer 2 (ITS2), the 28S rRNA gene and terminating with the intergenic spacer (IGS) (Gerbi, 1985). Typically, several hundreds of copies are found per genome, at one or more chromosomal sites termed nucleolus organizer regions (NORs) (Long & Dawid, 1980; Mindell & Honeycutt, 1990).

The ITS region (ITS1, 5.8S and ITS2) can easily be amplified by PCR, using primers annealing in the conserved flanking genes, and constitutes one of the most widely applied molecular markers in phylogenetics and species differentiation. The 5.8S gene shows a slow rate of evolutionary change, but the level of sequence variation of the spacers is higher (Hillis & Dixon, 1991) and they can be used to infer phylogenetic relationships from populations to families and even higher taxonomic levels (e.g. González *et al.*, 1990; Vogler & DeSalle, 1994; Coleman & Vacquier, 2002). As members of a sequence family, the multiple copies of the ITS do not evolve independently. They tend to evolve in a concerted fashion, which means that in a species the repeats evolve together, maintaining high similarities among themselves, as they diverge from repeats in other species (Dover, 1982; Arnheim, 1983). Unequal crossing-over and gene conversion are the prominent mechanisms responsible for the homogenization of sequences. Nevertheless, variation among repeats within genomes has been documented in a range of taxa (e.g. Vogler & DeSalle, 1994; Tang *et al.*, 1996; Harris & Crandall, 2000; Gandolfi *et al.*, 2001; Hartmann, Nason & Bhattacharya, 2001; Mayol & Rosselló, 2001; von der Schulenburg *et al.*, 2001; Leo & Barker, 2002; Xu *et al.*, 2009), showing that the level of intraindividual variation should be investigated to interpret accurately the information provided by the ITS region.

In bivalve molluscs the ITS region, or one of its spacers, has been used (1) to infer phylogenetic relationships (e.g. Insua *et al.*, 2003; Riginos *et al.*, 2004; He *et al.*, 2005; Lee & Ó Foighil, 2005; Han-Liang *et al.*, 2006; Wood *et al.*, 2007); (2) to differentiate hybrids (Masaoka & Kobayashi, 2005); and (3) to separate species (e.g. White, McPheron & Stauffer, 1994; Heath, Rawson & Hilbish, 1995; Heath, Hatcher & Hilbish, 1996; Fernández *et al.*, 2001; López-Piñón, Insua & Méndez, 2002). However, given that usually only few clones per individual are examined and that direct sequencing is often applied, little is known about the degree of homogenization of the ITS repeats. Wang *et al.* (2007), using denaturing high-performance liquid chromatography, screened indel polymorphisms in a population of the scallop *Chlamys farreri*, and reported that most of the individuals examined had a heterogeneous ITS composition. Thus, intraindividual variation in bivalves may be more common than usually assumed.

In this study we examined the ITS region in two species of the bivalve family Cardiidae, the common cockle *Cerastoderma edule* and the lagoon cockle *C. glaucum*. Both are found along the European coast: *C. edule* inhabits the west Atlantic coast, from the Barents Sea to Senegal, and *C. glaucum* occurs from the northern Baltic Sea to the Black Sea, the Caspian Sea and adjacent lakes. Although partial niche overlap exists, *C. glaucum* is more euryhaline and eurythermic than *C. edule* and typically inhabits closed brackish lagoons and estuaries (Brock, 1991). The divergence of the two cockles seems to have

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occurred in the Late Miocene/Early Pliocene as a consequence of the isolation of the Atlantic and Mediterranean basins. Once the Strait of Gibraltar reopened, *C. glaucum* expanded its distribution from the Mediterranean Sea, colonizing the areas that it occupies today (Brock, 1991; Hummel, Wolowicz & Bogaards, 1994). Although *C. edule* and *C. glaucum* have similar shell characters in sympatric populations (Machado & Costa, 1994), they are considered good species according to different species concepts (Gosling, 1994). Nevertheless, it is debated whether the Mediterranean and the Atlanto-Baltic forms of *C. glaucum*, referred individually to as *C. glaucum* and *C. lamarcki*, respectively, or collectively as the *C. glaucum* complex, should be considered separate species or subspecies (Brock, 1991; Hummel *et al*., 1994; Mariani, Ketmaier & de Matthaeis, 2002; Nikula & Väinolä, 2003). Here, we sequenced the ITS region in *C. edule* and *C. glaucum* to investigate its basic characteristics and to assess its variation. Based on these sequences, the divergence among *C. glaucum* from the Baltic and Mediterranean Seas and *C. edule* was also assessed.

