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Cytological and molecular analysis in the rare discoglossid species, *Alytes muletensis* (Sanchiz & Adrover 1977) and its bearing on archaeobatrachian phylogeny

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Abstract

Cytogenetic and molecular data on *Alytes muletensis* (Amphibia: Discoglossidae) are compared with other representatives of archaeobatrachian frogs: *Bombina variegata pachypus, Pelobates cultripes, Pelodytes punctatus, Xenopus laevis*, and *Discoglossus. A. muletensis* has the karyotype typical for the genus *Alytes*, 38 elements with either one or two arms, some of which can be considered as 'microchromosomes'. The NORs are located on the telomeres of the tenth chromosome pair which agrees with the state in *A. obstetricians* but differs from *A. cisternasii* reflecting phylogenetic affinities. C-banding and staining with DAPI and chromomycin A_3 revealed important blocks of telomeric CMA-positive heterochromatin on the smaller chromosomes of *Alytes*, similar to the state found in *Discoglossus*. Phylogenetic analysis of 750 bp of fragments of the mitochondrial 16S and 12S rRNA genes corroborated that *Discoglossus* and *Alytes* are sister taxa which together probably form the sister group of the Bombinatorinae. Centromeric heterochromatin in *Alytes* may be responsible for the retention of a plesiomorphic asymmetric karyotype which independently has evolved into a symmetric karyotype through centric fusions in *Bombina* and *Discoglossus*. The *Hind*III satellite DNA family was present in all archaeobatrachians studied but absent in hyloid and ranoid neobatrachians.

Introduction

Alytes muletensis is a rare anuran species, endemic to the Balearic island Mallorca. First described from fossil remains (Sanchiz & Adrover 1977), its last extant populations live along localized temporary brooks in steep canyons and were only discovered at the beginning of the 1980s (Mayol & Alcover 1981). The species belongs to the midwife toads (*Alytes*) which consist of four species: *A cisternasii, A dickhilleni, A. muletensis, A. obstetricians* (Arntzen & García-Paris 1995).

Alytes is generally included in the family Discoglossidae (Duellman & Trueb 1986, Sanchiz 1998). Together with a number of other primitive families (Ascaphidae, Leiopelmatidae, Megophryidae, Pelobatidae, Pelodytidae, Pipidae, Rhinophrynidae), they form the suborder Archaeobatrachia (see Feller & Hedges 1998) which is monophyletic according to molecular data (Hay *et al.* 1995). Archaeobatrachians are relict groups which together make up only about 4% of the approximately 4400 extant anuran species (Glaw *et al.* 1998).

The family Discoglossidae has, in the past, generally been understood as comprising the four genera *Alytes, Barboroula, Bombina* and *Discoglossus* (Duellman & Trueb 1986). *Barboroula* is an enigmatic South-East Asian genus with unknown relationships but it may be close to *Bombina* (Sanchiz 1998). The remaining three genera have a basically Eurasian distribution. Their relationships have been disputed. Lanza *et al.* (1976), based on immunological studies, recognized affinities between *Alytes* and *Bombina*, which they placed in a separate family (leaving *Discoglossus* in the Discoglossidae). In contrast Maxson & Szymura (1984), also based on immunology, found closer affinities of *Bombina* to *Discoglossus* than to *Alytes*. Finally, Ford & Cannatella (1993) considered *Alytes* and *Discoglossus* as sister taxa, and placed *Bombina* in a separate family, Bombinatoridae. The only molecular data (Hay *et al.* 1995) indicate a sister-group relationship of *Bombina* and *Discoglossus* within the Archaeobatrachia, but these authors did not include *Alytes*. Sanchiz (1998) recognized one family, Discoglossidae, with three extant subfamilies, Alytinae (*Alytes*), Bombinatorinae (*Barboroula, Bombina*), and Discoglossinae (*Discoglossus*).

