The 18S rRNA from *Odontophrynus americanus* 2*n* and 4*n* (Amphibia, Anura) reveals unusual extra sequences in the variable region V2

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Abstract: The nucleotide sequence of the rDNA 18S region isolated from diploid and tetraploid species of the amphibian *Odontophrynus americanus* was determined and used to predict the secondary structure of the corresponding 18S rRNA molecules. Comparison of the primary and secondary structures for the 2*n* and 4*n* species confirmed that these species are very closely related. Only three nucleotide substitutions were observed, accounting for 99% identity between the 18S sequences, whereas several changes were detected by comparison with the *Xenopus laevis* 18S sequence (96% identity). Most changes were located in highly variable regions of the molecule. A noticeable feature of the *Odontophrynus* 18S rRNA was the presence of unusual extra sequences in the V2 region, between helices 9 and 11. These extra sequences do not fit the model for secondary structure predicted for vertebrate 18S rRNA.

Key words: Odontophrynus americanus, Amphibia, polyploidy, 18S ribosomal DNA, molecular evolution.

Résumé : La séquence de l'ADNr 18S d'espèces diploïdes ou tétraploïdes du batracien *Odontophrynus americanus* a été déterminée et employée pour prédire la structure secondaire des molécules correspondantes d'ARNr 18S. Une comparaison des structures primaire et secondaire chez les espèces 2*n* et 4*n* a confirmé que ces espèces sont très apparentées. Seules trois substitutions nucléotidiques ont été observées entre ces espèces (résultant en une identité de 99 % entre les séquences 18S), tandis que plusieurs différences ont été détectées par rapport à la séquence du *Xenopus laevis* (96 % d'identité). La plupart des changements étaient situés dans les régions hypervariables de la molécule. Une caractéristique notable de l'ARNr 18S de l'*Odontophrynus* est la présence de séquences additionnelles inhabituelles dans la région V2, entre les hélices 9 et ll. La présence de ces séquences additionnelles discorde avec le modèle prédit de la structure secondaire de l'ARNr 18S chez les vertébrés.

Mots clés : Odontophrynus americanus, batraciens, polyploïdie, ADN ribosomique18S, évolution moléculaire.

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Introduction

The ribosomal gene family has been extensively studied in eukaryotes in view of the important role the rRNA molecules exert in ribosomal structure and function. This is underscored by the partial conservation of their sequences across species. The 18S rRNA molecule is a mosaic structure composed of several highly conserved domains and eight variable regions, which accounts for the species specificity of secondary structure patterns (Wuyts et al. 2000; 2001; Van de Peer et al. 2000). These secondary structure features greatly enhance the quality of rRNA molecule alignments and comparisons among different species (Eddy 2002; Dandekar and Hentze 1995). The 18S molecule is folded into 50 helical regions, interspersed with single-stranded areas. The conservation of sequence and (or) structure reflects functional constraints on the rRNA molecules related to translation efficiency. Since different regions of the 18S

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Fig. 1. Restriction map of the diploid and tetraploid *O. americanus* 18S rDNA region. Clone DNAs were digested with *Bam*HI (B), *Dra*I (D), *Sac*I (S), and *Xba*I (X) restriction enzymes. Black rectangle, sequences coding for 18S rRNA (1889 bp); IGS, intergenic spacer; ETS, external transcribed spacer; ITS1, internal transcribed spacer 1.

