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S1 satellite DNA confirms the species rank of *Rana pseudodalmatina* Eiselt & Schmidtler, 1971

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Abstract. S1 satellite from Palaearctic brown frogs, genus *Rana* (Ranidae) is the only satellite DNA family in which the 'most common sequence' (MCS) and overall variability of repetitive units in the genome has been determined. Both features are species-specific in all European frogs and the same applies to species including their subspecies. This indicates that they were established when each species was forming and remained unchanged to the present. We previously found that both features were the same in all Anatolian taxa (*Rana macrocnemis, R. camerani, R. holtzi, R. tavasensis*), suggesting that they belong to a single species, *R. macrocnemis*. In this study we characterized S1 satellite DNA from *R. pseudodalmatina* from Iran. Southern blots, quantitative dot blots, and FISH analyses produced results similar to those obtained from Anatolian taxa. However, all *R. pseudodalmatina* specimens contain S1a repetitive units (477 bp) with the same MCS and overall genomic variability that are both different from those of Anatolian taxa, indicating that they belong to a distinct species. S1a MCS from *R. pseudodalmatina* is more homologous to the corresponding MCSs from all European species than that of *R. macrocnemis* from which it differs in 22 positions. Our results provide relevant conclusions on the usefulness and limitations of the use of satellite DNA sequences in taxonomic and phylogenetic analyses.

Key words. Amphibia, Anura, Satellite DNA, repetitive units, molecular evolution, taxonomy, brown frogs, Iran.

Introduction

Satellite DNAs in eukaryotes are formed by repeated sequences organized in tandem to form arrays that sometimes span across several megabases (Beridze 1986, Schueler & Sullivan 2006). This repetitive DNA occurs in heterochromatic regions, located mostly at the centromeric, pericentromeric and subtelomeric positions of the chromosomes (Miklos 1985, Charlesworth et al. 1994). Evolutionarily related organisms may present species-specific varieties of the same satellite DNA or share a common library of satellite sequences, whose quantitative changes may generate species-specific satellite compositions (Mestrovic et al. 1998).

Western Palaearctic brown frogs, genus *Rana* (Ranidae), include species of similar morphology. The systematics of brown frogs living in the Middle East has been particularly debated. Boulenger (1885) described a brown frog species from Bursa (*R. macrocnemis*), and one year later a second species from Georgia (*R. camerani*). Werner (1898) described a third species of brown frog from the Taurus Mountains (*R. holtzi*). However, more recent morphological analyses have indicated overlapping morphological characters, suggesting that these three taxa could belong to a single polymorphic species (Mensi et al. 1992, Picariello et al. 1999).

Phylogenetic studies based on allozymes (Mensi et al. 1992) and on mitochondrial and nuclear genes (Veith et al. 2003) indicated a Plio-Pleistocene diversification of Western Palaearctic brown frogs from a common ancestor, while Oosterbroek & Arntzen (1992) proposed an origin of these frogs in the Miocene. According to Veith et al. (2003), Western Palaearctic brown frogs underwent a basal post-Messinian radiation from a common ancestor about 4 mya into five major clades: three monotypic lineages (*R. dalmatina*, *R. latastei*, *R. graeca*) and two polytypic lineages, including the other European species (*R. temporaria* group) and the Anatolian species (*R. macrocnemis* group), respectively. These studies indicated that within the Anatolian lineage *R. macrocnemis tavasensis* Baran & Atatür, 1986 and *R. macrocnemis pseudodalmatina* Eiselt & Schmidtler, 1971 radiated from the basal clade 2 mya. On the basis of this study, Frost (2013) assigned species rank to the latter two taxa, while the other three taxa should correspond to a single polymorphic species (*R. macrocnemis*).

A more recent study proposes a different model of evolution of Western Palaearctic brown frogs (Najibzadeh et

al. 2017). According to this study, these frogs underwent a basal radiation in two main monophyletic clades, the European brown frogs plus the Asian *R. asiatica* and the Anatolian-Hyrcanian brown frogs, during the Early Miocene (ca. 20 mya). The Hyrcanian (*R. pseudodalmatina*) and the Anatolian lineages diverged approximately 16.6 mya. Moreover, the latter clade diverged into two subclades, *R. tavasensis* and *R. macrocnemis*, during the Middle Miocene, 14.5 mya.

The S1 satellite from western Palaearctic brown frogs is the only satellite DNA family in which the most common sequence (MCS) and the overall genomic variability of S1a and, when present, S1b repetitive units were identified and found to be species-specific and not to vary within each species, including subspecies (Picariello et al. 2002, Feliciello et al. 2005, 2006). Therefore, S1 satellite DNA constitutes an *absolute marker* of the species in brown frogs (Picariello et al. 2002), and we have used this feature to verify the systematics of brown frogs of the Middle East.

