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Phylogenetic relationships and biodiversity in Hylids (Anura: Hylidae) from French Guiana

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Abstract

We evaluated two biodiversity criteria, higher taxonomic diversity and phylogenetic diversity in French Guiana. For this, we used a recent assessment of the knowledge accumulated since 30 years of study on the amphibian species currently known in French Guiana. We focused on two well-represented genera, *Hyla* and *Scinax*, belonging to the subfamily Hylinae. We used partial sequences of two mitochondrial genes (16S rDNA and 12S rDNA, 813 bp) and two nuclear genes (tyrosinase and 18S rRNA, 1590 bp) covering a total of 2403 bp. According to the high bootstrap support in phylogenetic analysis of the complete dataset, the genus *Scinax* is a homophyletic clade formed by two species groups (*rubra* and *rostrata*) in French Guiana. The genus *Hyla* was confirmed to be a paraphyletic group formed by two species groups as well (30 chromosomes and the 'gladiator frogs'). We confirmed that these genera should be taxonomically reconsidered. Moreover, at the genus, subfamily and family levels, the use of only morphological characters or only molecular DNA markers would hamper estimations of biodiversity. Thus, we strongly advise the combined use of both morphology and molecular data (nuclear and mitochondrial markers). ***To cite this article: M.-D. Salducci et al., C. R. Biologies 328 (2005).***

Résumé

Relations phylogénétiques et biodiversité chez les Hylidés (Anura : Hylidae) de Guyane française. Nous avons évalué deux critères de biodiversité, la diversité taxonomique et la diversité phylogénétique en Guyane française. À cet effet, nous nous sommes appuyés sur une étude récente faisant le bilan de toutes les espèces d'Amphibiens de cette région. Nous avons choisi deux genres bien représentés, *Hyla* et *Scinax*, appartenant à la sous-famille des Hylinae. Nous avons analysé les séquences partielles de deux gènes mitochondriaux (16S ADNr et 12S ADNr, pour un total 813 pb) et de deux gènes nucléaires (tyrosinase et 18S ADNr,) l'ensemble représentant 2403 pb. Les proportions de *bootstrap* élevées pour le jeu de données complet montrent que le genre *Scinax* est un clade homophylétique en Guyane. Le genre *Hyla* s'est avéré être un groupe paraphylétique, constitué de deux groupes (« 30 chromosomes » et *gladiator frog*). Nous avons confirmé que ces genres doivent être reconsidérés sur un plan taxonomique. De plus, pour les unités taxonomiques (genre, sous-famille et famille), la seule utilisation des caractères morphologiques ou des marqueurs moléculaires ADN biaiserait l'estimation de la biodiversité. Ainsi, nous conseillons fortement l'utilisation à la fois des données

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morphologiques et moléculaires (marqueurs nucléaires et mitochondriaux). ***Pour citer cet article : M.-D. Salducci et al., C. R. Biologies 328 (2005).***

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Mots-clés : *Amphibia* ; *Scinax* ; *Hyla* ; Centrolenidae ; Diversité phylogénétique ; Guyane française

1. Introduction

Some of the greatest challenges for biologists are to understand and to evaluate biodiversity [1]. One of the measures commonly used to assess biodiversity is species richness, as defined by the number of species at a site or habitat. However, the evaluation of species richness is strongly dependent on the definition of a species, an intensively debated question [2]. Therefore, higher taxon richness, mostly on morphological criteria, is often used as a surrogate to evaluate biodiversity rather than species richness [3,4]. This approach is valid only if there is no systematics bias, i.e., when only monophyletic groups are used and when the taxonomic boundaries are similar among groups.

Another way of measuring biodiversity involves the screening of genetic variability among populations. Genetic variability may be at least partly reflected by the phylogenetic diversity (PD), which is estimated as the sum of the branch lengths in a phylogenetic tree [5]. The genetic and morphological approaches generate different assessments of biodiversity values, perhaps reflecting differences in the temporal and spatial scales of the determining processes [6]. It is therefore necessary to clarify their practical utility for the assessment of biodiversity in taxa in which an exhaustive inventory cannot be compiled due to the high number of species and/or the environmental conditions.

This is often the case in tropical environments, where the greatest species diversity is recorded [7–10], as for amphibians. The rate of description of new species is also higher in these zones than anywhere else [11].

The Guianan region is one of the last wild areas in South America and among the three richest tropical regions on Earth (Guiana Shield Conservation Priority Setting Workshop, 5–9 April 2002, Paramaribo, Surinam). However, it is extremely difficult to assess the diversity of many taxa in this region for practical reasons. This is the case for amphibians, for which biologists are extremely concerned since few years. Indeed, this group is subject to a fast worldwide decline particularly worrying in South America [12–16]. Even though collecting efforts have intensified in many previously unknown areas, especially in this part of the world [17], large regions remain unknown and many reduced areas of potentially high diversity have not been examined [18]. There is therefore an urgent need to characterise the diversity of amphibians by using rigorous approaches. It is unclear whether this should involve higher taxonomic biodiversity analysis, phylogenetic diversity analysis or both.

Here, we used a recent taxonomical, geographical and ecological assessment based on 30 years of work on the species present in French Guiana. It concerns 110 amphibian species over 90 000 km^2 [19]. We considered taxonomy as a surrogate of morphological diversity. Thus, we chose a group for which we know the number of morphologically distinct species, their distribution area and basic aspects of their ecology (the habitat). We concentrated on amphibians belonging to the second largest frog family in South America: the Hylidae (neotropical tree frogs), with 25 genera in this part of the world [17]. The family Hylidae is divided into four subfamilies [20], only two of which are present in French Guiana: the Phyllomedusinae, with four species, and the Hylinae, with 38 species [19,21,22].