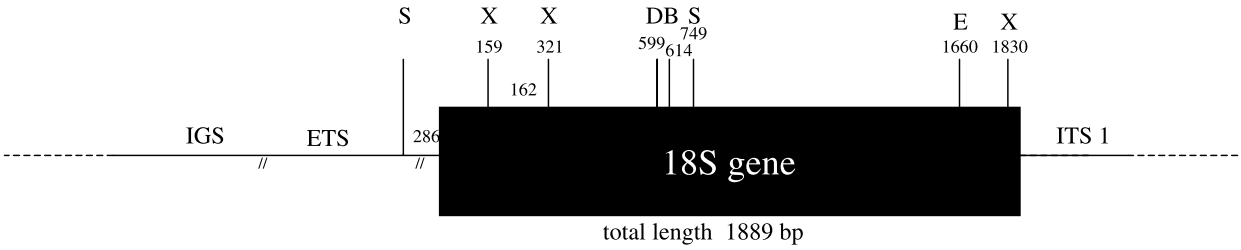

Fig. 2. Alignment of 18S sequences from *Odontophrynus americanus* 2*n* and 4*n* and *Xenopus laevis*. Identical nucleotides are denoted by dots; point mutations and extra-sequences are indicated in bold characters.

```
Oa 2n 18s       TACCTGGTTGATCCTGCCAGTAGCATATGCTTGTCTCAAAGATTAAGCCATGCACGTGTA    60
Oa 4n 18s       ............................................................    60
Xl 18s          ............................................................    60
                ************************************************************

Oa 2n 18s       AGTACACACGGCCGGTACAGTGAAACTGCGAATGGCTCATTAAATCAGTTATGGTTCCTT   120
Oa 4n 18s       .....A......................................................   120
Xl 18s          .....G......................................................   120
                ***** ******************************************************

Oa 2n 18s       TGATCGCTCCAACCGTTTCCTCGGATAACTGTGGTAATTCTAGAGTCACCAAGGCGGCCG   180
Oa 4n 18s       ...........A.C...T.C.C.....................TC.CC.AGGCG....   180
Xl 18s          ...........T.T...A.T.-.....................CT.AT.CA--T....   177
                *********** * *** * * **********************  *  *     ****

Oa 2n 18s       GGGAGAGCGGAGGGGAGGACGACGGCGGCCCCGAGAGGTCGCCGAGCGCACCACGCACCC   240
Oa 4n 18s       GG...A..GGAGGGGAGGACGACGGC.G....G..A.G.CGCCGAGCGCACCA..CA.CC   240
Xl 18s          AC...C..-----------------T.A....C..G.A.-------------G..TG.AT   207
                  *** **                  * **** ** * *             **  *

Oa 2n 18s       CCGCCCGGGAACCCCGGATTGGTTTTGGTCTGATAAATGCACGCATCCCTGGGGGTCAGC   300
Oa 4n 18s       CCGC.CGGG.AC..CGG.TTGG.TTT..T.TGATAAATGCA...AT...T.GGGGTCAG.   300
Xl 18s          TTAT.----.GA..AAA.CCAA.CCG..G.C--------CC...GC...G.CC------.   248
                    *    *  **   *    *   ** *         * ***  *** *       *

Oa 2n 18s       GCTCGTCGGCACGTATTAGCTCTAGATAACCTCGGGCCGATCGCACGTCCTCGTGACGGC   360
Oa 4n 18s       ...CGTCGGCACG.AT.AG.....................................T....G....   360
Xl 18s          ...T---------.GG.GA.....................................C....A....   299
                ***          *  *  ******************************** **** ****

Oa 2n 18s       GACGATCCATTCGGGTGTCTGCCCTATCAACTTTCGATGGTACTTTCTGCGCCTACCATG   420
Oa 4n 18s       ......C.......G.............................................   420
Xl 18s          ......A.......A.............................................   359
                ****** ******* *********************************************

Oa 2n 18s       GTGACCACGGGTAACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCT   480
Oa 4n 18s       ............................................................   480
Xl 18s          ............................................................   419
                ************************************************************

Oa 2n 18s       ACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCACTCCCGACTCGGGGAGGTAGT   540
Oa 4n 18s       ...........................................T................   540
Xl 18s          ...........................................G................   479
                ******************************************* ************
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Fig. 2 (*continued*).

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Oa 2n 18s       GACGAAAAATAACAATACAGGACTCTTTCGAGGCCCTGTAATTGGAATGAGTACACTTTA  600
Oa 4n 18s       ............................................................  600
Xl 18s          ............................................................  539
                ************************************************************

Oa 2n 18s       AATCCTTTAACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCA  660
Oa 4n 18s       ..................C.........................................  660
Xl 18s          ..................T.........................................  599
                ****************** ****************************************

Oa 2n 18s       GCTCCAATAGCGTATATTAAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATCTTGGGAT  720
Oa 4n 18s       ............................................................  720
Xl 18s          ............................................................  659
                ************************************************************