In a previous study, we have shown that the MCS and overall genomic variability of S1a repetitive units are the same in all four Anatolian taxa, indicating that they all belong to the species *R. macrocnemis* (Picariello et al. 2016). In the present study, we characterized S1 satellite DNA from *R. pseudodalmatina*. Results from Southern blots, quantitative dot blots, and FISH analyses were similar to those obtained from *R. macrocnemis*, but all tested specimens of the taxon *pseudodalmatina* contain S1a repetitive units with a different structure and overall genomic variability, demonstrating that this taxon is a distinct species. Our results indicate that satellite DNA can be a fundamental tool for the correct taxonomic ranking of closely related taxa.

Materials and methods

Sampling

The analysed specimens from various populations from the south coast of the Caspian Sea, Iran, were sampled by B.S.-M. in June of 2014, by permit from the Iranian Department of Environment (licence number 95/36437). The samples of *R. pseudodalmatina* were collected from the following Iranian localities: R.ps1a: Pol-sefid, Mazandaran Province (36.0929°N, 53.0300°E); R.ps1b: Palang-dareh, Mazandaran Province (36.0929°N, 53.0300°E); R.ps2a: 10 km west of Asalem Jungle, Gilan Province (37.4131°N, 48.5131°E); R.ps2b: Bareh-sar jungle, Damash, Gilan Province (36.4516°N, 49.4518°E); R.ps3: Shir Abad Cave, Khanbebin, Golestan Province (36.5705°N, 55.0211°E). The specimen R.ma1 of *R. macrocnemis* originated from Oshnaviyeh, West Azerbaijan Province (37.0648°N, 45.0205°E).

DNA extraction

DNA was extracted from skin or tadpole tail samples (R.ps2a), stored in 85° ethanol-5mM EDTA, and purified as described by Picariello et al. (2016).

PCR amplification

Whole S1a repeats were amplified by PCR using three pairs of primers with overlapping origin and opposite orientation, located in a different, highly conserved part of S1a repeat sequence. The pairs used and corresponding locations are:

pair A: GTTTCCCCATAGACTTCCAT-GAAACAG-GGTCAGCTTGA (bp 460-15)

pair B: AAAGTTATAGCCCAAAAAC-TTTGAACG-GAGCRTGCTA (bp 249-284)

pair C: CCACATTGTAGCCCCATA-TGGCGAA-GAAAGTAAGTCGC (bp 361-395)

The procedures for the amplification of S1 satellite repetitive units from genomic DNA and the isolation of the amplified S1a monomers after purification by preparative agarose gel electrophoresis were described by Picariello et al. (2002). As verified in previous studies, these procedures yield mixtures of amplified S1a repeats that are representative of the whole genomic population (Feliciello et al. 2005, 2006).

Aliquots (5–20 ng) of the purified S1a monomer mixtures obtained from specimens Ps1a and Ma2 with the primer pair B were re-amplified to obtain quantitative standards for dot blots and biotinylated probes for Southern blot and dot blot analyses. In both cases PCR amplifications were carried out as indicated: the initial denaturation step at 94°C for 2 min was followed by 30 cycles of 30 s at 94°C, 30 s at 50°C and 1 min at 68°C, with a final extension step of 2 min at 72°C. Reaction mixtures for standards (2×100 µl) contained 0.2 µM primers, 30 U/ml of TAQ polymerase, and 0.2 mM dNTP. In reaction mixtures for biotinylated probes (75 µl) 0.2 mM dTTP were replaced by 0.02 mM dTTP+0.02 mM 11 biotinyl dUTP. Amplified S1a standards were purified by using the GenElute PCR Clean-up Kit (Sigma), and the purity of isolated S1a monomers was verified through agarose gel electrophoresis.

Southern blot

DNA samples were digested overnight with the appropriate restriction enzyme (3 units/µg of DNA). Aliquots containing 3 µg of digested DNA were used for gel electrophoresis and Southern blot analysis as described by Picariello et al. (2002).

Quantitative dot blot

Dot blot analyses were performed as described by Picariello et al. (2002). For each sample the amounts of DNA loaded on nitrocellulose were 100, 50, 25, 12.5, 6.2 and 3.1 ng, respectively. Hybridisation for Southern blots and dot blots was carried out in 10×SSC at 68°C overnight with a biotin-labelled sample of S1a repeats amplified from *R. pseudodalmatina* or *R. macrocnemis* genomic DNA. After two wash-

ings under medium stringency conditions for 15 min. in 0.1×SSC at 56°C, filters were exposed to streptavidin-alkaline phosphatase (Sigma), and hybridisation signals were developed by means of a BCIP-NBT staining procedure.

FISH

Preparation of metaphase plates from testes, intestine, lungs and spleen of a male specimen R.ps2b was carried out as described by Odierna et al. (1999). FISH were performed as described by Cardone et al. (1997) with a biotinylated sample from genome-amplified S1a repetitive units from *R. pseudodalmatina* or *R. macrocnemis*. Images of metaphase plates of *R. pseudodalmatina* were acquired by using a Leica DM 6000 B epifluorescence microscope, whereas metaphase plates of *R. macrocnemis* were taken with a Zeiss fluorescence microscope Axioskop HBO-50 equipped with a camera.