Members of the subfamily Phyllomedusinae are widely distributed in tropical parts of Middle and South America [23]. In this group, the constricted pupil is vertically elliptical. These tree frogs, with a green dorsal coloration, are adapted to an arboreal lifestyle. Only one genus, *Phyllomedusa*, among the three genera constituting this group, is present in French Guiana [19].

Members of the subfamily Hylinae are found throughout the temperate and tropical parts of North and South America, in temperate Eurasia, Japan, extreme northern Africa, and the West Indies [24]. Five genera are found in French Guiana: *Hyla*, *Scinax*, *Osteocephalus*, *Phrynohyas* and *Sphaenorhynchus*. The two largest Hyline genera are *Hyla* and *Scinax*, containing respectively 45 and 7 species groups (or currently five species groups [25]).

The genus *Hyla* does not share any unique features and two major groups within *Hyla* are present in French Guiana, the Gladiator frogs sensu lato and the $2n = 30$-chromosome lineage [24,26]. The Gladiator frogs are represented by seven species groups in French Guiana and the 30-chromosome Hyla by six

species groups [19]. This is the only genus within hylines found in North, Central and South America, Eurasia and the extreme north of Africa [24,27]. We have previously shown that some representatives of the genus *Hyla* form a monophyletic group according to partial 16S rRNA gene sequences, but the bootstrap proportion was low [28]. In contrast, morphological analysis revealed that this genus was paraphyletic [27]. These results seem to be contradictory and require clarification.

The genus *Scinax* is divided into seven [19] or five groups [25,29]. Two of these are represented in French Guiana. The first is the *rostrata* group, which includes *Scinax jolyi*, *Scinax proboscideus* and *Scinax nebulosa*. The second is the *rubra* group, which includes *Scinax rubra*, *S x-signatus*, *Scinax boesemani* and *Scinax cruentomma* [19,25]. Faivovich [29] found the *rostrata* group to be nested within the *rubra* group, making it non-monophyletic. *Scinax* species were initially informally grouped in a *Hyla rubra* group [29], mainly on the basis of sperm cell morphology, then included in a genus named *Ololygon*. It was only in 1992 that the name *Scinax* took priority over *Ololygon* [30]. In a previous study based on 16S rRNA gene sequences and a few species, we showed that the *Scinax* genus was homophyletic and supported by a high bootstrap proportion [28]. Moreover, based on morphology, this homophyly is supported by ten synapomorphies [29].

In the light of this systematic framework, we use a subset of species constituted by 33 specimens belonging to the family Hylidae (genera *Scinax*, *Osteocephalus*, *Hyla*, and *Phrynohyas*), three specimens from the family Centrolenidae (*Hyalinobatrachium* and *Cochranella* genera) were used to verify the impact of outgroup on Hylinae phylogenetics relationships [28,31,32], and two *Eleutherodactylus* (Leptodactylidae) specimens were used as outgroups. Phylogenetic diversity was estimated by sequencing two mitochondrial genes (16S and 12S rDNA) and two nuclear genes (18S rDNA and tyrosinase). These sequences have been successfully used to assess the relationships among some orders, families and species of amphibians [31–40].

In this paper, we tested congruence between molecular and morphological/taxonomic data at the species, genus and family levels in one subset representative of amphibian diversity in the Guianan region.

If there is congruence between molecular markers and morphology, the morphological criteria will be sufficient for the study of neotropical frog biodiversity and the higher taxonomic diversity criteria will be a rigorous alternative for the conservation of divergent lineages.

If there is no congruence between molecular markers and morphology in this representative subset, morphology must be systematically coupled to molecular markers when studying the biodiversity of neotropical frogs and the higher taxonomic diversity must be coupled to phylogenetic diversity for the conservation of divergent and reticulate lineages.

2. Materials and methods

2.1. Biological samples

Twenty-nine new species were added to those studied by Salducci et al. [28]. Thus, a total of 143 new sequences were obtained from 33 Hylidae constituted by 11 individuals belonging to the genus *Scinax* (four species assigned to the *rubra* group and four species to the *rostrata* group), 14 to the genus *Hyla* (Gladiator frogs: four species of the *geographica* group, two of the *albopunctata* group and one for the *boans* group and one for the *granosa* group; Hyla 30 chromosomes: two species of the *nana* group, one of the *leucophyllata* group, one of the *luteocellata* group 1 of the *minuta* group, one of the *parviceps* group), three to the genus *Osteocephalus*, two to the genus *Phrynohyas*, and three to the genus *Phyllomedusa*. Three specimens from the Centrolenidae (*Hyalinobatrachium* and *Cochranella* genera) and two Leptodactylidae (*Eleutherodactylus*) were used as outgroups. Thirty-six specimens were collected in French Guiana over a four-year period. Samples of *Scinax rostrata* and *Scinax elaeochroa* were collected respectively in Venezuela and in Costa Rica (National Museum of Natural History of The Netherlands). The geographical ranges of the specimens examined are given in Table 1, and a map of collecting localities is given in Fig. 1. Tissue samples were derived from muscle by performing a biopsy. From preserved specimens in collections, we used liver tissues preserved in ethanol.