Oa 2n 18s       CGAGCTGGCGGTCCGCCGCAAGGCGAGCTCACCGCCTGTCCCAGCCCCTGCCTCTCGGCG  780
Oa 4n 18s       ..................A.........C...............................  780
Xl 18s          ..................G.........-...............................  718
                ****************** ********* ****************************

Oa 2n 18s       CCCCCCCGATGCTCTTGACTGAGTGTCCCGGGGGCCCGAAGCGTTTACTTTGAAAAAATT  840
Oa 4n 18s       ..C..T......................................................  840
Xl 18s          ..T..C......................................................  778
                ** ** ******************************************************

Oa 2n 18s       AGAGTGTTCAAAGCAGGCCGGGTCGCCTGAATACTTCAGCTAGGAATAATGGAATAGGAC  900
Oa 4n 18s       .........A..........G........A...............................  900
Xl 18s          .........C..........C........G...............................  838
                ********* ********** ******** ****************************

Oa 2n 18s       TCCGGTTCTATTTTGTTGGTTTTCGGAACTGGGGCCATGATTAAGAGGGACGGCCGGGGG  960
Oa 4n 18s       ............................................................  960
Xl 18s          ............................................................  898
                ************************************************************

Oa 2n 18s       CATTCGTATTGTGCCGCTAGAGGTGAAATTCTTGGACCGGCGCAAGACGAACCAAAGCGA 1020
Oa 4n 18s       ...C........................................................ 1020
Xl 18s          ...T........................................................  958
                *** ********************************************************

Oa 2n 18s       AAGCATTTGCCAAGAATGTTTTCATTAATCAAGAACGAAAGTCGGAGGTTCGAAGACGAT 1080
Oa 4n 18s       ............................................................ 1080
Xl 18s          ............................................................ 1018
                ************************************************************
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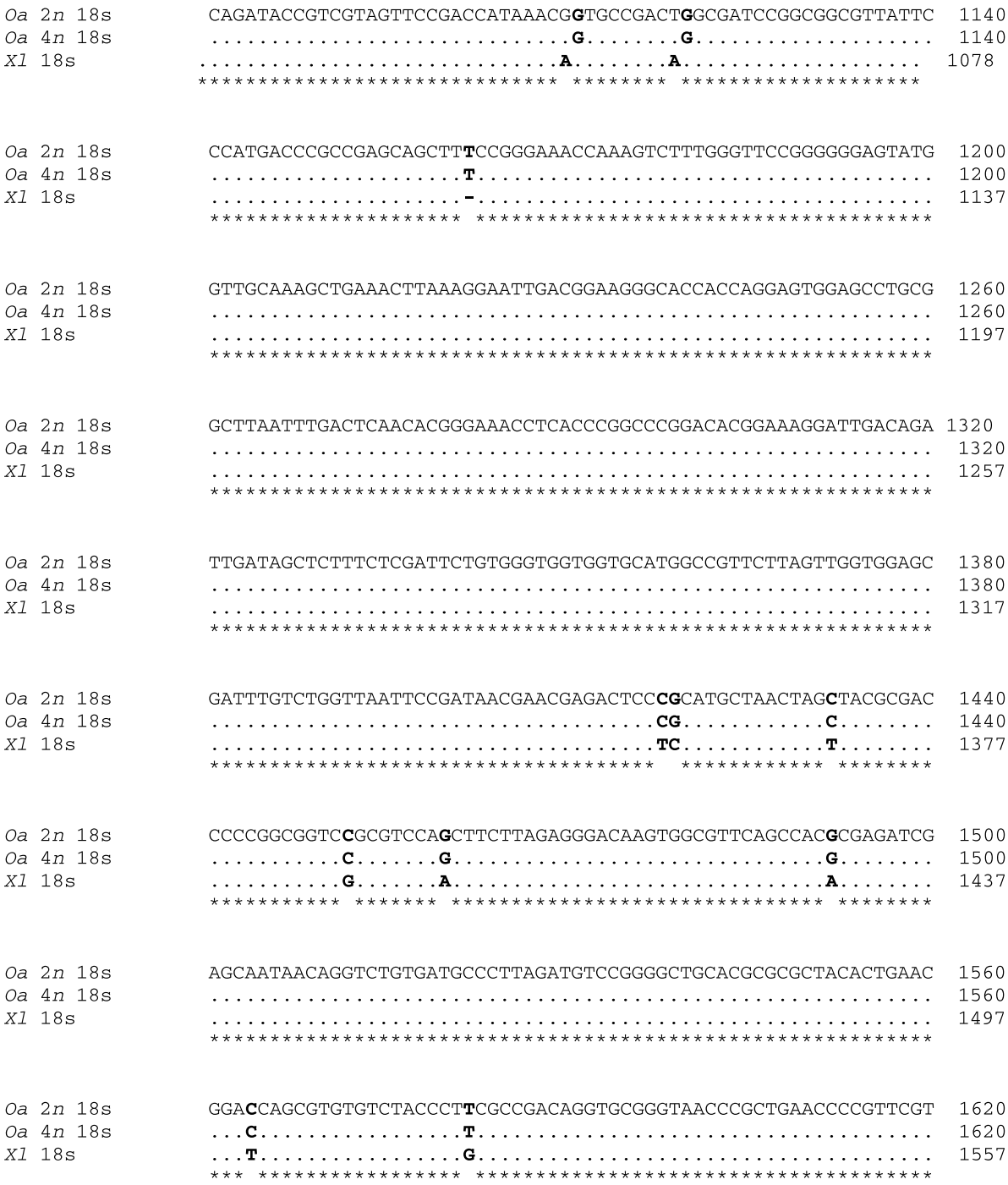
molecule evolve at different rates, evolutionary relationships may be inferred from a broad taxonomic range, as well as from closely related species (Mallat and Sullivan 1998; Bernardi et al. 1992). Even so, some studies have found a negative correlation between the number of nucleotide substitutions and the phylogenetic distance for the 18S rRNA molecule in several metazoa and plants (Abouheif et al. 1998; Soltis et al. 1999).

The diploid and tetraploid species of the anuran amphibians *Odontophrynus americanus* are an important model for evolutionary and gene expression studies in polyploid species. Morphological, cytogenetic, and biochemical studies have shown the close relationship between these two species (review in Ruiz et al. 1981). Further studies on ribosomal cistrons using Southern blotting (Cortadas and Ruiz 1988), cloning (Ruiz and Brison 1989), and sequencing methods (Alvares et al. 1998; 2002) revealed high sequence similarities between them, reinforcing the assumption that the tetraploid *O. americanus* species arose by a recent auto-polyploidy event.

In this paper, the molecular cloning and sequencing of the 18S region of rDNA from 2*n* and 4*n* specimens are reported. Secondary structures inferred from sequencing data were

Fig. 2 (*continued*).



similar to those predicted for *Xenopus laevis* and other eukaryote 18S molecules, with the exception of some extra sequences found in *O. americanus* 18S rRNA that could not fit to the predicted secondary structures.

Material and methods

Cloning and sequencing of diploid and tetraploid 18S rDNA

Genomic DNA was extracted from blood of *O. americanus* 2*n* and 4*n* specimens as described (Ruiz and Brison 1989), in accordance with international principles and guidelines. *Eco*RI restriction fragments were ligated to the *Eco*RI arms of vectors EMBL4 (2*n* DNA) or λgtWESλC (4*n* DNA). The recombinant phages were used to infect *E. coli* LE392 and screened with a ^{32}P-labeled HM456 probe containing the intergenic spacer and the 18S region of *X. laevis* rDNA. One 2*n* and one 4*n* *Eco*RI phage insert was subcloned into plasmid pGEM4 and restriction mapped with *Bam*HI, *Dra*I, *Sac*I, and *Xba*I endonucleases. To isolate the sequences cor-

Fig. 2 (*concluded*).

