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Identification of COI partial sequences in two closely related frog species, *Rana dalmatina* and *Rana temporaria*

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Abstract. Herein we report the characterization of cytochrome oxidase subunit 1 gene (COI) partial sequence in two closely related European *Rana* species, *Rana dalmatina* and *Rana temporaria*. The sequences should be useful for both phylogenetic and phylogeographic in these two species.

Key words: COI, phylogeny

Introduction

Phylogeny of the European brown frogs was already investigated using amplification and sequencing of 16S RNA coding mitochondrial sequence (Veith et al. 2003). However, other mitochondrial markers might be even more sensitive for this purpose (Kuzmin & Poyarkov 2009, Crawford et al. 2010). Application of multiple mitochondrial markers to determine species phylogeny and phylogeography may lead to more precise phylogenetic trees.

The COI gene (cytochrome oxidase subunit I) can be used as a mitochondrial marker for species barcoding, phylogeny and phylogeography (Kuzmin & Poyarkov 2009). COI sequences have been reported for North American *Rana* species (Smith et al. 2008). However, corresponding information for European frogs including the genus *Rana* is missing with the notable exception of *Rana pyrenaica* (EU746402.1). The common frog (*Rana temporaria*) and the agile frog (*Rana dalmatina*) are two closely related European brown frog species with partially overlapping distribution ranges (IUCN 2010). From a morphological point of view the two species are difficult to distinguish, especially in sub-adult and tadpole stages. Morphological characters used to distinguish the adults of these two species are the following: In *Rana dalmatina* the tibio-tarsal joint stretched forward passes over the nose and the internal metatarsal tuber is oval and very proeminent. In contrast, in *Rana temporaria* the tibio-tarsal joint only reaches to the eyes and the internal metatarsal tuber is blunt (Ovenden 2002).

Here we present COI partial sequences of the two species and analyze the phylogenetic relationships between these sequences and those of other *Rana* species available in the NCBI database. In order to corroborate species assignment based on morphology we also analyzed 16S RNA partial gene sequences of the respective species.

Methods

Two individuals each of *Rana dalmatina* (dalmatina1 lat. 46.734327° long. 23.569173°, dalmatina2 lat. 44.942989° long. 28.443808°) and *Rana temporaria* (temporaria1 lat. 44.618517° long. 22.253228°, temporaria2 lat. 44.643086° long. 22.291728°) were used in our study. The *Rana dalmatina* individuals were adults and the *Rana temporaria* individuals were tadpoles since adults were unavailable. Distinguishing tadpoles was based on the positions of the spiraculum and of the eyes (Miaud & Muratet 2004). In case of adults genomic DNA was extracted from toe clips while in case of tadpoles from the tail clip, using the MACHEREY NAGEL Nucleospin®Tissue Kit standard protocol for DNA extraction. We used the universal primers 16Sar and 16Sbr (Palumbi 1996) to amplify a sequence of approximately 590 bps of the 16S RNA coding mitochondrial gene. PCR products were sequenced at Macrogen Inc. Korea using the same primers. For construction of the 16S RNA-based phylogenetic tree sequences from the following species were used: *Rana temporaria aragonensis* AY147951.1, *Rana temporaria*, AF275735.1, *Rana temporaria canigonensis* AY147952.1, *Rana temporaria honnorati* AY147954.1, *Rana temporaria parvipalmata* AY147955.1, *Rana arvalis arvalis* AY147938.1, *Rana iberica* AY147944.1, *Rana pyrenaica* AY147950.1, *Rana macrocnemeis pseudodalmatina* AY145732.1, *Rana dalmatina* AY014381.1 AY0147941.1, *Rana sylvatica*, AY779199.1, DQ283387.1,, *Rana clamitans* DQ283185.1, DQ347320.1, *Rana septentrionalis* AY779200.1, *Rana caetsbeiana* DQ283257, DQ289127.1 and *Ambystoma laterale* NC006330.1

In order to obtain partial COI gene sequence we used the degenerate primer pair V1F and V1R published by Smith et al (2008). Initially a 710 bps fragment was amplified on dalmatina1 genomic DNA. The PCR product was extracted from an agarose gel using the MACHEREY NAGEL Nucleospin® Extract Kit and subcloned into pTZ57R/T (InsTAclone™ PCR Cloning Kit Fermentas). Plasmid DNA was extracted by GeneJET™ Plasmid Miniprep Kit (Fermetas). Two clones were sequenced at Macrogen Inc. Korea, using the universal M13 F primer. This way, 704 bps of COI sequence could be obtained. Based on this sequence specific primers for Rana COI were designed (RanaCOIF: 5′ TTCTCTACTAACCACAAAGACATTGG 3′ and RanaCOIR: 5′ TAGACTTCTGGGTGGCCGAAAAATCA 3′). Amplification on genomic DNA of the four Rana individuals yielded products of 710 bps that were subsequently sequenced at Macrogen Inc. Korea using RanaCOIF. The length of the sequences obtained was around 640 bps.