DNA cloning

Purified S1a repeat monomers amplified with primer pair C from DNA of specimen Ps2b were cloned in a T-easy vector (Promega). After transformation, white colonies were screened by PCR amplification with M13 direct and reverse primers, and analysed by means of agarose-gel electrophoresis. The bands of S1a-inserts amplified from 23 positive colonies were excised from gel, purified and used to sequence the corresponding repetitive unit as described by Picariello et al. (2016).

DNA sequencing

Each mixture of purified S1a repeat monomers produced by PCR from genomic DNA was sequenced in both orientations with each of the two primers used for amplification and the two sequences were compared with the Sequence Navigator software of the automatic sequencer. For each specimen, we identified the 'most common sequence' (MCS) and the 'consensus sequence' (GCS) of S1a repetitive units present in the genome, as described by Feliciello et al. (2005, 2006). This requires sequencing of two amplification products generated by two primer pairs located in different positions of the repetitive unit sequence, because the sequence around each primer pair can be identified by sequencing the amplification product obtained by the other primer pair. In this case, and in addition to primer pairs A and B previously used to characterize S1a-repeats from Anatolian brown frogs, we also analysed S1a repeat mixtures amplified with primer pair C, to confirm the presence of the same sequence differences between the two Iranian taxa in all three amplification products.

Each mixture of amplified repetitive units contains a very large number of sequence variants. Consequently, electropherograms showed the presence of more than one base in many positions of the sequence. The MCS of the repetitive units in the genome of a specimen takes into account only the main base occurring in each position of the repeat sequence. The few positions in which two bases were present in about the same amounts (50±10%) are indicated by IUBMB one-letter codes (e.g. Y for C and T). In the GCS, the main variable positions of the sequence are indicated by replacing the main base in the MCS with the letter of the IUBMB code corresponding to the main and minor base(s) occurring in that position. The main variable positions are those in which the minor base(s) display a signal at least one-tenth of that of the major base in both the direct and reverse sequencing orientation.

Purified S1a clones were sequenced in direct and reverse orientations by an automatic sequencer (Applied Biosystems, Foster City, CA), using the Big-Dye Terminator kit by the same manufacturer and M13 direct and reverse primers. From the 23 sequenced S1a repeats, we identified the clone most common sequence (cMCS) and the clone consensus sequence (cCS) with the same thresholds for base substitutions employed for MCS (40%) and GCS (10%). Therefore, cMCS indicates all bases found in at least 10 clones and cCS those found in at least 3 clones.

We also calculated the average sequence divergence (ASD) of these cloned repeats from the MCS as the percentage of S1a repetitive unit sizes by dividing the sum of the base changes from MCS occurring in these repeats by their number (23) and by the size of the S1a repeat (477 bp).

Sequence divergence of S1a MCS among samples

Sequence divergence of the MCS of S1a repetitive units from *R. pseudodalmatina* and *R. macrocnemis* from the MCS of the same repeat from European brown frogs was calculated as the percentage of total bases changed, either considering all base changes (A) or only base substitutions (B). Positions that contained two bases in the MCS of a species and one of these two bases in the other species were considered 0.5 changes. In A, the total bases correspond to the size of S1a repeat in the European brown frog species considered (494 or 485 bp), and in B, to the size of S1a repeat from *R. pseudodalmatina* and *R. macrocnemis* (477 and 476 bp, respectively).

Results

Southern blot analysis

Each European brown frog species presents a species-specific S1 satellite DNA. The S1a repeats show differences in restriction sites, and (when present) S1b repeats show differences in both size and restriction sites. Consequently, after DNA digestion with specific enzymes, each species presents a different, species-specific pattern of S1 satellite fragments on Southern blots. S1 satellite DNA from Anatolian brown frog taxa was previously found to contain only S1a repetitive units that are slightly smaller than those

found in European brown frogs (476 vs. 494 bp), and have one site for the restriction enzymes *Eco*RV, *Nde*I and *Nhe*I and two sites for *Kpn*I (Picariello et al. 2016). One sample of *R. macrocnemis* and one of *R. pseudodalmatina* from Iran were digested with each of these four restriction enzymes and analysed by Southern blot (Fig. 1). The resulting patterns of S1 satellite hybridisation bands indicate that the taxon *pseudodalmatina* also contains only S1a repetitive units that have the same size and sites for these four restriction enzymes as S1a repeats from *R. macrocnemis*. This indicates that the repetitive units of S1 satellite are very similar or identical in both taxa.

Quantitative dot blot analysis

We have previously found that samples obtained from a single cloned repetitive unit do not produce complete hybridisation of all satellite DNA repeats present in a genome and that this can be overcome by using samples derived from the mixture of satellite DNA repeats amplified from genomic DNA (Amor et al. 2009). For this reason, the dot blot analysis illustrated in Fig. 2 was carried out using a biotin-labelled sample derived from purified S1a monomers amplified from *R. macrocnemis* and two quantitative standards. Standard A contained 2% of purified S1a monomers amplified from DNA of the same species and 98% *Escherichia coli* DNA as carrier, while standard B was a DNA sample from *R. italica*. Standard B was found to contain 160±30 fmol of S1 satellite repeats per microgram of DNA, a value corresponding to about 4% of the frog genome.