```
Oa 2n 18s       GATAGGGATCGGGGATTGCAATTATTTCCCATGAACGAGGAATTCCCAGTAAGTGCGGGT  1680
Oa 4n 18s       ............................................................  1680
Xl 18s          ............................................................  1617
                ************************************************************

Oa 2n 18s       CATAAGCTCGCGTTGATTAAGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGA  1740
Oa 4n 18s       ............................................................  1740
Xl 18s          ............................................................  1677
                ************************************************************

Oa 2n 18s       TTGGATGGTTTAGTGAGGTCCTCGGATCGGCCCCGCCGGGGTCGGCGACGGCCCTGGCGG  1800
Oa 4n 18s       ...........................................G................  1800
Xl 18s          ...........................................C................  1737
                ******************************************* ************

Oa 2n 18s       AGCGCCGAGAAGACGATCAAACTTGACTATCTAGAGGAAGTAAAAGTCGTAACAAGGTTT  1860
Oa 4n 18s       ............................................................  1860
Xl 18s          ............................................................  1797
                ************************************************************

Oa 2n 18s       CCGTAGGTGAACCTGCGGAAGGATCATTA  1889
Oa 4n 18s       ............................  1889
Xl 18s          ............................  1826
                ****************************
```

Your guide tree:

625629.451519-176576.dnd