For construction of the COI-based phylogenetic tree the following sequences were used: *Rana caetsbeiana* EF525855.1 EF525856.1, *Rana clamitans* EF525864.1, EF525869.1, *Rana septentrionalis* EF525893.1, EF525896.1, *Rana pipiens* EF525901.1, *Rana sylvatica* EF525888.1, EF525903.1., *Rana pyrenaica* EU746402.1 and *Ambystoma laterale* EF525709.1.

Sequences were aligned using VectorNTI 10. Phylogenetic trees were constructed with MEGA5 (maximum-likelihood method with 1000 bootstraps) as outgroup for both markers we used the respective homologous sequence from *Ambystoma laterale* (Ambystomatidae, Ambystoma).

Results and discussion

Species assignment

To corroborate assignment of the specimens to *R. temporaria* and *R. dalmatina*, respectively, partial 16S RNA sequences were amplified. Analysis using BLAST and subsequent comparison with sequences available in the NCBI database demonstrates correct assignment to the two species (Fig. 1).

Analysis of *R. dalmatina* and *R. temporaria* COI partial sequences

Mitochondrial cytochrome oxidase subunit I sequences provide a reliable means especially in species barcoding. For the two closely related European brown frogs *Rana dalmatina* and *Rana temporaria*, however, COI sequences and primer pairs permitting parallel amplification and sequencing of larger sample sets have not been reported to date. We therefore initially used a degenerate primer pair described in the literature to obtain *R. dalmatina* COI sequence. BLAST search using the amplified and subcloned sequence of 710 bps (uppermost sequence in Fig. 2) as query revealed closest homology to the COI sequence reported for *Rana pyrenaica* (alignment not shown). Specific primers developed on the basis of the initial sequence then yielded amplification products of around 710 bps in both *Rana dalmatina* and *Rana temporaria*, which could be sequenced directly using the forward primer of the pair.

Alignment of the *Rana dalmatina* and *Rana temporaria* sequences indicates that there is considerable difference between the two species at the molecular level (Fig. 2). Based on the multiple alignment of *R. temporaria*, *R. dalmatina* and database-derived COI sequences of other *Rana* species a Maximum likelihood phylogenetic tree was constructed (Fig. 3). The COI-based tree presents a topology similar to that obtained with 16S RNA sequences as far as *R. dalmatina* and *R. temporaria* are concerned. As in the case of 16S RNA sequences, *R. pyrenaica* is found to be more closely related to *R. temporaria* than to *R. dalmatina*. The clear separation of the

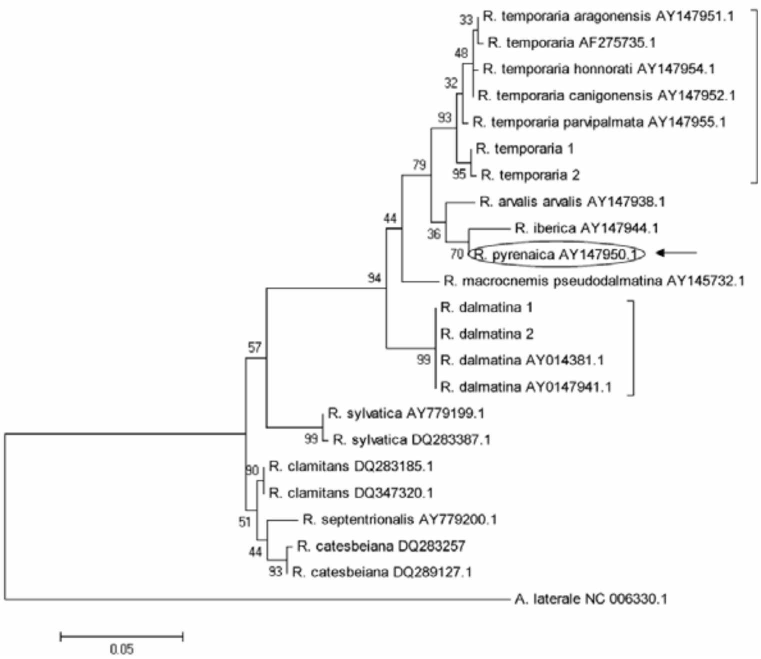

Figure 1: Maximum likelihood phylogenetic tree of 16S partial coding sequences of different *Rana* species. The sequences derived from the four individuals used for amplification of COI sequences (dalmatina1, dalmatina2, temporaria1, temporaria2) cluster with sequences of the species they were assigned to based on morphological criteria. *R. temporaria* and *R. dalmatina* sequences are marked with brackets, *R. pyrenaica*, for which closest homology to *R. temporaria* and *R. dalmatina* was found in the COI sequence analysis is indicated with an oval. Bootstrap values are shown to the left of nodes.