Chromosomal studies on *Alytes muletensis* have been conducted by Herrero (1984) and Schmid *et al.* (1987). In the present paper, we report further results, obtained using a variety of banding techniques. The data are complemented by karyological information on four other archaeobatrachian taxa, namely *Pelobates fuscus* (Pelobatidae), *Pelodytes punctatus* (Pelodytidae), *Bombina variegata pachypus* (Discoglossidae) and *Xenopus laevis* (Pipidae), and with our own data on *Discoglossus* (Odierna *et al.* 1999). We also sequenced fragments of mitochondrial DNA in the three Eurasian discoglossid genera. Our main goals were to analyse the position of *Alytes muletensis* (and thus the genus *Alytes*) within the Discoglossidae, and within the Archaeobatrachia in general. We will use the molecular phylogeny to develop scenarios of karyological evolution within the Archaeobatrachia.

Materials and methods

Specimens studied

For karyological analysis, the following specimens were available: one male and one female of *Alytes muletensis* (from the captive breeding programme carried out in Mallorca, Spain), four males and three females of *Pelobates cultripes* (Soria, Spain), four males and one female of *Pelodytes punctatus* (Vallvidrera, Barcelona, Spain), three males and one female of *Bombina variegata pachypus* (Monti Picentini, Campania, Italy), two males of *Xenopus laevis* (no locality). Mitochondrial DNA was sequenced from one specimen of *Bombina orientalis* (no locality), one specimen of *Alytes muletensis* (see above), one specimen of *Alytes obstetricans boscai* (Spain), and one specimen of *Discoglossus galganoi* (Spain). Voucher specimens of *Alytes muletensis* and *Discoglossus galganoi* were deposited in the Zoologisches Forschungsinstitut Museum Koenig, Bonn, Germany.

Chromosome analysis

Each specimen was injected with a dose (0.01 ml/g body weight) of a 0.5-mg/ml colchicine solution and sacrificed 2 h later, after anaesthesia with tricaine metasulphonate. Chromosomes were taken from intestines, spleens, lungs and, in males, testes. The air drying plus scraping method (Odierna *et al.* 1999) was used in all species but *B. v. pachypus* and *X. laevis*, whose chromosomes were obtained by means of blood cultures (Miura 1995).

Both conventional staining (5% Giemsa at pH 7) and the following banding methods were used: Ag-NOR banding (Howell & Black 1980);

Q-banding (Schmid 1978); DAPI- and chromomycin A_3 (CMA) banding (Schweizer 1976); C-banding (Sumner 1972), employing $Ba(OH)_2$ at 45°C, sequential CMA- and DAPI-banding (Odierna *et al.* 1999); digestion with the restriction enzyme Alu I (Mezzanotte *et al.* 1983).

Satellite DNA studies

DNA was extracted from blood and/or liver of the studied specimens by means of the SDS + K proteinase digestion and chloroform–isoamyl alcohol extractions (see Capriglione *et al.* 1994).

The monomeric unit of the *Discoglossus pictus* *Hind*III satellite DNA family (named pDS) was extracted from low melting agarose gel. The fragment was ligated to the *Hind*III cloning site of pUC 18 plasmid. A 0.1-μg sample of the clone was labelled by random primer extension using digoxigenin–dUTP (Boehringer-Mannheim Kit; see also Odierna *et al.* 1999). By means of Southern blotting, the probe was hybridized to *Hind*III digested DNA of the studied species. *In-situ* hybridization with pDS was performed on the chromosomes of *D. pictus* as described in Capriglione *et al.* (1994).