2.2. Molecular data

Total DNA was extracted from muscle tissue samples by digestion with proteinase K for 12 h at 37 °C followed by standard phenol/chloroform extraction [41]. Two partial mitochondrial (mt) and two nuclear (nu) DNA fragments were amplified by standard PCR techniques. The mt DNA fragments were: 430 bp of the 16S rDNA gene and 397 bp of the 12S rDNA gene. These fragments were amplified using the primers listed in Table 2. The nuclear DNA fragments were: 403 bp of exon 1 of the tyrosinase gene and 1187 bp of the 18S

Table 1
List of batrachians used for this study, with the locality number (see Fig. 1)

Family	Subfamily	Species	Genbank accession number 16S	Genbank accession number 12S	Genbank accession number 18S	Genbank accession number tyrosinase	Origin locality number
Hylidae	**Hylinae**	*Scinax rubra 1*	AF467264	–	–	–	Guiana Antecum Pata 6
		Scinax rubra 2	–	–	–	–	Guiana Grand Santi 7
		Scinax cruentomma 1	AF467263	–	–	–	Guiana Mountain of Kaw 3
		Scinax cruentomma 2	–	–	–	–	Guiana Mountain of Kaw 3
		Scinax jolyi 1	AF467261	–	–	–	Guiana Swamp of Kaw 3
		Scinax jolyi 2	AF467261	–	–	–	Guiana Creek of Gabrielle 2
		Scinax nebulosa	AF467262	–	–	–	Guiana Regina Road St Georges 4
		Scinax proboscideus	–	–	–	–	Guiana Mountain of Kaw 3
		Scinax boesemani	–	–	–	–	Guiana Grand Santi 7
		Scinax rostrata	–	–	–	–	Venezuela
		Scinax elaeochroa	–	–	–	–	Costa Rica
		Hyla leucophyllata	–	–	–	–	Guiana Mountain of Kaw 3
		Hyla sp1	–	–	–	–	Guiana Mountain of Kaw 3
		Hyla minuscula	–	–	–	–	Guiana Mana 9
		Hyla ornatissima	–	–	–	–	Guiana ChutesVoltaires 8
		Hyla raniceps	AF467269	–	–	–	Guiana Yi-Yi's Creek 1
		Hyla dentei	AF467270	–	–	–	Guiana Mountain of Kaw 3
		Hyla brevifrons	–	–	–	–	Guiana Mountain of Kaw 3
		Hyla multifasciata	–	–	–		Guiana Mountain of Kaw 3
		Hyla nana	–	–	–	–	Guiana Mountain of Kaw 3
		Hyla minuta	–	–	–	–	Guiana Mountain of Kaw 3
		Hyla boans	–	–	–	–	Guiana Mont Bakra 11
		Hyla geographica	–	–	–	–	Guiana Grand Santi 7
		Hyla fasciata	–	–	–	–	Guatemala 14
		Hyla calcarata	–	–	–	–	Guiana Creek of Margot 13
Hylidae	Hylinae	*Osteocephalus oophagus*	AF467267	–	–	–	Guiana Mountain of Kaw 3
		Osteocephalus taurinus	–	–	–	–	Guiana Saül 12
		Osteocephalus leprieurii	–	–	–	–	Guiana Creek of Margot 13
		Phrynohyas coriacea	–	–	–	–	Guiana Mountain of Kaw 3
		Phrynohyas venelosa	–	–	–	–	Guiana Guatemala 14
	Phyllomedusinae	*Phyllomedusa tomopterna*	–	–	–	–	Guiana Mountain of Kaw 3
		Phyllomedusa hypochondrialis	–	–	–	–	Guiana Grand Santi 7
		Phyllomedusa bicolor					Guiana Saül 12
Centrolenidae		*Hyalinobatrachium taylori*	AF467268	–	–	–	Guiana Creek of Gabrielle 2
		Hyalinobatrachium taylori 2	–	–	–	–	Guiana Tibourou 10
		Cochranella sp	–	–	–	–	Guiana Mont Bakra 11
Leptodactylidae	**Eleutherodactylinae**	*Eleutherodactylus zeuctotylus1*	–	–	–	–	Guiana Mountain of Kaw 3
		Eleutherodactylus zeuctotylus 2	–	–	–	–	Guiana Tibourou 10

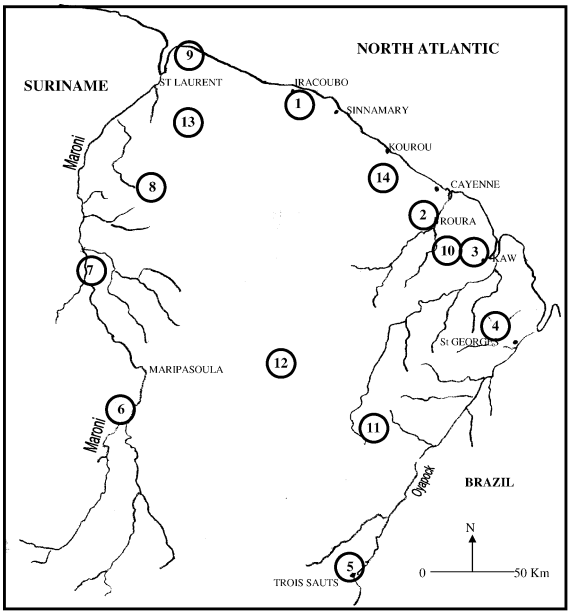

Fig. 1. Map of French Guiana showing the localities of the samples collected.

rRNA gene. The primers used for amplification were as described by Bossuyt and Milinkovitch [42] for tyrosinase and by Miquelis et al. [43] for 18S rDNA.

PCR was performed in a 50-µl total volume as in Gilles et al. [44]. Cycle parameters were as follows:

- for 16S rDNA, 2 min at 92 °C; then five cycles of 15 s at 92 °C, 45 s at 46 °C, 1 min 30 s at 72 °C; then 30 cycles of 15 s at 92 °C, 45 s at 48 °C, 1 min at 72 °C; and finally 7 min at 72 °C;
- for 12 S rDNA and 18S rDNA, 2 min at 92 °C; then 5 cycles of 15 s at 92 °C, 45 s at 48 °C, 1 min 30 s at 72 °C; then 30 cycles of 15 s at 92 °C, 45 s at 52 °C, 1 min at 72 °C; and finally 7 min at 72 °C;
- for tyrosinase, 2 min at 92 °C; then five cycles of 15 s at 92 °C, 45 s at 50 °C, 1 min 30 s at 72 °C; then 30 cycles of 15 s at 92 °C, 45 s at 54 °C, 1 min at 72 °C; and finally 7 min at 72 °C.