We estimated that the average content of S1a satellite repeats per microgram of genomic DNA in the samples analysed (Fig. 2) is 14±5 fmol per microgram of genomic DNA in the three samples of *R. pseudodalmatina*, and 10±2 fmol in the *R. macrocnemis* sample from Iran. The latter value corresponds to the value of 5±1 fmol previously calculated for *R. macrocnemis* specimens from Anatolia, assuming that S1 satellite repeats account for 2% of the *R. italica* genome as previously determined using a standard from a single repetitive unit (Cardone et al. 1997, Picariello et al. 2016). As expected, because of their high mutual homology, the same results were obtained when both biotinylated samples and unlabelled quantitative standards from *R. pseudodalmatina* were used (not figured).

R. macrocnemis has a C-value of 6.36 pg / nucleus (MacCulloch et al. 1996). In this species, a content of 10 fmol per microgram of genomic DNA corresponds to 6×10^4 copies of the S1a repeat per nucleus. If *R. pseudodalmatina* has about the same C-value, its quantitative dot blot indicates the presence of about 8×10^4 copies of the S1a repeat per nucleus. Because of the limited precision of the technique and some variability among specimens, these results indicate that the two taxa from Iran as well as all tested brown frog taxa from Anatolia contain about the same amount of S1 satellite repeats in their genomes.

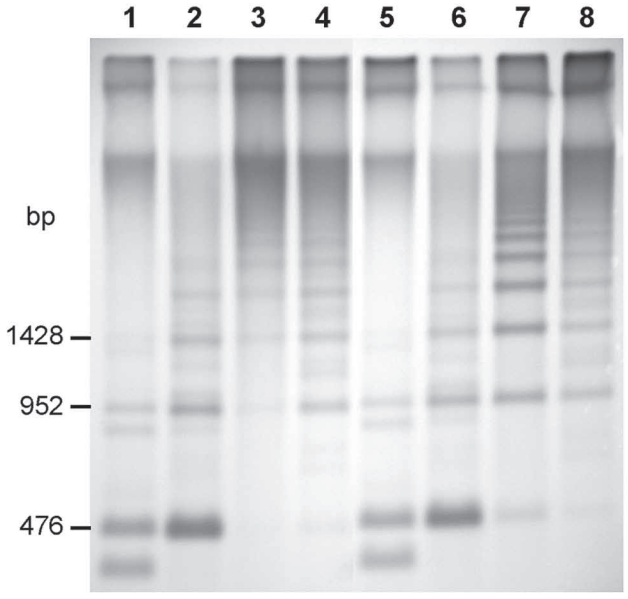

Figure 1. Southern blot analysis of the S1 satellite DNA from Iranian brown frog taxa. 3 µg of DNA from *Rana macrocnemis* specimen R.ma1 (rows 1–4) or from *R. pseudodalmatina* specimen R.ps3 (rows 5–8) were digested overnight at 37°C with 10 units of *Kpn*I (rows 1, 5), *Eco*RV (rows 2, 6,), *Nde*I (rows 3, 7), *Nhe*I (rows 4, 8). Following electrophoresis, samples were transferred to nitrocellulose filters and hybridised with a S1a biotinylated sample obtained from *R. macrocnemis* as described in the Material and methods section.

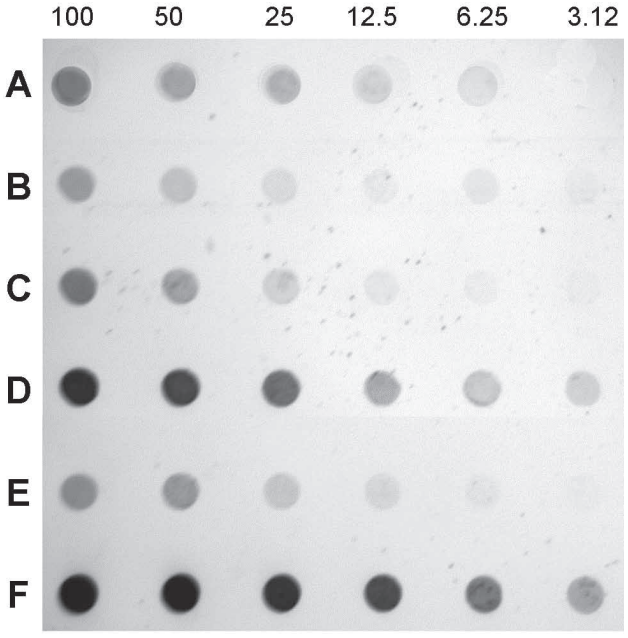

Figure 2. Quantitative dot blot hybridisation. Denatured DNA samples (from 100 to 3.12 ng) were loaded as indicated: (A) *Rana pseudodalmatina* R.ps2b; (B) R.ps1a; (C) R.ps3; (E) *R. macrocnemis* R.ma1. Quantitative standards are: 2% purified S1a monomers amplified from R.ma1 DNA diluted with *Escherichia coli* DNA (D); DNA from *R. italica* (F).