mtDNA sequencing

DNA was extracted from muscle tissue samples using Qiamp tissue kits. We amplified two fragments of the mitochondrial 12S and 16S rRNA genes using primers and cycling protocols given in Vences *et al.* (2000). Sequences were obtained using an automatic sequencer (ABI 377). Sequences were submitted to Genbank (accession numbers AF224726–AF224729). 12S sequences of *Bombina orientalis* and *Discoglossus pictus*, as well as 16S and 12S sequences of *Xenopus laevis*, were obtained through Genbank (accessions X86227, X86235, M10217). The salamanders *Salamandra salamandra* and *Euproctus asper* were used as outgroup (accessions AF224732, U04694). Sequences were aligned using the Clustal option of the program SEQUENCE NAVIGATOR (Applied Biosystems); alignments were adjusted manually. Phylogenetic analyses of sequences were carried out using PAUP 4.0 (Swofford 1998). We calculated Neighbor-joining (NJ) trees using Jukes–Cantor distances, and Maximum Parsimony (MP) trees.

Results

Alytes muletensis

In accordance with Herrero (1984) and Schmid *et al.* (1987), both specimens of *A. muletensis* had metaphase plates of 38 chromosomes (Figure 1). The first four pairs were metacentric and distinctly larger than the remaining 15 pairs which gradually decreased in size. Of these smaller pairs, ten were subtelo- or telocentric, and five submeta- or metacentric. The smallest chromosome pairs had sizes which qualified them as 'microchromosomes' (*sensu* Olmo *et al.* 1982). The NORs were located on the tenth chromosome pair (by decreasing size).

The C-banding and Alu-I digestion produced similar results and revealed a complex heterochromatin distribution. Centromeric heterochromatin was restricted to the subtelo- and telocentric chromosomes. In some of these pairs, the heterochromatin extended further than the centromeric regions and formed the small arm or the elements. This heterochromatin was positive to DAPI after the C-banding procedure on seven chromosome pairs.

On the other hand, telomeric heterochromatin was also observed. C-bands were present on the telomeric regions of the four large chromosome pairs as well as most smaller elements. In seven of the smaller elements, important regions of heterochromatin (CMA positive) were present on the long arm. Four of these elements were identical to those with DAPI-positive centromeric heterochromatin.

Bombina variegata pachypus

All specimens studied had 24 chromosomes with two arms (Figure 2), which is in accordance with Morescalchi (1965). After C-banding, faint bands were recognizable in the centromeric regions. After DAPI and CMA staining, the chromosomes appeared uniformly coloured, except for an