MATERIAL AND METHODS

Sample collection and DNA extraction

Specimens of *Cerastoderma glaucum* were collected in the Lagoon of Thau on the French Mediterranean coast and in the Gulf of Gdansk in the Baltic Sea, and those of *C. edule* in Pontevedra, northwestern Spain. Genomic DNA from fresh or ethanol-preserved specimens was extracted from a 30-mg piece of tissue according to Winnepenninckx, Backeljau & De Wachter (1993). In the case of ethanol-preserved specimens, two 15-min washes in phosphate-buffered saline and sterile deionized water were performed before proceeding to DNA extraction.

PCR amplification, cloning and sequencing

The set of primers (forward, 5′-GTTTCCGTAGGTGAACCTG-3′; reverse, 5′-CTCGTCTGATCTGAGGTCG-3′) designed by Heath *et al*. (1995) for *Mytilus* species was used. Amplification reactions were performed in volumes of 25 μl containing 10 ng of genomic DNA/μl, 1 μmol/l of each primer, 200 μmol/l dNTPs, 0.025 U/μl *Taq* polymerase (Roche Diagnostics) and the buffer recommended by polymerase suppliers. The thermal cycling protocol consisted of an initial denaturation of 3 min at 94°C, followed by 30 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 45 s, and a final extension for 5 min at 72°C.

The ITS PCR product of two individuals from *C. edule* and six from *C. glaucum* (three from each population) was ligated into the plasmid pGEM-T Easy, using pGEM-T Easy Vector System II (Promega), and transformed into *Escherichia coli JM109* cells. Plasmid DNA was purified as described by Sambrook & Russell (2001). Sequencing of both strands of the insert of each clone was performed using the ALFexpress AutoRead Sequencing kit and an ALFexpress automatic sequencer (Amersham Biosciences). The nucleotide sequences have been deposited in the EMBL database under accession numbers AM229680–AM229712.

Sequence analysis

A BLAST analysis (Altschul *et al*., 1997) was performed to corroborate the identity of the sequences obtained and to delimit the boundaries of coding and spacer regions. Sequences were aligned using CLUSTALX (Thompson *et al*., 1997) with default values for the parameters 'gap open' and 'gap extension'. Alignments were edited with GeneDoc (Nicholas, Nicholas & Deerfield, 1997). In all analyses, identical sequences from one individual were reduced to a single sequence. Differences between sequence pairs were expressed as a percentage (100 × number of variable sites/alignment length) with gaps counted as variable sites, independently of the number of nucleotide sites involved. The program DnaSP 4.0 (Rozas *et al*., 2003) was used to calculate nucleotide diversity and the amount of sequence divergence. Phylogenetic trees were constructed using MEGA 3.1 (Kumar, Tamura & Nei, 2004), calculating distances according to Jukes & Cantor (1969) for the neighbour-joining tree and using the close-neighbour-interchange as search method for the maximum parsimony trees. Bootstrap analysis (Felsenstein, 1985) with 1,000 replicates was applied to assess the reliability of nodes on trees.

RESULTS

PCR amplification using primers annealing at the 3′ end of the 18S gene and 5′ end of the 28S gene yielded a single product in at least 30 individuals of each population. After sequencing 10 clones of *Cerastoderma edule* and 33 of *C. glaucum*, the length of the PCR product was 762–783 bp. Table 1 shows the size and GC content of the ITS region delimited according to the BLAST analysis results. Both *C. edule* and *C. glaucum* showed slight size differences among clones due to the variation occurring in the ITS1 and ITS2, although Mediterranean *C. glaucum* also displayed size differences in the 5.8S gene. The GC content was similar in the two species, with the highest values in the ITS2 followed by the 5.8S gene and ITS1, except some clones of *C. glaucum* that displayed similar contents in ITS1 and ITS2.

The alignment of *C. edule* sequences showed 56 variable sites (Fig. 1). The largest differences were found in ITS1 (33 variable sites), followed by ITS2 (20 variable sites) and 5.8S gene (three variable sites). The percentage of differences in pairwise comparisons ranged from 0.00 to 3.57% in intraindividual comparisons and from 1.04% to 3.92% in interindividual comparisons. Overall nucleotide diversity was 0.007 (0.01 in ITS1 and 0.005 in ITS2) with values of 0.003–0.005 in each individual.

The aligned sequences of *C. glaucum* from the Mediterranean (Fig. 1) revealed 141 variable sites (one in 18S gene, 59 in ITS1, 11 in 5.8S gene and 70 in ITS2). The percentage of pairwise sequence differences ranged from 0.00 to 10.03% within individuals and 0.90% to 10.46% between individuals. Overall nucleotide diversity was 0.03 (0.06 in ITS1 and 0.03 in ITS2) and 0.0005–0.0480 in each individual.

The alignment of the Baltic sequences yielded 117 variable sites (one in 18S, 48 in ITS1, four in 5.8S and 64 in ITS2) (Fig. 1). The percentage of pairwise sequence differences ranged from 0.0 to 1.8% in intraindividual comparisons, and from 0.13% to 10.04% in interindividual comparisons. Overall nucleotide diversity was 0.03 (0.06 in ITS1 and 0.04 in ITS2) and 0.001–0.070 in each individual.