FISH

As frequently observed in other brown frog species, we could obtain only very few metaphase plates of poor quality from the available samples of *R. pseudodalmatina*. Interphase nuclei showed two strong signals and a few weaker ones after hybridisation with an S1a satellite sample (not shown). All metaphase plates indicated the location of strong signals in the pericentromeric position of a chromosome pair. The best available metaphase plates (Fig. 3A) allowed us to obtain a complete karyotype and locate the strong signals in the pericentromeric position of the short arm of chromosome pair 2. The karyotype of *R. macrocnemis* consists of 2n=26 bi-armed chromosomes (Birstein 1984); *R. pseudodalmatina* has a very similar karyotype (present paper). Previously unpublished FISH experiments on a sample from the intestine of a *R. macrocnemis* specimen also located these signals on the short arm of chromosome pair 2 (Fig. 3B). These results indicate that S1 satellite DNA conserved the same main location in both taxa.

Sequence analysis of S1a repetitive units

As in the case of the European and Anatolian brown frog species, we were able to sequence the mixture of S1a satellite repeats amplified from genomic DNA of brown frogs from Iran. This analysis allowed us to define the most common sequence (MCS) and the consensus sequence (GCS) of the satellite repetitive units in the genome.

In all brown frog species previously analysed, MCS is species-specific and identical in all populations of the species. The same is true for the overall genomic variability of repetitive units, the relative amount of main and minor bases in each variable position of the sequence differ even among specimens collected from the same place. Consequently, the genomic consensus sequences (GCS), which indicate the major and minor base(s) found in the main variable positions of the repetitive units in a genome, usually vary even within the same population. S1a repetitive units from five specimens of the taxon *pseudodalmatina* from the Mazandaran, Gilan and Golestan Provinces had a single MCS and the same overall variability, which both were different from those found in Anatolian brown frogs. The MCS and the GCS of these five specimens are illustrated in Fig. 4, row 1 and rows 2–6, respectively (GenBank accession numbers LT630056-LT630060). In rows 8 and 9 are shown the MCS and GCS (GenBank accession number LT630061) of the frog specimen from West Azerbaijan Province, which correspond to those found in specimens from all Anatolian varieties of *R. macrocnemis* previously characterized, as expected (Picariello et al. 2016).

The MCS of S1a repeats from *R. macrocnemis* and *R. pseudodalmatina* share the same 8 and 9 bp deletions from the 494 bp-S1a repeat of European brown frogs, but the deletion of C in position 284 occurs only in *R. macrocnemis*. The MCS from *R. macrocnemis* (row 8) shows 22 base changes from that of *R. pseudodalmatina*. Only the C-T mismatch in position 196 is uppercase, because it is the only complete base change. All the other 21 changes are incomplete. The MCS from *R. pseudodalmatina* and

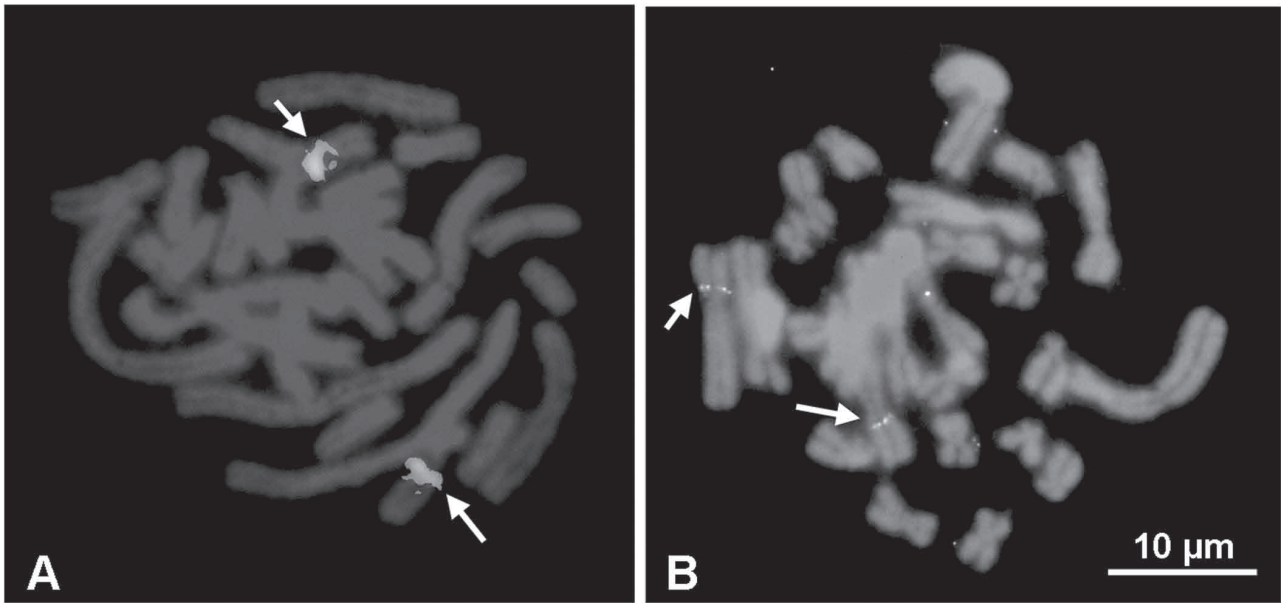

Figure 3. A) Metaphase plate from *Rana pseudodalmatina* hybridised with an S1a satellite sample from the same species; B) Metaphase plate from *R. macrocnemis* hybridised with an S1a satellite sample from the same species. Arrows indicate the main S1 satellite DNA localisation.