The purified PCR products were sequenced using an automated sequencer (Genome Express S.A.) and the same PCR primers as used in amplifications. Fifteen new sequences were obtained for the 410-bp fragment of 16S rDNA and 24 new sequences were obtained for the 403-bp fragment of 12S rDNA, for the 1197-bp fragment of 18S rDNA and for the 393-bp fragment of tyrosinase. See Table 1 for accession numbers.

2.3. Data analysis

The 16S rDNA and 12S rDNA gene sequences were aligned using Clustal X [45] and compared with the alignment based on secondary structure [46]. The 16S secondary structure was visualised as described in [28]. We carried out a similar procedure with the *Rana pipiens* sequence as a model for the 12S secondary structure. The amplified 12S fragments corresponded to the helices 1 to 26. For this study, we used the software SOAP [47] to identify unstable blocks in alignments for the 16S and the 12S datasets. Saturation was visualised using the software package MUST [48].

Phylogenetic analyses were performed using three different approaches:

(1) the Neighbour-Joining (NJ) method [49], using a matrix based on the Tamura–Nei distance with gamma parameter [50];
(2) a cladistic approach using the Maximum-Parsimony (MP) criterion and the heuristic search with 10 random additions of taxa and TBR branch-swapping as implemented in PAUP 4b10 [51];
(3) the maximum-likelihood (ML) method using the evolutionary model retained by MODELTEST [52]:
   - Lset mitochondrial (GTR + I + G selected by AIC) Base = (0.2671 0.1631 0.2136) Nst = 6 Rmat = (0.1829 5.4352 1.1539 0.2491 1.7544) Rates = gamma Shape = 0.6890 Pinvar = 0.2813;
   - Lset nuclear Base = equal Nst = 2 TRatio = 1.9683 Rates = gamma Shape = 0.6991 Pinvar = 0;
   - Lset combined (GTR + I + G selected by AIC) Base = (0.2427 0.2246 0.2452) Nst = 6 Rmat = (0.2603 4.6592 1.3927 0.3127 1.7642) Rates = gamma Shape = 0.5049 Pinvar = 0.5292.

The procedure described by Rogers and Swofford [53] was used to reduce the preliminary computing time. The robustness of nodes was estimated by bootstrap analysis with 1000 replicates for the NJ and MP methods [51], and 500 replicates for the ML method. Differences in topology between trees based on conserved versus variable regions of the 16S rDNA sequences were tested as described in Salducci et al. [28] and were shown to be non-significant. We used the Incongruence Length Difference (ILD) test [54] as implemented in PAUP 4b10 with $\alpha = 0.05$ to compare the trees obtained in stable and unstable zones. This

Table 2
Primers used for amplifying and sequencing segments of mitochondrial genes F at the end of the primer name = forward, R at the end of the primer name = reverse

Name	Gene	Sequence 5′-3′	Reference
N-934F	16S	CGCCTGTTTACCAAAAACATCG	[72]
3259R	16S	CCGCTTTGAGCTCAGATCA	[73]
N16F	16S	TATCCCTAGGGTAACTTG	This study
N16R	16S	TTACCAAAAACATCGCCT	This study
N316F	16S	GGAGGTTATTTTTTGTTC	This study
N316R	16S	CGAGAAGACCCTATGGAG	This study
N12F	12S	GGTATCTAATCCCAGTTTGT	This study
N12R	12S	AGGTTTGGTCCTAGCCTT	This study
12LPF tRNA phe-L	12S	GCRCTGAARATGCTRAGATGARCCC	[74]
12HSR 12SF-H	12S	CTTGGCTCGTAGTTCCCTGGCG	[74]
Tyr1A	Tyrosinase	AGGTCCTCTTRAGCAAGGAATG	[42]
Tyr1E	Tyrosinase	GAGAAGAAAGAWGCTGGGCTGAG	[42]
18SF	18S1	CCACATCCAAGGAAGGCAGCAGGC	[43]
18SR	18S1	CCCGTGTTGAGTCAAATTAA	[43]
18S2F	18S2	CGTAGTTCCGACCATAAA	This study
rev-Hillis18g-S	18S2	GGAAACCTTGTTACGACTT	[74]

test is useful to detect incongruence between different partitions [43,55–57], but has only limited power to detect incongruence caused by differences in the evolutionary process or in the tree topology [58] or when two datasets differ markedly in size [59]. The Wilcoxon sign-rank test [60] and the Shimodaira–Hasegawa test [61] were also calculated to assess the significance of topology differences respectively in parsimony and maximum-likelihood frameworks. Finally, departure from the molecular clock was tested using relative rate tests as implemented in PHYLTEST [62]. We studied the mismatch distribution between the sequences by using the Kimura two-parameter distance as genetic distance. The average distance between two groups is the arithmetic mean of all pairwise distances between taxa in the inter-group comparisons and the within-group means are arithmetic means of all individual pairwise distances between taxa within groups belonging to: the genus *Hyla*, the genus *Scinax*, the subfamily Hylinae, the family Hylidae, and between the families *Hylidae* and *Centrolenidae*, using the morphological hierarchical level and under the hypothesis that there is no departure from the molecular clock. Under this assumption, the mismatch distribution between two comparable hierarchical levels will not be significantly different and the mismatch distribution between two different levels (i.e., genus versus subfamily) will be significantly different. Due to the difference in sample size, the Kolmogorov–Smirnov test was used to test the homogeneity between the different distributions with $\alpha = 0.05$.