The alignment of the sequences from Mediterranean and Baltic individuals revealed 152 variable sites, with no fixed differences between the two populations (Fig. 1). The percentage of pairwise sequence differences between both populations ranged from 0.26% to 10.63%. Taking into account the differences observed in the intra-, interindividual and interpopulation comparisons, two sets of sequences were distinguished, one designated type I (25 sequences from five individuals) and the other named type II (eight sequences from two individuals). Table 2 shows the values of the percentage of pairwise sequence differences within and between the two ITS types. Irrespective of the region considered (ITS1, 5.8S gene or ITS2), the greatest differences were always found in comparisons between both types of sequences. Moreover, each ITS

Table 1. Size (bp) and GC content (%) of the ITS region in *Cerastoderma* species.

Species	No. of individuals	No. of clones per individual	ITS1		5.8S gene		ITS2		ITS Region	
			Length	GC	Length	GC	Length	GC	Length	GC
C. edule	2	5	226–244 (234)	52–53 (52)	160	56–57 (57)	316–322 (319)	62–63 (62)	762–775 (768)	57–58 (57)
C. glaucum Mediterranean population	3	5/6	244–251 (248)	54–61 (59)	158–161 (160)	56–57 (57)	306–324 (314)	61–62 (61)	764–783 (776)	58–59 (59)
C. glaucum Baltic population	3	5/6	242–244 (243)	54–62 (59)	160	56–57 (57)	305–325 (316)	61–63 (62)	763–783 (773)	58–60 (59)

In parentheses, average values.

type was associated with a characteristic GC content in ITS1 (54% in type I and 57–61% in type II) and three fixed differences in the 5.8S gene. To determine if the two ITS types coexist within individual cockles, reverse primers for each ITS type were designed (TypeI-R: 5′-AATTCGCCATCGTCGG-3′ and TypeII-R: 5′-ACTTCTCGCAGCTACCTGG-3′) and assayed in 10 individuals of each population to generate specific PCR products of type I (469–475 bp) and type II (341 bp). All individuals examined showed the expected PCR product for each ITS type (data not shown).

The ITS alignment of *C. edule* and *C. glaucum* combined consisted of 833 bp (Fig. 1), with 299 variable sites, 116 of them fixed between the two species (42 in ITS1 and 74 in ITS2). The number of parsimony-informative sites was 82 in ITS1 and 97 in ITS2. The 5.8S gene, however, remained invariable.

Table 3 summarizes the sequence divergence in the ITS region. The divergence value between the two types of ITS sequences was similar in the two populations of *C. glaucum*, with ITS2 values being lower than those of ITS1. Irrespective of the sequences considered, the sequence divergence between *C. glaucum* populations was always lower than that observed between ITS types. All comparisons between *C. edule* and *C. glaucum* showed higher divergence values than those observed within *C. glaucum*. When *C. edule* sequences were compared with each ITS type of *C. glaucum*, comparisons including type I showed lower divergence than those including type II, suggesting that type I could be closest to *C. edule* and therefore to the *Cerastoderma* ancestral ITS.

Phylogenetic analyses were carried out ignoring the 5.8S gene and considering the sequences of ITS1, ITS2 and the combined datasets (ITS1 + ITS2) separately. No outgroup was included because the available bivalve ITS sequences, including *Trachycardium flavum* (Cardiidae), were too divergent for a reliable alignment with the *Cerastoderma* sequences. In all cases tree topology was identical, irrespective of the set of sequences (ITS1, ITS2 or ITS1 + ITS2) considered. Typical results are illustrated by the tree obtained from ITS1 + ITS2 sequences (Fig. 2). Both the neighbour-joining and the maximum parsimony consensus trees showed two clusters, one containing the sequences of *C. edule*, the other those of *C. glaucum*. Within *C. glaucum*, the sequences were further separated into two groups corresponding to type I and II sequences as defined by the percentage differences.

DISCUSSION

The ITS region of *Cerastoderma edule* and *C. glaucum* was successfully amplified by PCR and 43 sequences were examined. The length deduced for ITS1 (226–251 bp) and ITS2 (305–325 bp) in the two cockles fell within the values described for other bivalves. The ITS1 and ITS2 of scallops, for example, are 209–277 and 270–294 bp (Insua *et al*., 2003; Wang *et al*., 2007) and those of Unionidae are 367–514 and 336–446 bp (Han-Liang *et al*., 2006), respectively. Just as in scallops and some other bivalves, the two cockle spacers differ by <100 bp (Kenchington *et al*., 2002; Källersjö *et al*., 2005), but in others such as clams ITS1 can be twice or more as long as ITS2 (Fernández *et al*., 2001; Han-Liang *et al*., 2006). As bivalve data accumulate, it seems that there are few constraints affecting the variation in spacer length because all kinds of situations may occur, e.g. ITS1 > ITS2, ITS1 < ITS2, and ITS1 and ITS2 of similar size. The length deduced for the 5.8S gene showed some variation in *C. glaucum* individuals from the Mediterranean (158–161 bp), but all the cockles examined displayed units with a 160 bp gene, indicating that this may be the size of the *bona fide* gene. This length is 1–4 bp longer than that deduced for other bivalves examined (Freire, 2002; Kenchington *et al*., 2002; Källersjö *et al*., 2005; Han-Liang