```
(
oa2n18s:0.00000,
oa4n18s:0.00162,
xl18s:0.03289);
```

responding to the 18S rRNA the plasmids were digested with appropriate enzymes and the fragments eluted from agarose gel were subcloned into the pGEM4 plasmid vector.

The 230 nucleotides located between the *Eco*RI site and the 3′ end of the 18S molecule were obtained from other clones containing diploid or tetraploid *Eco*RI inserts covering the 18S 3′ end, ITS1, 5.8S, ITS2, and most 28S sequences (I.R.G. Ruiz, E.P. Silva, and O. Brison, unpublished results). After restriction enzyme digestion of these clones, diploid and tetraploid *Eco*RI–*Kpn*I subfragments containing the 3′ end of 18S plus part of the ITS1 region were sequenced (Sequenase USB, Macrophor Pharmacia Sequencer). Most of the 18S subclones were sequenced in the ABI Prism 377 automated sequencer using Big Dye reagent (Perkin Elmer, Foster City, Calif.) based on the dideoxy chain-termination method (Sanger et al. 1977).

Prediction of 18S rRNA secondary structure

The 18S sequences from 2*n* and 4*n* clones were used to infer rRNA secondary structures and to construct a phylogenetic tree with the aid of the DCSE (De Rijk and De Wachter 1993), RnaViz (De Rijk et al. 2003), or Treecon (Van de Peer and De Wachter 1994) softwares, respectively.

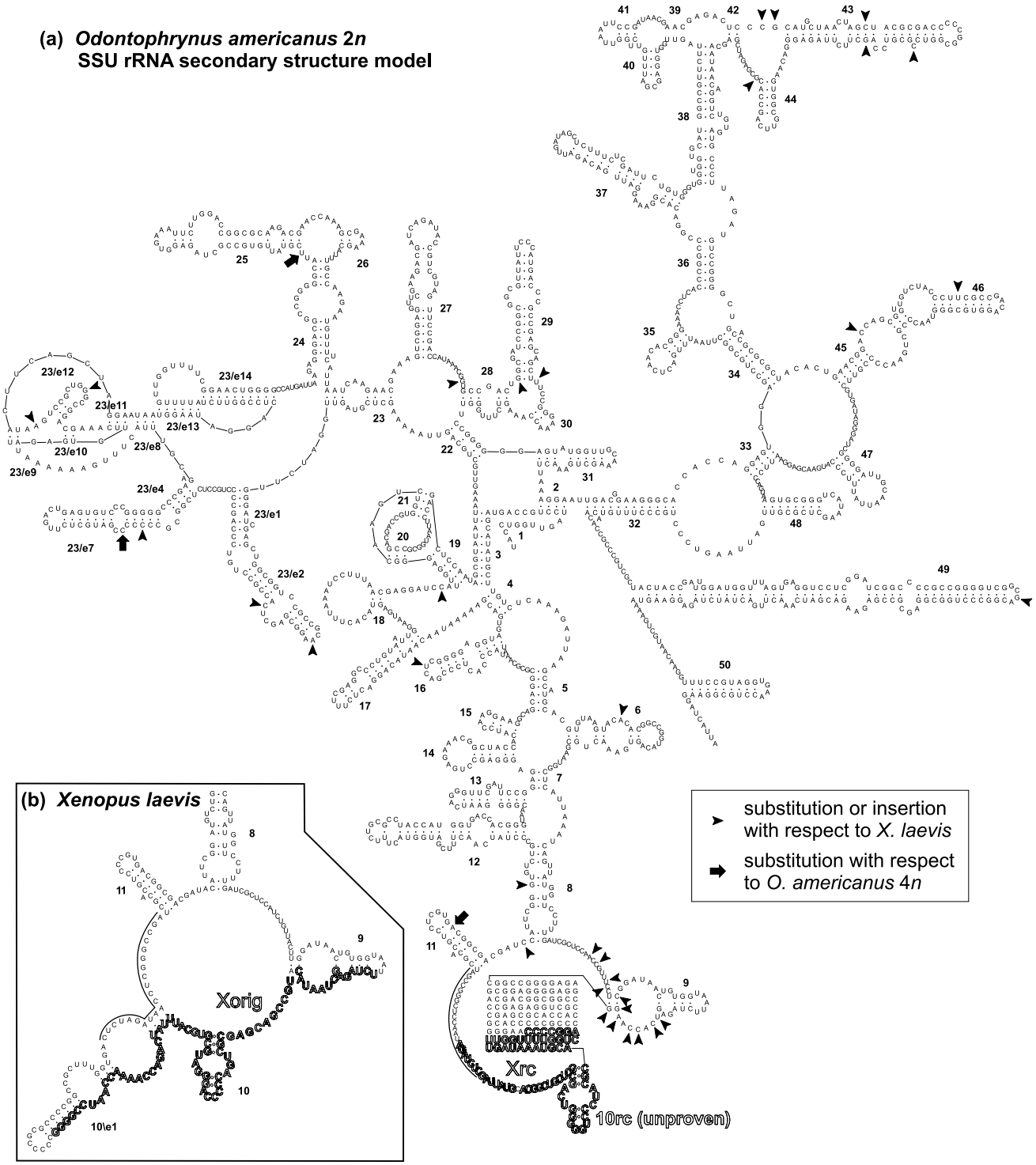
Results and discussion

Clones containing most of the 18S sequences from *O. americanus* 2*n* and 4*n* were digested with the restriction enzymes *Bam*HI, *Dra*I, *Sac*I, and *Xba*I. The same restriction patterns were observed for the 2*n* and 4*n* species, as shown in Fig. 1. *Dra*I was the only restriction site common to *O. americanus* and *X. laevis* 18S rDNAs.

Sequencing of *O. americanus* 2*n* and 4*n* clones revealed that 18S rRNA is 1889 bp long in both cases (EMBL accession Nos. AJ555449 and AJ555450). Three point mutations at positions 356 (A→G), 786 (C→T), and 964 (T→C) were the only differences found between diploid and tetraploid sequences. Comparison of *O. americanus* 2*n* and 4*n* sequences with the *X. laevis* 18S sequence (1826 bp) revealed 38 dissimilarities, which amounts to 97% identity (Fig. 2).

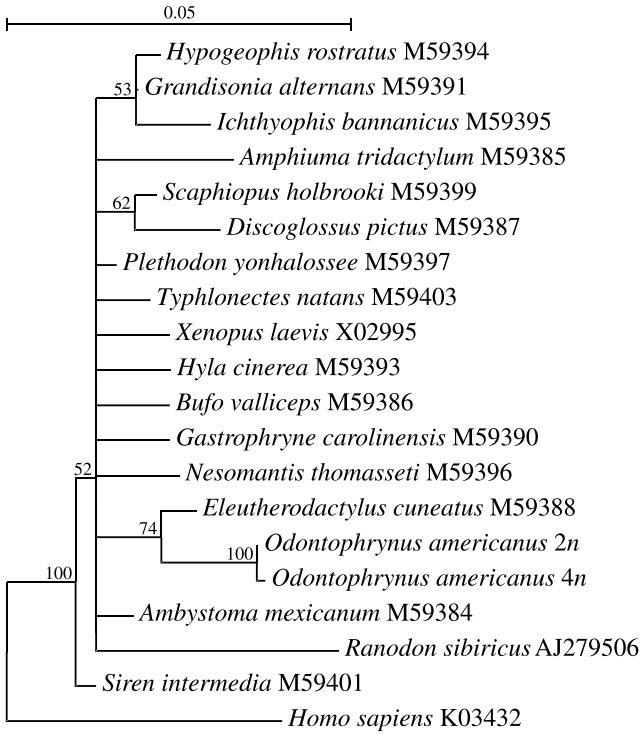
The basic secondary structure of eukaryotic 18S rRNA has been conserved throughout evolution by compensatory and neutral base changes (De Rijk et al. 1992). Comparison of the predicted secondary structures for *O. americanus* 2*n* and 4*n* 18S rRNAs (Fig. 3) shows that the observed differences between 2*n* and 4*n* 18S sequences consist of base changes located in the following regions of the corresponding secondary structures: the double-stranded region in helix