3. Results

3.1. Combined analysis of mitochondrial datasets

3.1.1. Phylogenetic analysis

The incongruence test detected no significant heterogeneity between the 12S and 16S datasets ($p = 0.018$). Indeed, according to the Shimodaira–Hasegawa and Wilcoxon sign-rank tests, the 16S constraint trees were significantly different to the 12S and total data trees (considering the 12S rDNA dataset and the total dataset). However, the 12S and total data trees did not differ significantly from the 16S rDNA tree according to the Shimodaira–Hasegawa test (considering the 16S rDNA dataset). This pattern seems to be due to the lack of phylogenetic information in the 16S dataset since few nodes were supported.

Saturation was observed from the transition and transversion substitution patterns (Fig. 2A, C, and E). The homophyly of the Hylinae genera was tested using an 813-bp region of the total dataset. Of the 813 positions, 394 were informative (gaps were treated as missing for the unweighted parsimony analysis). We found one single parsimonious tree (2035 steps). The g1 value was −0.76, the CI was equal to 0.387, the RI was equal to 0.620.

The combination of the two datasets showed phylogenetic relationships within the French Guianan Hylidae at a better resolution than fragments analysed separately (Fig. 3). The genus *Scinax* constituted a homophyletic group with a high bootstrap support (BP = 100/100/97, ML/MP/NJ). We detected two groups: the first one, the *rostrata* species group was constituted by