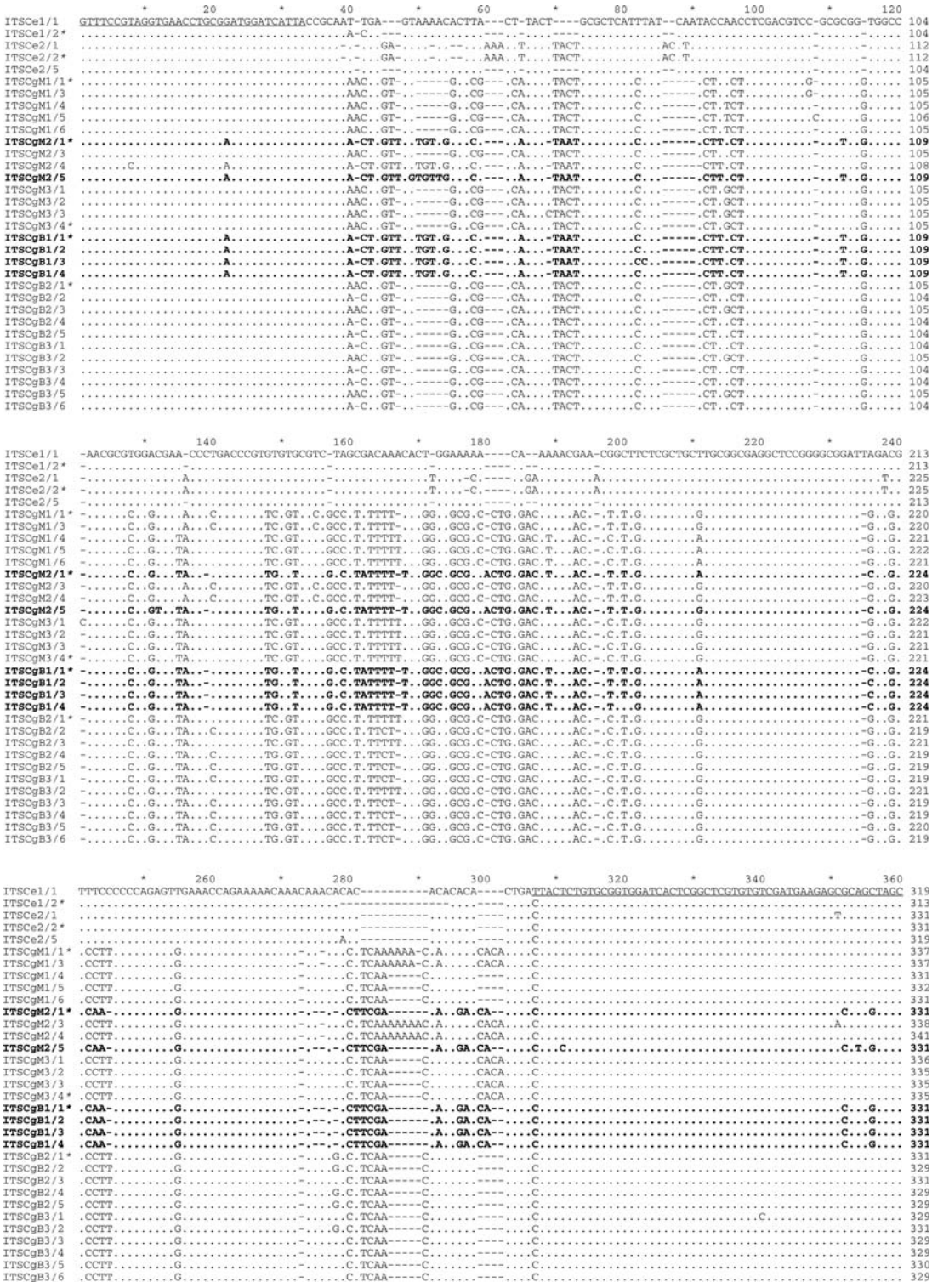

Figure 1. Alignment of the ITS sequences of *Cerastoderma edule* (Ce1/1–2/5) and *C. glaucum* from the Mediterranean (CgM1/1–3/4) and Baltic (CgB1/1–3/6) Seas. Coding regions are underlined. Sequences type II are in bold. Dots represent residues identical to those of the reference sequence, dashes indicate alignment gaps and base substitutions are indicated by the respective bases. Asterisk denotes a representative sequence of several clones.

et al., 2006) but is in line with the average length of eukaryote 5.8S rRNA (∼160 bp) deduced from direct sequencing (Nazar, 1984).