Fig. 3. (*a*) Secondary structure models for *Odontophrynus americanus* 2*n* 18S rRNA. Arrows indicate dissimilarities with respect to the *Odontophrynus americanus* 4*n* sequence, whereas arrowheads indicate dissimilarities with respect to the *Xenopus laevis* sequence. (*b*) Secondary structure model for helices 8–11 of 18S rRNA in *Xenopus laevis*. Nucleotides marked with a line are identical in *Xenopus laevis* and *Odontophrynus americanus*, whereas the sequence in large outline letters in *Odontophrynus americanus* (Xrc) is the reverse complement of the sequence in large outline letters in *Xenopus laevis* (Xorig). Note that in *Odontophrynus* these areas overlap.



11 (A→G), the loop region of helix 23/e7 (C→T), and the single-stranded region between helices 24 and 25 (T→C). These changes are compatible with identical predicted secondary structures for 2*n* and 4*n* 18S rRNAs. The secondary structure model and helix numbering are in accordance with a model published earlier (Wuyts et al. 2001). Fifty helices were observed in the 2*n* and 4*n* structures, as expected (Van de Peer et al. 1999). The few nucleotide substitutions

Fig. 4. Neighbor-joining tree of 19 amphibian rRNA sequences. The sequence of *Homo sapiens* was used as the outgroup. Branches with less than 50% bootstrap support are drawn unresolved; on other branches this percentage is indicated. The distance scale shows the length equivalent for 0.05 nucleotide changes per site.



do not disturb the common base-pairing pattern. The changes in helices 11 and 23 were located in the known highly variable and rapidly evolving regions of the 18S rRNA molecule. Such regions are informative about relatively recent divergences, whereas changes in more conserved regions provide signals occurring across ancient periods of the species history.

When the 18S rRNA sequence from *X. laevis* was compared to that of *O. americanus*, the differences concerning primary and (or) secondary structures appeared in helices or loops 6, 8, 9, 16, 18, 23/e1, 23/e7, 23/e11, 23/e12, 25, 28, 29, 42, 43, 44, 45, and 46. Comparison of the predicted secondary structures for *O. americanus* and *X. laevis* 18S rRNAs indicated that cores of sequence homology appear at the same place in both models (Fig. 3). On the other hand, the variable area V2, located between helices 9 and 11, is 59 bp longer in *O. americanus* (166 nt) than in *X. laevis* (107 nt) and it could not be reliably aligned. Consequently, no secondary structure pattern could be inferred for this area. Most nucleotides between helices 9 and 11 (including the extra sequences) are shown in condensed format, since no independent structure could be assigned to these