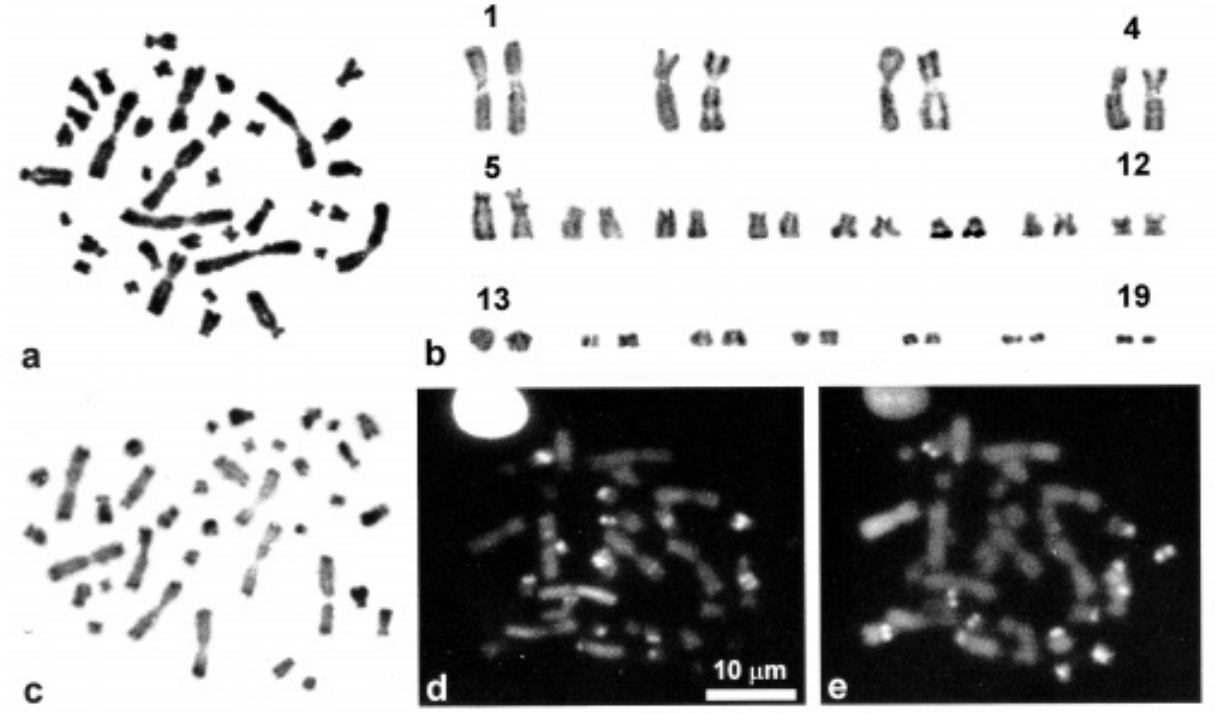

Figure 1. Giemsa stained (**a**) and C-banded (**c**) metaphase plates of *A. muletensis*. Ag-NOR stained karyotype (**b**) of this species sequentially stained with DAPI (**d**) and chromomycin A_3 (**e**). Scale bar (in **d**) applies to all plates in this figure.

interstitial band on the short arm of the seventh pair which was CMA positive. The NORs were located on the short arm of the seventh pair.

Pelodytes punctatus

All specimens had 24 chromosomes with two arms (Figure 2), which is in accordance with Morescalchi *et al.* (1977). Ag-NOR staining identified the NORs on the long arm of the seventh pair. C-banding showed solid centromeric bands on all chromosomes. On the first pair, there was a further subtelomeric band. After C-banding followed by fluorochrome staining, only the NOR regions were CMA positive, while the pericentromeric regions were DAPI positive on some chromosome pairs.

Pelobates cultripes

All specimens had 26 chromosomes with two arms (Figure 2), in accordance with Morescalchi *et al.* (1977). NORs were identified on the short arm of the seventh pair. All chromosomes had DAPI-positive centromeric heterochromatin, visible both with and without previous C-banding.

Xenopus laevis

The specimens had 36 chromosomes with two arms (Figure 2) which were all uniformly coloured by the fluorochromes both with and without previous C-banding. The NORs in this species are found on the 12th pair (Schmid *et al.* 1987).

Satellite DNA

The genomic DNA of *A. muletensis* and of the four other archaeobatrachians studied showed, after digestion with the endonuclease *Hind*III, a distinct banding pattern typical for satellite DNA (Figure 3). However, subsequent hybridization with pDS did not result in significant hybridization signals of the *Hind*III-digested DNA of *Alytes, Bombina, Pelodytes, Pelobates* or