While spacers of *C. edule* and some clones of *C. glaucum* differed by 7–11% in GC content, other *C. glaucum* clones displayed spacers with a more similar GC content. Thus, the ITS GC content appears to follow two different patterns in the cockles. A balanced GC content between the two spacers is often observed in other organisms (Torres, Ganal & Hemleben, 1990; Miller, Crabtree & Savage, 1997), including

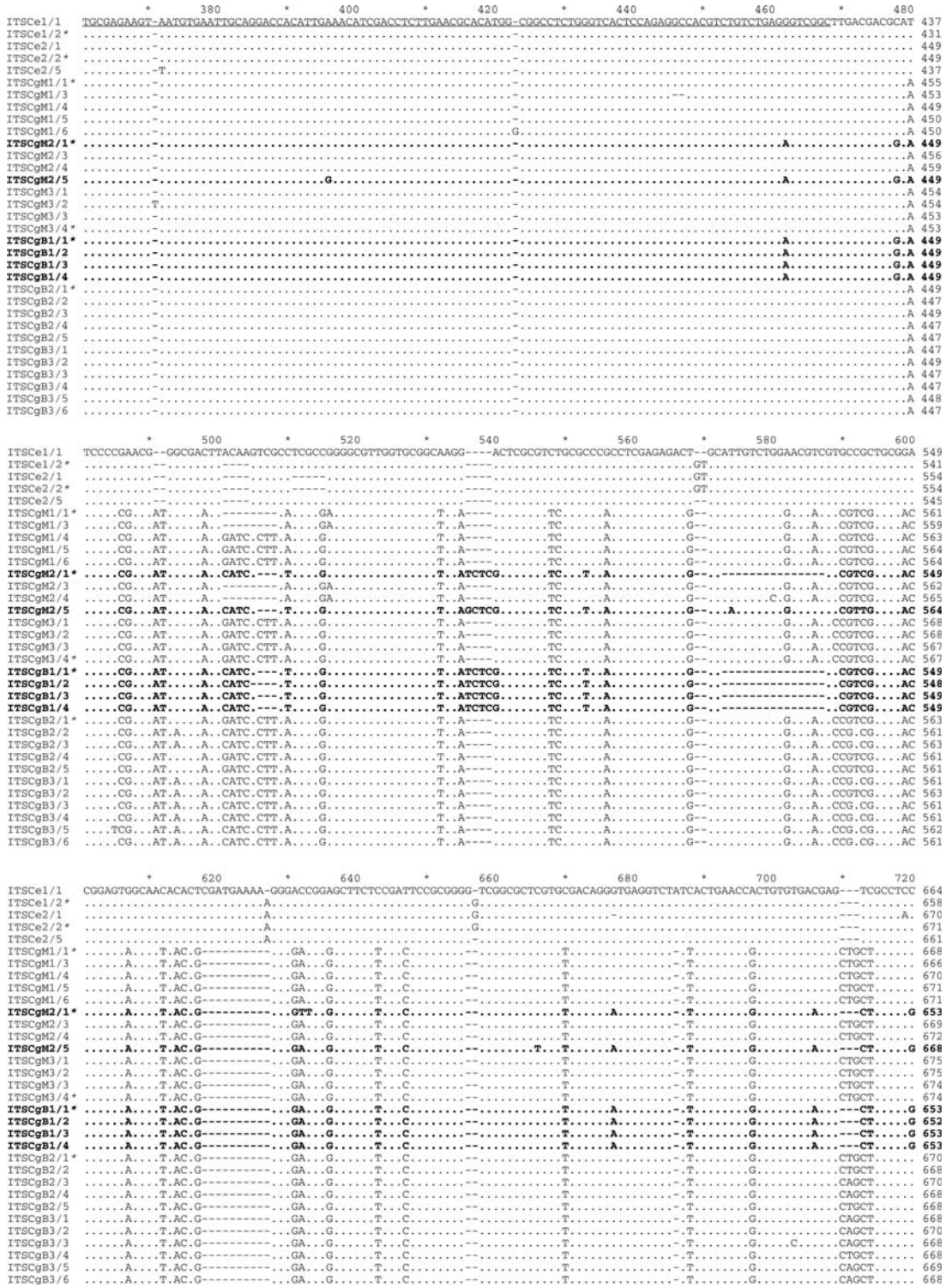

Figure 1. *Continued*

bivalves (Insua *et al*., 2003; Han-Liang *et al*., 2006), suggesting that the base composition of the two spacers may coevolve (Torres *et al*., 1990), at least in some repeats of *C. glaucum*. Compared to other bivalves, the GC content of cockle spacers is similar to that of the clam *Dosinia corrugata* (58% in ITS1 and 66% in ITS2, Han-Liang *et al*., 2006), but higher than in other species such as the scallop *Hinnites distortus* (43% in ITS1 and 44% in ITS2, Insua *et al*., 2003). As expected for a highly conserved sequence, the GC content of the 5.8S gene showed little variation and the values correspond to those observed in bivalves (Freire, 2002) and other animals (e.g. Wesson, Porter & Collins, 1992; Odorico & Miller, 1997).