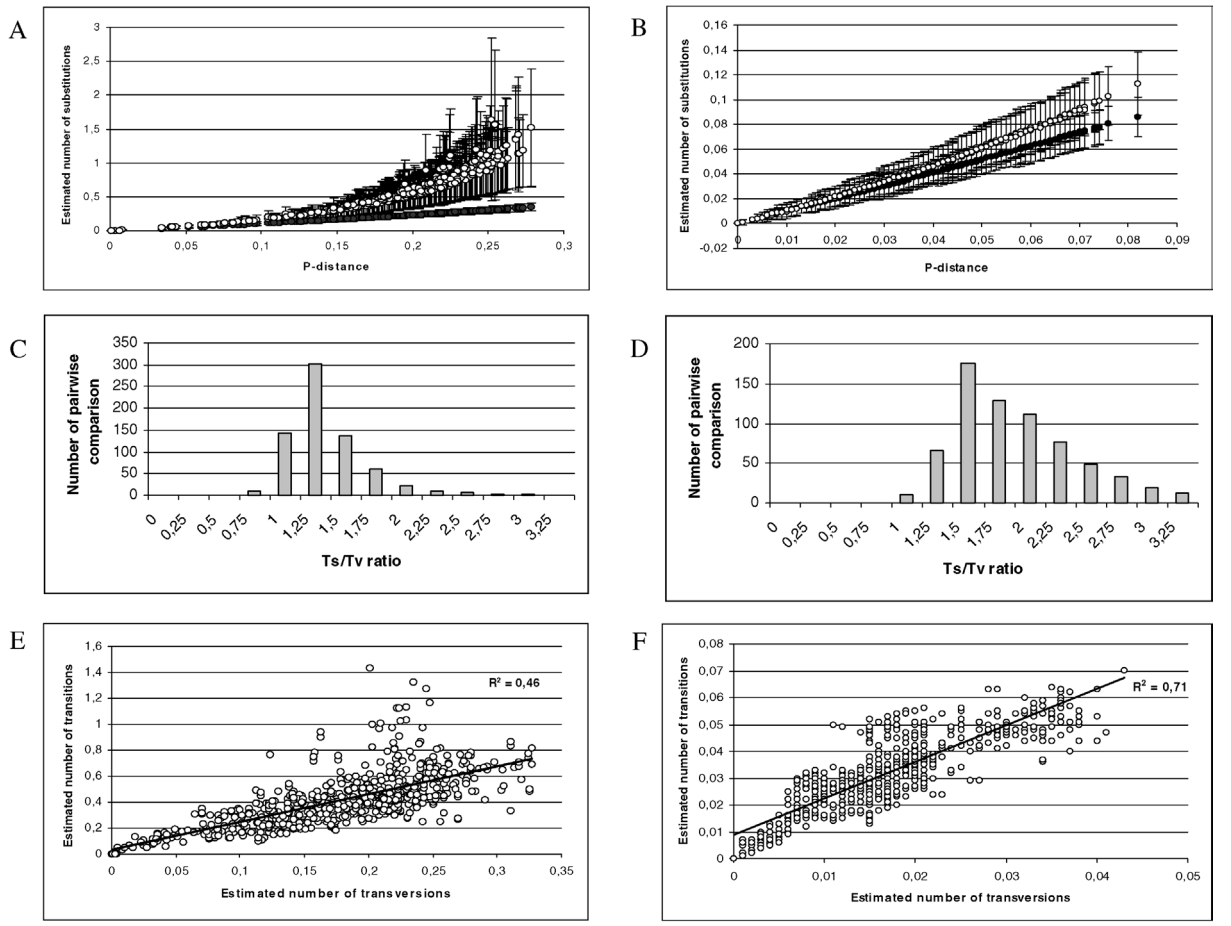

Fig. 2. (A) Scatter plots obtained from 813 positions of the 16S rDNA and 12S rDNA of 38 species. *X* axis: pairwise number of substitutions using the *p* distance; *Y* axis: pairwise number of substitutions using the Tamura–Nei model with alpha = 0.30 (open circle) and Jukes and Cantor (black circle). (B) Scatter plots obtained from 1590 positions of the tyrosinase and 18S rDNA of 38 species. *X* axis: pairwise number of substitutions using the *p* distance; *Y* axis: pairwise number of substitutions using the Tamura–Nei model with alpha = 0.21 (open circle) and Jukes and Cantor (black circle). (C) Distribution of the transition–transversion ratio using the 16S rDNA and 12S rDNA of 38 species ($\mu = 1.26$; $\sigma = 0.49$). (D) Distribution of the transition–transversion ratio using the tyrosinase and 18S rDNA of 38 species ($\mu = 1.81$; $\sigma = 0.57$). (E) Scatter plots obtained from 813 positions of the 16S rDNA and 12S rDNA of 38 species. *X* axis: pairwise number of substitutions using transversions; *Y* axis: pairwise number of substitutions using the transition. (F) Scatter plots obtained from 1590 positions of the tyrosinase and 18S rDNA of 38 species. *X* axis: pairwise number of substitutions using transversions; *Y* axis: pairwise number of substitutions using the transition. (G) Scatter plots obtained for 38 species, *X* axis: pairwise number of substitutions using the Tamura–Nei model with alpha = 0.21 from 1590 positions of the tyrosinase and 18S rDNA; *Y* axis: pairwise number of substitutions using the Tamura–Nei model with alpha = 0.30 obtained from 813 positions of the 16S rDNA and 12S rDNA. (H) Distribution of the transition–transversion ratio using the tyrosinase, 18S rDNA, 16S rDNA and 12S rDNA of 38 species ($\mu = 1.62$; $\sigma = 0.40$). (I) Scatter plots obtained from 2403 positions of the tyrosinase, 18S rDNA, 16S rDNA and 12S rDNA of 38 species. *X* axis: pairwise number of substitutions using transversions; *Y* axis: pairwise number of substitutions using the transition.

Scinax jolyi, *Scinax proboscideus*, *Scinax rostrata* and *Scinax nebulosa* (BP = 92/100/100) and the second one, the *rubra* species group by *Scinax rubra*, *Scinax cruentomma*, *Scinax boesemani* and *Scinax elaeochroa* (BP = 98/100/100).

The homophyly of genus *Hyla* was not supported (Fig. 3). The homophyly of the two major groups of *Hyla* was confirmed by the 'mitochondrial total evidence' approach (BP = 89/74/85 for the first 30 chromosomes group *H. minuscula*, *Hyla nana*, *H. sp1*, *H. leucophyllata*, *H. brevifrons*, *H. minuta*, and bp = 99/98/99 for the 'Gladiator frogs' group *H. multifasciata*, *H. raniceps*, *H. calcarata*, *H. fasciata*, *H. dentei*, *H. geographica*, *H. boans*, *H. ornatissima*). Furthermore, the species *Phrynohyas* was the sister group to *Osteocephalus* (BP = 61/86/99). We observed the homophyly of the Centrolenidae (BP = 83/90/100), which was the sister group to the Hylidae, but this topology

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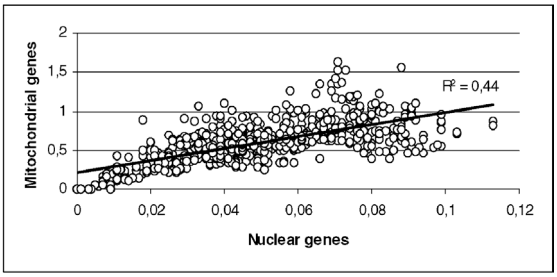

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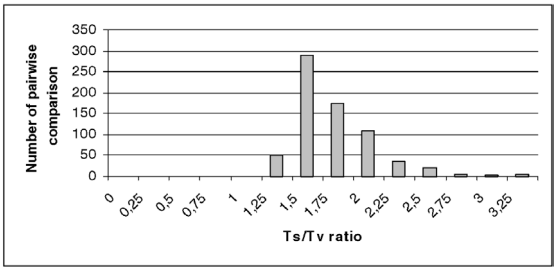

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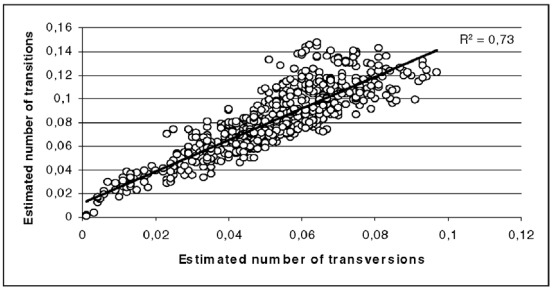

Fig. 2. *Continued.*

was only supported in NJ. On a lower systematic level, we noted that the *Hyla geographica* and the *H. nana* species group are paraphyletic. Indeed *H. geographica* clustered with *H. boans* and *H. sp.1* with *H. minuscula* with high bootstrap supports.

3.1.2. Genetic diversity of the 16S rRNA and 12S rRNA genes

Considering the homogeneity between the two partitions (12S and 16S) and the correlation coefficient between the two distance matrices (0.837), we estimated the genetic variability [63] using the complete dataset. According to the Tamura–Nei genetic distance alpha = 0.30 (Table 3), the variability between the different genera of family Hylidae ranged from 0.129 ± 0.019 to 0.852 ± 0.151. The genetic divergence between the Centrolenidae (*Hyalinobatrachium* and *Cochranella*) and the Hylidae ranged from 0.279 ± 0.041 with *Phrynohyas* to 0.685 ± 0.125 with *Phyllomedusa*. The genus *Scinax* displayed a within group average of 0.417 ± 0.067 (data not shown) and the genus *Hyla* yielded an average of 0.392 ± 0.05. Genetic diversity within the two clades was respectively 0.223 ± 0.026 (Hyla 30-chromosome group) and 0.278 ± 0.03 ('gladiator frogs'). The diversity within the genus *Hyla* is huge, but we need to reconsider this result in light of the polyphyletic pattern of this group. The genetic diversity between the two groups of *Hyla* was 0.512 ± 0.078 and the variability between the two groups of *Scinax* was 0.556 ± 0.109. By comparison, the genetic diversity between *Osteocephalus* and *Phrynohyas* is equal to 0.129 ± 0.019. These results were corroborated by the mismatch analyses (Table 5), which revealed no congruence between the taxonomic levels (subfamily and family) and the molecular features.