sequences. A BLAST (Altschul et al. 1996) search of this area against a database with all available 18S sequences revealed that a 75-nt segment of the 3′ part of this area is, with one mismatch, the exact reverse complement of the 5′ part of area V2 in *X. laevis* (large outline letters in Fig. 3) and most other amphibians if a few additional substitutions are accepted. Hereafter, this sequence will be called "Xorig" (*Xenopus* original), whereas the reverse complement of this sequence as found in *O. americanus* will be called "Xrc" (*Xenopus* reverse complement). Helix 10 of *X. laevis* is completely included in Xorig. It is intriguing to assume that Xrc could accommodate the reverse complement of this helix. This hypothetical helix was named 10rc and is included in Fig. 3. Of the remaining 91 nt in the area of V2 of *O. americanus*, 14 are clearly homologous to the 3′ region of V2 in *X. laevis* (marked with a line in Fig. 3). A BLAST search for the other 77 nt in the 5′ area of V2 turned up no significant results. Overall, this rearrangement of the rRNA molecule could have a deleterious effect on the secondary structure of area V2, but apparently this does not disturb the functioning of the ribosome too much. This is not too hard to accept, since this area is located at the periphery of the ribosome in the three-dimensional ribosome structures. In other eukaryotes, especially the Granuloreticulosea and the Euglenida, this area is also subjected to large insertions with both known and unknown secondary structures (Wuyts et al. 2001).

For molecular phylogenic analysis, 18S rRNA sequences from *O. americanus* 2*n* and 4*n* and 20 other amphibians were compared. Human sequence was used as an outgroup. Positions that could not be unambiguously aligned were excluded from the alignment. The phylogeny package Treecon was used to infer the neighbor-joining tree shown in Fig. 4. Sequence dissimilarities were converted to distances by the Kimura (1980) method with a transition:transversion ratio of 2. Bootstrap analysis was performed on 1000 resampled alignments. The 18S sequences of *O. americanus* 2*n* and 4*n* differ in only three positions, whereas human and mouse, which are separated by 80 million years of evolution (Gonzales and Schmickel 1988), already have 12 dissimilarities. This indicates that the divergence between the two *O. americanus* species must have occurred very recently, although the number of dissimilarities is too small to make a meaningful time estimation. In the phylogenetic tree, *Odontophrynus* clusters together with the other Leptodactylidae, *Eleutherodactylus cuneatus*.

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