Both cockles showed intraindividual variation mainly in the spacers, ITS1 being more variable than ITS2, as in other organisms (Schlötterer *et al*., 1994; Tang *et al*., 1996).

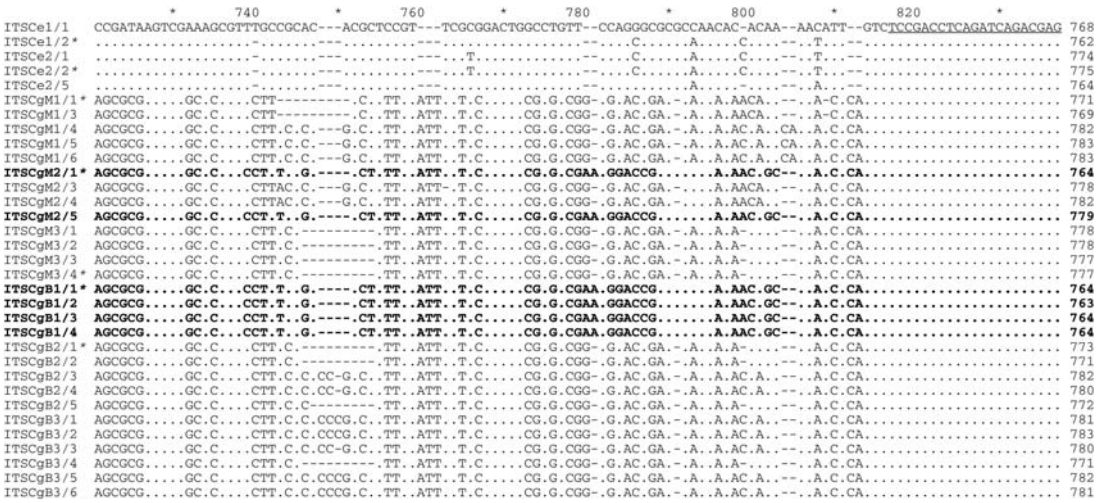

Figure 1. *Continued*

Table 2. Pairwise differences in *Cerastoderma glaucum* ITS sequences.

	Type I	Type II	Type I *vs* Type II
ITS region	0–4.26 (1.66)	0–1.7 (0.56)	7.7–10.63 (9.52)
ITS1	0–9.72 (2.98)	0–2.05 (0.62)	12.05–17.48 (15.41)
ITS2	0–3.78 (1.66)	0–1.95 (0.69)	8.47–11.73 (10.13)
5.8S gene	0–1.26 (0.25)	0–1.87 (0.47)	1.87–4.4 (2.23)

In parentheses, average values.

Table 3. Hierarchical analysis of DNA sequence divergence in ITS/ITS1/ITS2 region of *Cerastoderma* cockles.

Sequence groups	D_{xy}	D_a
C. glaucum Mediterranean population		
Type I *vs* Type II	0.07/0.14/0.07	0.06/0.12/0.06
	0.01/0.03/0.01	0.01/0.03/0.01
C. glaucum Baltic population		
Type I *vs* Type II	0.07/0.13/0.08	0.07/0.13/0.08
	0.01/0.02/0.01	0.01/0.02/0.01
Mediterranean *vs* Baltic*	0.03/0.06/0.03	0.0009/0.001/0.001
	0.006/0.01/0.006	0.006/0.001/0.006
Type I	0.01/0.02/0.01	0.005/0.007/0.005
	0.002/0.005/0.003	0.002/0.007/0.003
Type II	0.007/0.007/0.008	0.0009/0.00001/0.002
	0.004/0.006/0.003	0.004/0.006/0.003
*C. edule vs C. glaucum**	0.18/0.27/0.29	0.16/0.23/0.28
	0.01/0.02/0.02	0.01/0.02/0.02
C. edule vs Mediterranean population	0.18/0.26/0.29	0.16/0.23/0.28
	0.02/0.03/0.03	0.02/0.03/0.03
C. edule vs Baltic population	0.18/0.26/0.29	0.16/0.23/0.27
	0.02/0.03/0.03	0.02/0.03/0.03
C. edule vs Type I	0.17/0.25/0.28	0.17/0.24/0.27
	0.01/0.02/0.02	0.01/0.02/0.02
C. edule vs Type II	0.21/0.33/0.34	0.20/0.32/0.33
	0.03/0.05/0.05	0.03/0.05/0.05

Sequences are grouped by type (I and II), species (*C. glaucum* and *C. edule*) or populations. Each group is treated as a 'population' where each sequence represents an 'individual'. D_{xy} denote the average number of substitutions per site between populations and D_a the number of net substitutions between populations (Nei, 1987). Upper line, D_{xy} and D_a values; lower line standard deviations.