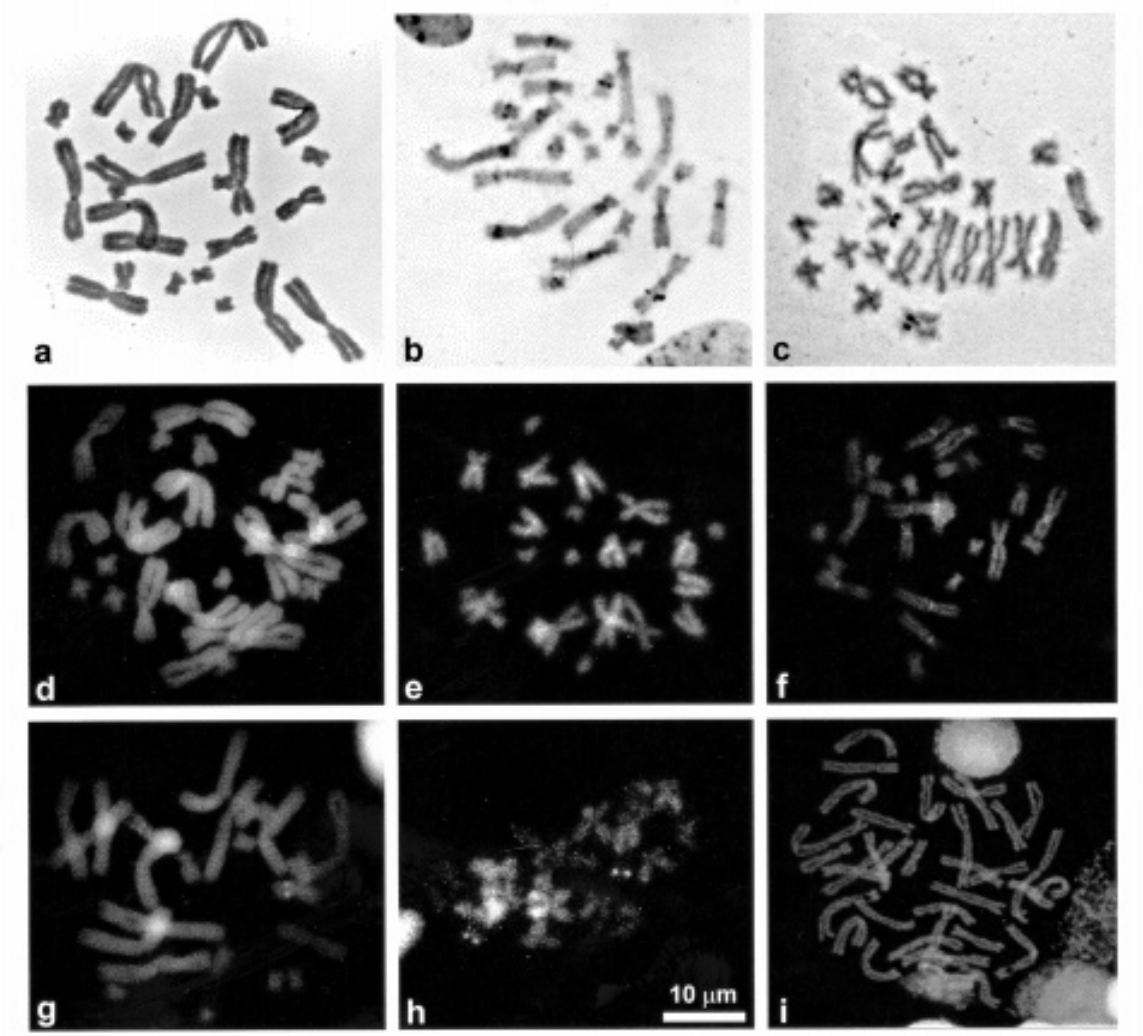

Figure 2. Metaphase plates of: *Bombina v. pachypus* (**a** = C-banded; **d** = C-banded + DAPI; and **g** = C-banded + chromomycin A_3); *Pelodytes punctatus* (**b** = C-banded; **e** = C-banded + DAPI; and **h** = C-banded + chromomycin A_3); *Pelobates cultripes* (**c** = Ag-NOR stained, and **f** = C-banded + DAPI); *Xenopus laevis* (**i** = C-banded + chromomycin A_3). Scale bar (in **h**) applies to all plates in this figure.

Xenopus. *In-situ* hybridization of pDS on chromosomes of *D. pictus* showed that labelling was prevalently interspersed along the arms of all chromosome pairs (Figure 4).

No banding pattern was obtained after *Hind*III digestion of the DNA of several neobatrachian anurans, belonging to both the hyloid and ranoid groups of families: *Rana, Mantidactylus, Mantella, Bufo* (results not shown).

Phylogenetic analysis

NJ and MP trees of 750 bp of the aligned 12S and 16S rRNA gene fragments had identical topologies. *Alytes* and *Discoglossus* were sister groups, and together formed the sister group of *Bombina*. Bootstrap support (2000 replications) of these two arrangements was 73% and 70% (MP), 76% and 66% (NJ).