R. macrocnemis contain three variable positions each. In all six instances, one of the two main bases present in the MCS variable position of one species corresponds to the main base occurring in the same position in the other species. In 12 of the residual 15 positions, the main base occurring in the MCS from *R. macrocnemis* was also found as a minor base in a main or minor variable position of S1a repeat from *R. pseudodalmatina*. In three positions, both species showed as minor base the main base occurring in the other species.

We also sequenced 23 clones of S1a repetitive units from one specimen from the Gilan Province (GenBank accession numbers LT630033-LT630055). These cloned repeats had an average sequence divergence from the MCS of only 2%, which confirmed the high homogeneity of S1 satellite repetitive units also in this taxon. About 3/4 of these base changes are due to substitutions of the main base with a minor base in variable positions, and 1/4 to other base changes.

From these sequences, we derived a clone most common sequence (cMCS) and a clone consensus sequence (cCS). As in the case of *R. macrocnemis,* cMCS was identical to genomic MCS (row 1), including the three variable positions (Picariello et al. 2016). Obviously, cCS is only related to the variability of the few repeat sequences analysed. As expected and already observed in other brown frog species, only part of the main genomic variable positions occur in cCS, while some minor variable positions in the genome may occur as main variable positions (row 7) as in the case of the C deletion in position 284 found in 3 of the 23 analysed clones.

In Table 1, we compared the percentages of sequence divergence of the MCS of *R. macrocnemis* and *R. pseudodalmatina* from the MCS of the European brown frog species as identified from the total base changes (A), or only from base substitutions (B). As is evident, the MCS of S1a repeats from *R. pseudodalmatina* has a divergence that is lower by 10–25% than the MCS from *R. macrocnemis* in the corresponding S1a MCS from all European brown frog species.

Discussion

S1 satellite from brown frogs is the only satellite DNA family in which the overall sequence variability of the repetitive units within a species and in several related species has been characterized and reported so far. In all European