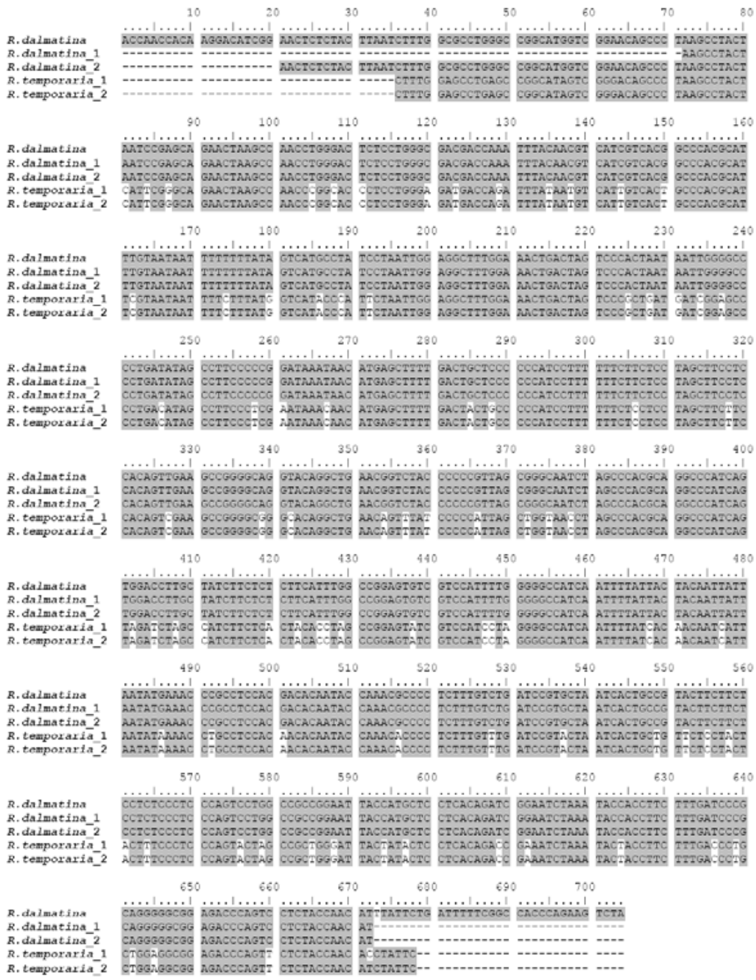

Figure 2: Alignment of *Rana dalmatina* and *Rana temporaria* COI partial coding sequences. The 704bps sequence obtained from the subcloned R.dalmatina COI fragment as well as the shorter sequences obtained from direct sequencing of PCR products were included in the alignment.

European species *Rana temporaria, Rana dalmatina* and *Rana pyrenaica* from the American ones is also in accordance with the data obtained using 16S RNA sequences.

To our knowledge this is the first report of COI sequences for *Rana temporaria* and *Rana dalmatina*. The specific primers developed for direct sequencing should facilitate a fast assessment of population variability in these two species.

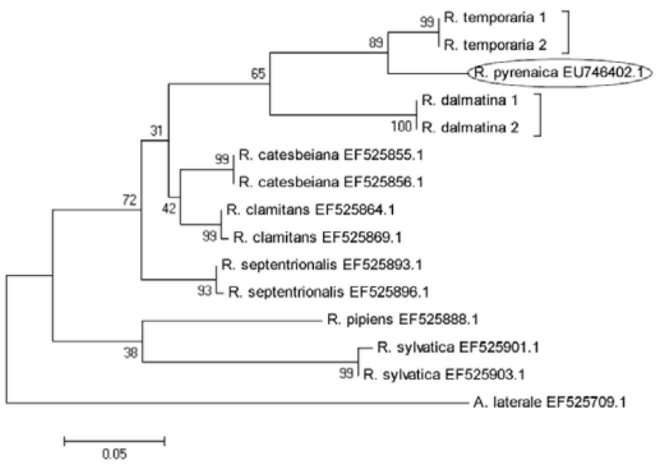

Figure 3: Maximum likelihood phylogenetic tree of COI partial coding sequences of different *Rana* species. The COI sequences reported here (dalmatina1, dalmatina2, temporaria1, temporaria2) together with *R. pyrenaica*, form a cluster apart from the other *Rana* species. Bootstrap values are shown to the left of nodes.

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