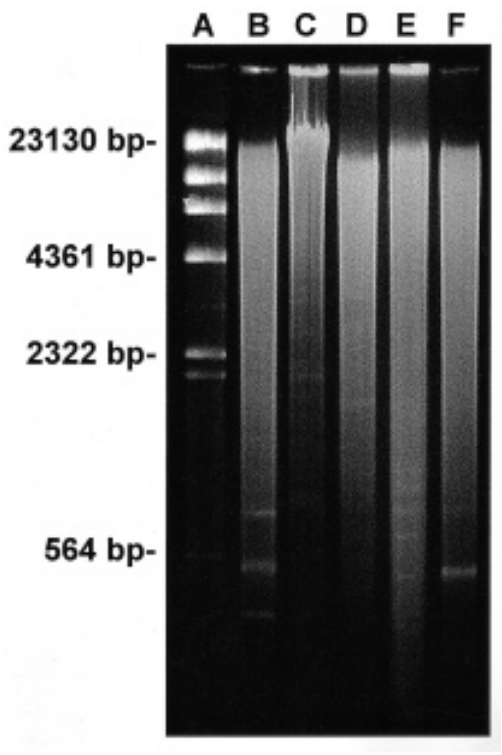

Figure 3. *Hind*III-digested DNAs of *A. muletensis* (**B**), *B. v. pachypus* (**C**), *P. cultripes* (**D**), *P. punctatus* (**E**) and *X. laevis* (**F**). Lane **A** is the λ *Hind*III marker.

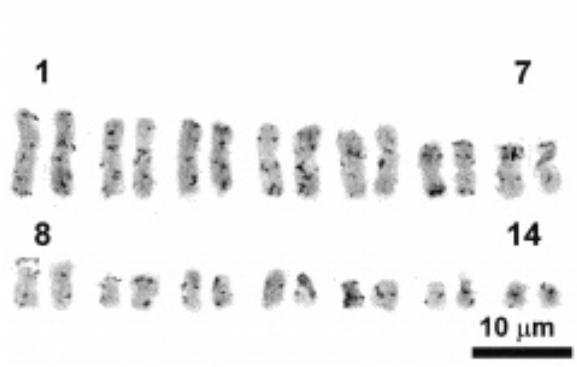

Figure 4. Karyotype of *Discoglossus pictus* showing *in-situ* hybridization of a digoxigenin-labelled pDS probe; note the interspersed chromosomal location of this satellite DNA family.

Discussion

Chromosome number and morphology of *Alytes muletensis* (2n = 38; four large metacentric chromosomes; 15 small chromosomes, eight of which are acrocentric) as found in this study and by Herrero (1984) and Schmid *et al.* (1987) is similar to that of other *Alytes: A. obstetricans obstetricans* (Morescalchi 1966, Vitelli *et al.* 1982, Herrero 1984, Schmid *et al.* 1987), *A. o. boscai* (Olmo *et al.* 1982) and *A. cisternasii* (Olmo *et al.* 1982, Herrero 1984). The C-banding pattern found in the present study was slightly different from that observed by Herrero (1984). This especially regards the telomeric heterochromatin bands which were not found by that author in the smallest elements, possibly due to a different incubation temperature and time in barium hydroxide.

The taxonomic and phylogenetic utility of the position of the ribosomal cistrons is well known (e.g. King 1990). *A. muletensis* (data herein) and *A. obstetricans* (Olmo *et al.* 1982, Vitelli *et al.* 1982, Schmid *et al.* 1987) have the NORs on the telomeres of one small telocentric chromosome pair (tenth pair), while, in *A. cisternasii*, they are located in an interstitial region of the long arm of the subtelocentric fifth pair. These data agree with the allozyme data of Arntzen & García-Paris (1995) which grouped *A. cisternasii* as outgroup to a clade containing *A. muletensis* and *A. obstetricans* (and *A. dickhilleni*).