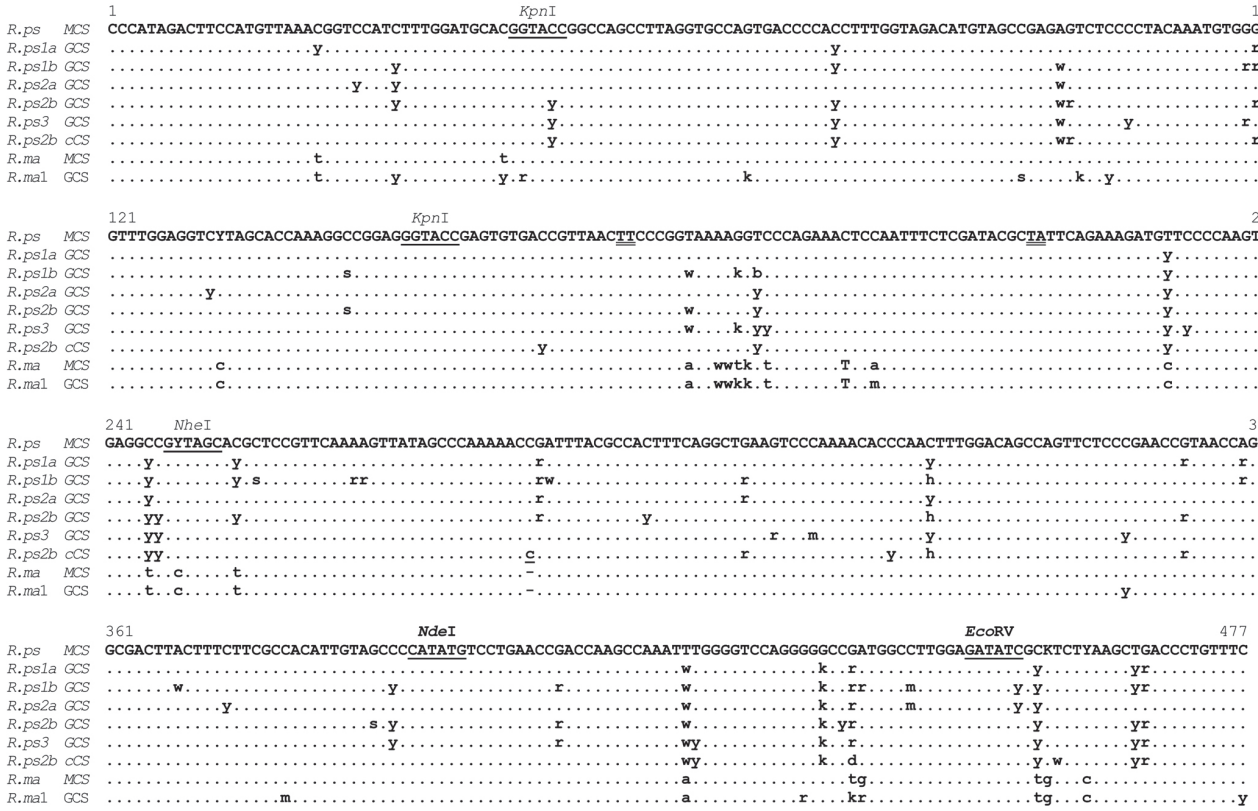

Figure 4. Genomic 'most common sequence' (MCS) and 'consensus sequences' (GCS) of the S1a repetitive units from Iranian brown frogs. Row 1: MCS of the S1a repetitive unit from *Rana pseudodalmatina*. Double underscoring indicates the position of the 8 and 9 bp deletions. Rows 2–6: genomic consensus sequence of the indicated specimens of *R. pseudodalmatina*. Row 7: clone consensus sequence from 23 S1a repeats cloned from specimen R.ps2b. MCS (row 8) and GCS (row 9) from *R. macrocnemis* specimen R.ma1. Recognition sites for the indicated restriction enzymes are underscored. – indicates one bp deletion.

Table 1. Sequence divergence of MCS of the S1a repetitive units from *Rana pseudodalmatina* and *R. macrocnemis* from MCS of the same repeat from European brown frogs. Sequence divergence was calculated as a percentage of total bases changes, either considering all base changes (A) or only changes due to base substitutions (B).

Rana	*pseudodalmatina*		*macrocnemis*	
	A	B	A	B
temporaria	13.3	10.2	15.2	12.0
dalmatina	10.9	7.8	13.1	9.8
arvalis	12.9	9.7	15.2	12.0
graeca	10.9	7.8	12.2	8.9
italica	12.6	9.4	14.5	11.2
latastei	13.1	10.0	14.5	11.2
iberica	12.4	10.8	14.3	12.5
pyrenaica	11.1	8.0	12.4	9.1

brown frog species, analysis of the variability of S1 satellite DNA at genomic level showed a remarkable property: the MCS of S1a and, when present, the S1b repetitive unit is identical in all individuals of a species and possible subspecies, but different from species to species. Therefore, this satellite DNA can be considered an absolute marker of species in brown frogs (Picariello et al. 2002).

The taxonomy of the brown frogs from Anatolia and neighbouring regions has been debated for a long time. These frogs were originally classified as three different species: *R. macrocnemis*, *R. camerani* and *R. holtzi*. However, more recent morphological studies (Mensi et al. 1992, Picariello et al. 1999) and the detailed phylogenetic analysis by Veith et al. (2003), using mitochondrial sequences, definitely confirmed that all three taxa belong to a single polymorphic species: *R. macrocnemis*. However, the latter study also indicated that brown frogs populations from Tavas (Turkey) and the northern Iranian provinces should represent two distinct species *R. tavasensis* and *R. pseudodalmatina*, respectively.

In a previous study, we demonstrated that specimens from all Anatolian populations tested, including a DNA sample of *R. tavasensis* received from Dr. Veith, display the same MCS and genomic variability of S1a repetitive units (Picariello et al. 2016). This was also confirmed in a *R. macrocnemis camerani* specimen from Georgia, and in this report in a *R. m. macrocnemis* specimen from Iran (Fig. 4, rows 8 and 9). The homogeneity of the S1a repetitive unit in all Anatolian taxa suggests that they belong to a single species. However, more recent studies based on mitochondrial sequences of CYTB and COI gene regions strongly support the species rank of *R. tavasensis* (Najibzadeh et al. 2017, Kalayci et al. 2017). Since it is unlikely that two species that formed and evolved independently can have an identical satellite DNA, the absolute homogeneity of S1 satellite DNA in all Anatolian frogs should be confirmed from other specimens of *R. tavasensis* in order to exclude a sampling error.

In this study, we characterized S1 satellite DNA from the brown frog taxon *pseudodalmatina* in specimens from all three Iranian provinces where these frogs are found. Initial results seemed to support the inference that this taxon is a variety of *R. macrocnemis*. In Southern blots, samples of the taxon *pseudodalmatina* display patterns of hybrid bands similar to those found in *R. macrocnemis* from Anatolia (Picariello et al. 2016) and Iran (Fig. 1). Moreover, quantitative dot blots indicate a similar genomic amount of S1 satellite DNA in all taxa from Anatolia (Picariello et al. 2016) and Iran (Fig. 2). FISH analyses also indicate that this satellite DNA in the taxon *pseudodalmatina* is mainly located in a pericentromeric position on the short arm of chromosome 2, as is the case in *R. macrocnemis*.