The genetic diversity between the different genera within the subfamily Hylinae was significantly higher than that estimated between the subfamilies Hylinae and Phyllomedusinae. A similar result was found when the genetic diversity within the subfamily Hylinae was compared with that estimated between the families Hylidae and Centrolenidae.

Moreover, we found no significant difference between the genetic diversity within the genus *Scinax* and the genetic diversity between the families Hylidae and Centrolenidae. However, this pattern could be due to saturation effects.

3.2. Combined analysis of nuclear datasets

The incongruence test did not detect significant heterogeneity between the two nuclear datasets ($p = 0.94$) and no saturation was observed for the transition and transversion substitution patterns (Fig. 2B, D, F). Of the 1590 positions, 221 were informative (gaps were treated as missing for the unweighted parsimony analysis). We found 192 equiparsimonious tree (623 steps). The g1 value was −0.68, the CI was equal to 0.594, the RI was equal to 0.797.

Considering the *Eleutherodactylus* species as outgroup, we observed the homophyly of the Centrolenidae (BP = 99/97/99) (Fig. 4). Moreover, these species were sister groups to the Hylidae, which constituted a homophyletic group (BP = 79/–/69). Within French Guiana Hylidae, we observed that the genus *Phyllomedusa* (Phyllomedusinae) was the sister group to the subfamily Hylinae (BP = 78/92/93). Within the Hylinae, the genus *Scinax* was homophyletic (BP = 100/100/100). The *rostrata* species groups constituted by *Scinax jolyi*, *Scinax proboscideus*, *Scinax rostrata* and *Scinax nebulosa* (BP = 100/100/100) and the *rubra* species group (BP = 61/58/–) were supported.

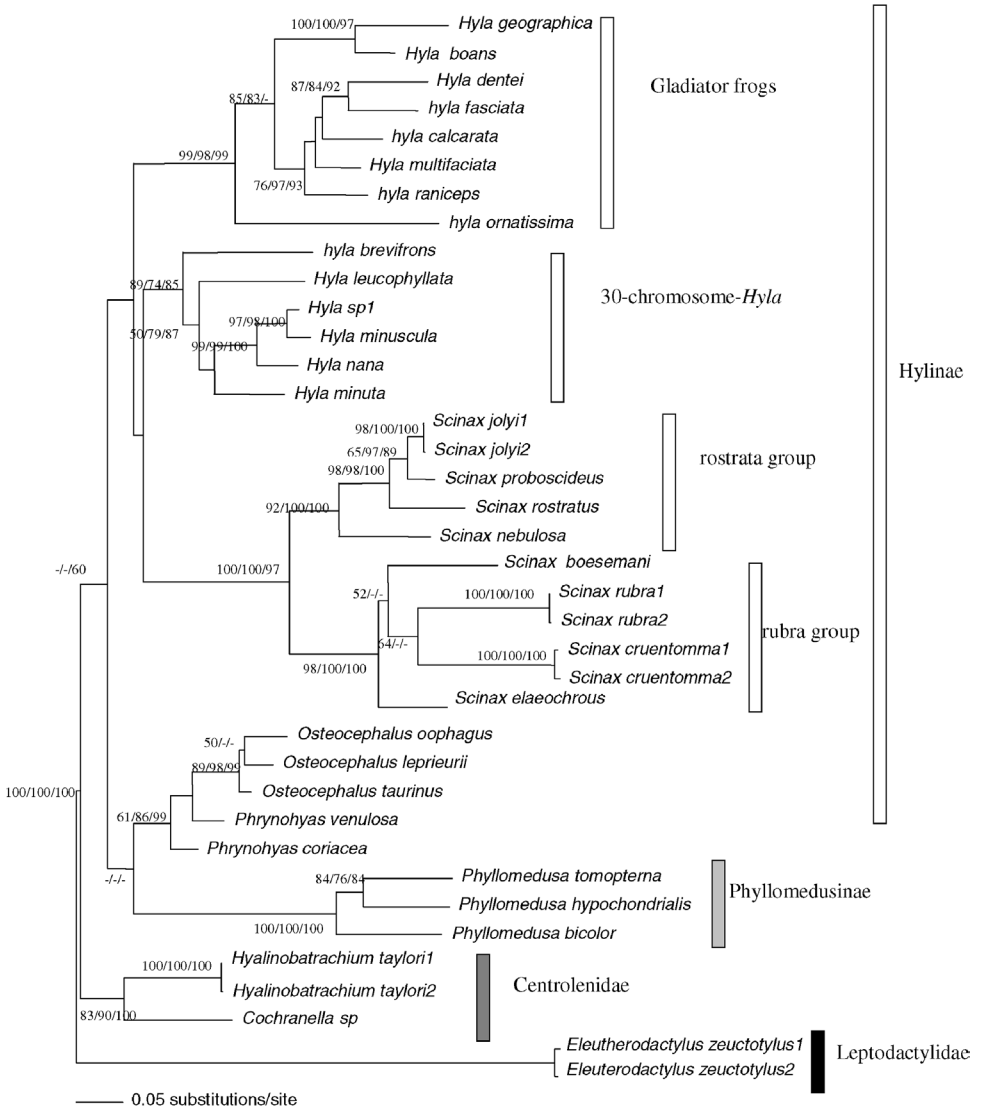

Fig. 3. Bootstrap analyses carried out with 1000 iterations using the 813 pb of the 16S rDNA and 12S rDNA among 38 'species' of *Hyla*, *Osteocephalus*, *Scinax*, *Phrynohyas*, *Phyllomedusa*, *Cochranella* and *Hyalinobatrachium* genera: using Maximum-Likelihood bootstrap value on the left, Maximum-Parsimony bootstrap value on the middle, Neighbour-Joining on a matrix of the Tamura–Nei (alpha = 0.30) model right bootstrap value. The Maximum-Likelihood tree is shown ($-\ln = 9450.77869$). The tree was rooted on *Eleutherodactylus* as outgroup.