Karyological diversification within *Alytes* further involved the number of subtelo- and telocentric elements. This accounts for the different FN values listed in King (1990): FN = 60 in *A. muletensis*, FN = 64 in *A. obstetricians obstetricans*, FN = 62 in *A. o. boscai*, and FN = 50 in *A. cisternasii*. Our results indicate that these differences could mainly be caused by a different degree of amplification of the centromeric heterochromatin in the subtelo- or telocentric elements. All these elements, in *A. muletensis*, had centromeric heterochromatin. In several of them, the heterochromatin was amplified sufficiently to make up the entire short arms. Variations of heterochromatin are considered to be evolutionarily neutral, and thus often polymorphic among species (Schmid 1978, Odierna *et al.* 1999).

It is generally accepted that, in anurans, chromosomal evolution mainly involved elimination of 'microchromosomes' or their incorporation into the macrochromosomes, and centric fusions of the telocentric elements (Morescalchi

1973, King 1990). This led to symmetrical karyotypes consisting mainly or exclusively of metacentric elements. Evidence exists that heterochromatin, and especially the highly repeated DNA sequences present in it, plays a relevant role in chromosome rearrangements. So, variations in its quantity and composition can induce or inhibit certain chromosomal mutations, such as inversions and centric fusions (Mayr *et al.* 1984, Luke *et al.* 1992, Garagna *et al.* 1995).

In *Alytes*, centromeric heterochromatin is present on the subtelo- and telocentric chromosomes, and on the 'microchromosomes' (actually better defined as supernumerary chromosomes or B-chromosomes). In anurans, and beside *Alytes*, 'microchromosomes' are only known in the archaeobatrachians, *Ascaphus* and *Leiopelma* (Duellman & Trueb 1986). In both genera, centromeric heterochromatin is present on almost all chromosomes (Schmid *et al.* 1987, Green 1988). It may therefore be hypothesized that this heterochromatin inhibited the translocation of the small chromosomal elements and thus preserved an asymmetrical karyotype in these taxa.

According to molecular data (Hay *et al.* 1995, this study), *Alytes* is the sister group of *Discoglossus*, and both taxa together form the sister group of the Bombinatorinae (see also Ford & Cannatella 1993). A hypothetical ancestor of *Discoglossus* with an *Alytes*-like karyotype might have reduced the centromeric heterochromatin from the uniarmed chromosomes. This event subsequently permitted a series of centric fusions which might have led to the symmetrical karyotype observed today in *Discoglossus*. A symmetrical karyotype was apparently achieved independently also in *Bombina* which has only faint centromeric bands.

The *Alytes–Discoglossus* sister group relationship allows to explain phylogenetically the GC-rich telomeric heterochromatin bands which are found both in *Discoglossus* (Odierna *et al.* 1999) and *A. muletensis*. Similar heterochromatin blocks are not found in the other Archaeobatrachia studied. However, considering the ancient divergence between both genera (more than 60 MYA according to Maxson & Szymura 1984), the observed bands may also be due to convergent accumulation of different families of highly repeated DNA with similar fluorochrome-staining characteristics.

Satellite DNA families generally tend to accumulate mutations, and thus to diverge rather fast (Miklos 1985, Charlesworth *et al.* 1994). Divergence of the *Hind*III satellite DNA family since the phylogenetic splits between archaeobatrachian lineages may account for the fact that hybridization with pDS (the monomeric *Hind*III unit in *Discoglossus pictus*) was not possible in genera different from *Discoglossus*. However, it is relevant that the *Hind*III family is present in all archaeobatrachians so far studied (as well as in salamanders; Nardi *et al.* 1999) but is absent in advanced frogs (suborder Neobatrachia). The *Hind*III family may be an old character of lissamphibians which was lost in the ancestor of neobatrachians.

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