*All sequences from both populations or species are considered.

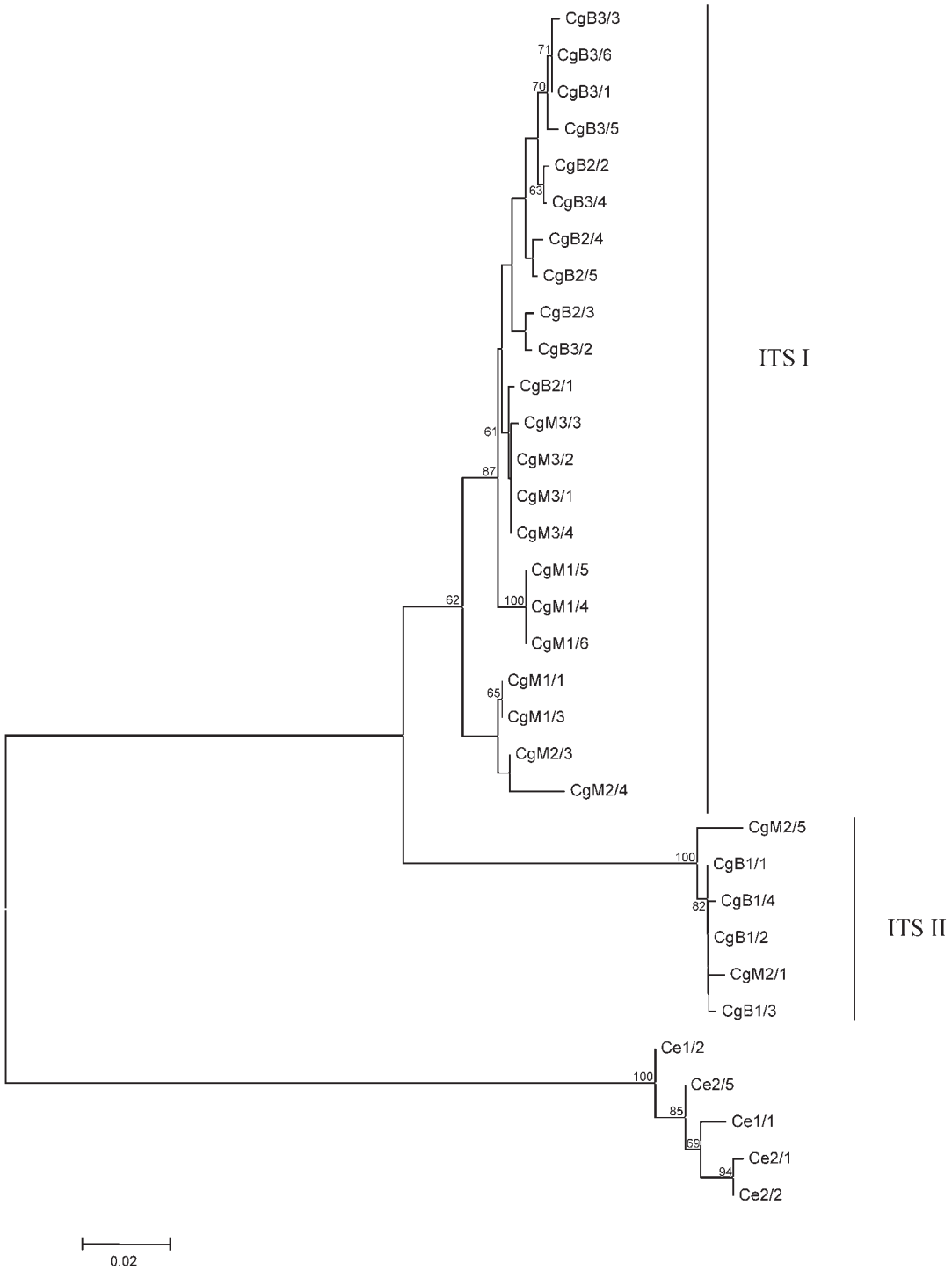

Figure 2. Neighbour-joining tree inferred from ITS sequences of *Cerastoderma edule* (Ce1/1–2/5) and *C. glaucum* from the Mediterranean (CgM1/1–3/4) and Baltic (CgB1/1–3/6) Seas. Bootstrap values >50% are shown at nodes. Note that type I and II sequences of *C. glaucum* are grouped separately.