However, all tested specimens representing the taxon *pseudodalmatina* displayed MCS and genomic variability of S1a repeats that were different from those found in *R. macrocnemis* specimens. This indicates that they belong to a closely related, but different species. This close relationship is also indicated by the presence in their S1a repetitive units of the same 8 and 9 bp deletions from S1a repeats from European brown frogs. MCS of the two species differ only in 22 positions, and most of these changes are not complete because the main base present in S1a MCS from *R. macrocnemis* frequently occurs as a minor base or deletion in the corresponding main or minor variable positions of S1a repeats from *R. pseudodalmatina*. Moreover, S1a MCS from *R. macrocnemis* also shows a 10–25% higher sequence divergence from S1a MCS from all European brown frog species than that from *R. pseudodalmatina* (Table 1). Both features suggest that *R. pseudodalmatina* could be the more ancient extant species of the Anatolian lineage and the progenitor species of *R. macrocnemis*. Alternatively, it is possible that both species originated from the common ancestor species of the Anatolian lineage, and that *R. pseudodalmatina* has maintained the original structure of the S1a repetitive unit to a greater extent than *R. macrocnemis*.

In conclusion, our results based on S1 satellite DNA from the brown frogs of the Anatolian lineage confirm that *R. pseudodalmatina* and *R. macrocnemis* are distinct species.

Our studies of S1 satellite DNA from Western Palaearctic brown frogs also provide relevant conclusions on the usefulness of satellite DNA in phylogenetic and taxonomic analyses. The definition of the structure of all other satellite DNAs has been based only on analysis of the sequences of cloned repetitive units. This analysis has indicated that related species frequently have satellite DNAs belonging to the same family.

In many cases, however, related species were shown to contain satellite DNAs of very similar structure (see e.g. Lohe & Brutlag 1987, De la Herran et al. 2001, Mravinac et al. 2002, Plohl et al. 2010), and sometimes the same species may contain two varieties of the same satellite DNA as human alpha A and B satellite DNA (Romanova et al. 1996). Probably the most extreme case is represented by the ZpS1 satellite DNA specific of, and present in,

all species of the old plant genus *Zamia* (Cafasso et al. 2009). Analysis of repetitive units cloned from 12 species representative of the whole geographic distribution of the genus in North, Central and South America indicated that all 12 species contain the same satellite variety, with an additional species-specific variety occurring in two of these species (Cafasso & Chinali 2014). This indicates that the common satellite variety is likely to have formed at the origin of the genus (65 mya) and has been persisted with little changes through intermediate species to most or all extant *Zamia* species. All satellite DNAs showing no evident differences between species are clearly poorly suitable for phylogenetic analyses.

Other satellite DNA families contain different satellite varieties in each related species, and could therefore be used to define the phylogeny of these species. This is the case of the S1 satellite family from brown frogs, whose repetitive units appear to have originated from a 494 bp S1a repetitive unit present in a common ancestor species. In this case, the species-specific variety is univocally defined by the genomic MCS. However, as has been analysed in a previous study (Picariello et al. 2016), phylogenetic trees obtained by four different statistical methods were neither reliable, as indicated by their low bootstrap values, nor resembled the tree obtained by Veith et al. (2003) who had used mitochondrial and nuclear sequences. In each brown frog species, the MCS and overall genomic variability of S1a repetitive units were the same in all specimens of the same frog species including possible subspecies. Taken together, these features indicate that different S1a satellite varieties were generated at the origin of each species by amplification of a specific subpopulation of repeats and that each variety has since maintained its structure and overall variability for millions of years to the present. The absence of evolution in satellite DNA in species represents one of the most amazing features discovered in the eukaryotic genome. Our results indicate that this exceptional stability is due to a specific repair mechanism that has the function to keep satellite DNA stably assembled in the constitutive heterochromatin (Feliciello et al. 2006). This mechanism is activated when the accumulation of mutations determines the inability of a part of a satellite DNA array to be correctly packed in constitutive heterochromatin, and replaces the defective part with a new array generated from non-mutated functional repetitive units by rolling-circle amplification. This mechanism periodically restores the original variability of repetitive units in satellite arrays and thus can maintain satellite DNA practically unchanged in a species as long as this species continues to exist. This also explains why some satellite DNAs, like the ZpS1 satellite, can be transmitted with little changes from species to species for many millions years.

Our conclusion is that satellite DNA should not be used for phylogenetic analyses, because its modality of evolution is completely different from that of the other components of the genome and therefore not suitable for phylogenetic analyses by statistical methods.

The species-specific structure and overall variability of satellite repetitive units could be relevant to assign closely related taxa to the same or different species. The main limitation of this analysis is that sequences of cloned repetitive units do not make provision for the overall genomic variability of the repetitive units and, consequently, the identity of two closely related satellite DNAs to be determined. However, depending on repetitive unit variability, in many satellite DNAs, 10–30 repeat sequences are usually sufficient to define a clone most common sequence (cMCS), which contains very little or no variable positions and should be identical or quasi identical to the genomic MCS.

The presence of satellite DNAs of the same family with different cMCSs in two related taxa provides strong evidence that these taxa contain different satellite DNA varieties and therefore are distinct species. By contrast, the presence of very similar or even identical cMCSs does not necessarily imply that the two taxa belong to the same species.

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