For *C. glaucum*, two types of ITS sequences, defined by the percentage of differences observed in the spacers, the sequence of the 5.8S gene and the GC content of the ITS1, were distinguished. Their existence was also supported by the phylogenetic trees, where the sequences of each type form a clade. Judging from the sequences examined, individuals from the Mediterranean contain either type I or both ITS types, whereas specimens from the Baltic contain either type I or type II. However, a PCR assay provided evidence that the two types coexist within individuals in the two populations, indicating that a higher number of sequences should be examined to characterize better the distribution of the ITS types in *C. glaucum* individuals. While the variation detected in *C. edule* is similar to that described in ITS2 in pearl oysters (He *et al.*, 2005), that of *C. glaucum*, with its two ITS types, is described here for the first time in bivalves, although this type of variation in not unusual in other organisms (e.g. Vogler & DeSalle, 1994; Xu *et al.*, 2009).

Variation at the ITS region is often associated with repeated sequences, including elements with comparatively long repeat units (Tang *et al*., 1996; Kenchington *et al*., 2002) or, more commonly, with simple repetitive sequence motifs (Wesson *et al*., 1992; Fritz *et al*., 1994; King *et al*., 1999; Harris & Crandall, 2000; von der Schulenburg *et al*., 2001). In cockle ITS, although indels were detected, only positions 280–296 of the alignment were associated with a CA microsatellite sequence (Fig. 1), and types I and II of *C. glaucum* were not defined by repetitive sequence motifs. Taking into account that the coding region of type I corresponds to that of *C. edule* and that type II displayed three fixed substitutions, it cannot be discounted that this represents a nonfunctional copy (pseudogene) given that these substitutions may disrupt the secondary structure and functionality of the rRNA. Irrespective of their functional significance, the two types of ITS probably originated after *C. glaucum* and *C. edule* diverged (4–5 million years ago, Brock, 1991; Hummel *et al*., 1994), since evidence for the occurrence in *C. edule* of two types of sequences was not found.

The existence of ITS variants is related in several organisms with the occurrence of ribosomal DNA on different chromosomes, because it is conceivable that rDNA homogenization proceeds more efficiently within homologous chromosomes than among nonhomologous chromosomes (Vogler & DeSalle, 1994). However, cytogenetic analyses carried out in *C. glaucum* demonstrated that NORs carrying the rDNA are located at one chromosome pair (Thiriot-Quiévreux & Wolowicz, 1996). It is possible that one of the ITS types, presumably type II, represents orphons or repeats organized in small clusters not detectable by conventional techniques. But other scenarios compatible with the existence of both ITS types in the same array may be considered. These include the occurrence of chromosomal arrays homogenized for different variants by intrachromosomal recombination as observed in other species (Schlötterer & Tautz, 1994; Polanco *et al*., 1998; Wang *et al*., 2007). Another is the distribution of the ITS types in different regions within the array because in terminal regions the degree of possible misalignment required for unequal crossing-over is more limited than in the centre (Ruiz Linares, Bowen & Dover, 1994; Trontin, Grandemange & Favre, 1999) and therefore divergence between terminal and central members may be expected.

Phylogenetic analyses revealed that *C. edule* and *C. glaucum* sequences always group separately, supporting that both taxa are good species under different species concepts (Gosling, 1994) and in line with previous studies based on different molecular markers (Brock, 1978; Brock & Christiansen, 1989; André *et al*., 1999; Freire, Insua & Méndez, 2005). However, *C. glaucum* sequences from Mediterranean and Baltic individuals are intermixed in the clades grouping the ITS types, instead of being grouped geographically as would be expected if they were independent lineages. This finding is in contrast with the separation of *C. glaucum* Atlanto-Baltic and Mediterranean forms into two distinct taxa inferred in light of immunoelectrophoretic analysis, DNA hybridization and some allozyme studies (Brock, 1987; Brock & Christiansen, 1989; Hummel *et al*., 1994) and supports the conclusion derived from mitochondrial COI gene sequences and genetic variation at several enzyme loci that refuted the Atlanto-Baltic *vs* Mediterranean systematic division (Mariani *et al*., 2002; Nikula & Väinolä, 2003). The analysis of a reduced number of populations in the studies undertaken may explain the discrepancy observed between conclusions derived from different markers or loci. The ITS sequences examined here provide additional data about *C. glaucum* populations, but further investigations are still required to resolve the debate on their taxonomic status.

ACKNOWLEDGEMENTS

We thank Dr Philippe Borsa and Dr Maciej Wolowicz for supplying samples, Rosa García Díaz for the technical assistance in the laboratory and anonymous reviewers for constructive comments. This work was funded by the Ministerio de Ciencia y Tecnología and Ministerio de Educación y Ciencia of Spain and Xunta de Galicia through projects IFD97-1295, RZ2006-00005-00-00 and XUGA 10300B97, respectively.

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