Table 3
Between group average using the Tamura–Nei (alpha parameter for the gamma shape equal to 0.30) for the seven genera standard errors estimated by bootstrap method (1000 replications) for the 16S and 12S mitochondrial DNA (813 bp)

	1	2	3	4	5	6	7
[1] Hyla		0.048	0.068	0.064	0.102	0.124	0.153
[2] Phrynohyas	0.371		0.019	0.041	0.092	0.078	0.111
[3] Osteocephalus	0.466	0.129		0.066	0.102	0.081	0.156
[4] Centrolenidae	0.490	0.279	0.382		0.102	0.125	0.123
[5] Scinax	0.683	0.529	0.590	0.646		0.151	0.258
[6] Phyllomedusa	0.785	0.453	0.495	0.685	0.852		0.218
[7] Eleutherodactylus	0.866	0.624	0.707	0.648	1.188	0.985	

Above diagonal: **Hylidae** [Hylinae *Hyla Phrynohyas Osteocephalus Scinax* Phyllomedusinae *Phyllomedusa*]; **Centrolenidae** *Hyalinobatrachium, Cochranella*; **Leptodactylidae** *Eleutherodactylus*.

Table 4
Between group average using the Tamura–Nei (alpha parameter for the gamma shape equal to 0.21) for the seven genera standard errors estimated by bootstrap method 1000 replications for the 18S and Tyrosinase nuclear DNA (1590 pb)

	1	2	3	4	5	6	7
[1] Hyla		0.004	0.003	0.006	0.005	0.008	0.008
[2] Phrynohyas	0.032		0.003	0.006	0.005	0.010	0.008
[3] Osteocephalus	0.032	0.014		0.006	0.005	0.010	0.009
[4] Centrolenidae	0.049	0.045	0.046		0.007	0.009	0.006
[5] Scinax	0.051	0.042	0.039	0.058		0.009	0.009
[6] Phyllomedusa	0.079	0.083	0.085	0.080	0.089		0.011
[7] Eleutherodactylus	0.066	0.063	0.066	0.055	0.073	0.091	

Above diagonal: **Hylidae** [Hylinae *Hyla Phrynohyas Osteocephalus Scinax* Phyllomedusinae *Phyllomedusa*]; **Centrolenidae** *Hyalinobatrachium, Cochranella*; **Leptodactylidae** *Eleutherodactylus*.

Table 5
Test of the homogeneity between the different mismatch distributions using the Kolmogorov–Smirnov test with alpha equal to 0.05 (see text for more details)

2 **1**	Within Genus *Hyla*	Within Genus *Scinax*	Within Hylinae	Between Hylinae-Phyllomedusinae	Between Hylidae-Centrolenidae
Within Genus *Hyla*		**1 > 2**	1 < 2	1 < 2	1 < 2
Within Genus *Scinax*	NS		1 < 2	1 < 2	1 < 2
Within Hylinae	1 > 2	1 > 2		1 < 2	1 < 2
Between Hylinae-Phyllomedusinae	1 > 2	1 > 2	**1 < 2**		**1 > 2**
Between Hylidae-Centrolenidae	1 > 2	NS	**1 < 2**	**NS**	

NS: non significant, 1 > 2: distance distribution of the first group significantly higher than the second, 1 < 2: distance distribution of the first group significantly lower than the second. In bold: result not congruent with the morphological taxonomic level. The results for the mitochondrial genes are below the diagonal and for the nuclear genes above the diagonal.

The paraphyly of genus *Hyla* was supported by ML, MP, and NJ methods (BP = 52/56/55), due to the relationship between the homophyletic group constituted by *Osteocephalus* and *Phrynohyas* species and the *Hyla* 30-chromosome group bp = 97/94/99). The homophyly of the two groups of *Hyla* was confirmed by the 'nuclear total evidence' approach (BP = 95/88/97 for the 30-chromosome *Hyla* and bp = 90/93/99 for the Gladiator frogs. Paraphyly of the *H. geographica* species group and the *nana* species group are confirmed. For the second group, this paraphyly is not supported.

3.2.1. Genetic diversity of the Tyrosinase and 18S rDNA genes

According to the Tamura–Nei distance with alpha = 0.21 (Table 4), the variability between the different genera of Hylidae ranged from 0.014 ± 0.003 to 0.089 ± 0.009. The genetic divergence between the Centrolenidae (*Hyalinobatrachium* and *Cochranella*) and the Hylidae ranged from 0.045 ± 0.006 with *Phrynohyas* to 0.080 ± 0.009 with *Phyllomedusa*. The genus *Scinax* displayed a within group average of 0.026 ± 0.003 (data not shown) and the genus *Hyla* yielded an average of 0.030 ± 0.003. Genetic diversity within the two groups of *Hyla* was respectively 0.016 ± 0.002 for 30-chromosome Hyla, and 0.018 ± 0.003 for Gladiator frogs. The genetic diversity between the two groups was 0.04 ± 0.006 and the variability between the two groups of *Scinax* was equal to 0.032 ± 0.004. By comparison, the genetic diversity between *Osteocephalus* and *Phrynohyas* is only 0.014 ± 0.003.

Pairwise comparison showed that genetic diversity was significantly higher between the two different subfamilies Hylidae (Hylinae and Phyllomedusinae) than between the families Hylidae and Centrolenidae, which is another incongruence between morphology and molecular data (Table 5).

3.3. Combined analysis of total datasets (nuclear and mitochondrial)

The incongruence test did not detect significant heterogeneity between the two partition (mitochondrial versus nuclear $p = 0.09$) and weak saturation was observed for the mitochondrial versus nuclear substitution patterns (Fig. 2G–I). Of the 2403 positions, 615 were informative (gaps were treated as missing for the unweighted parsimony analysis). We found one single par-

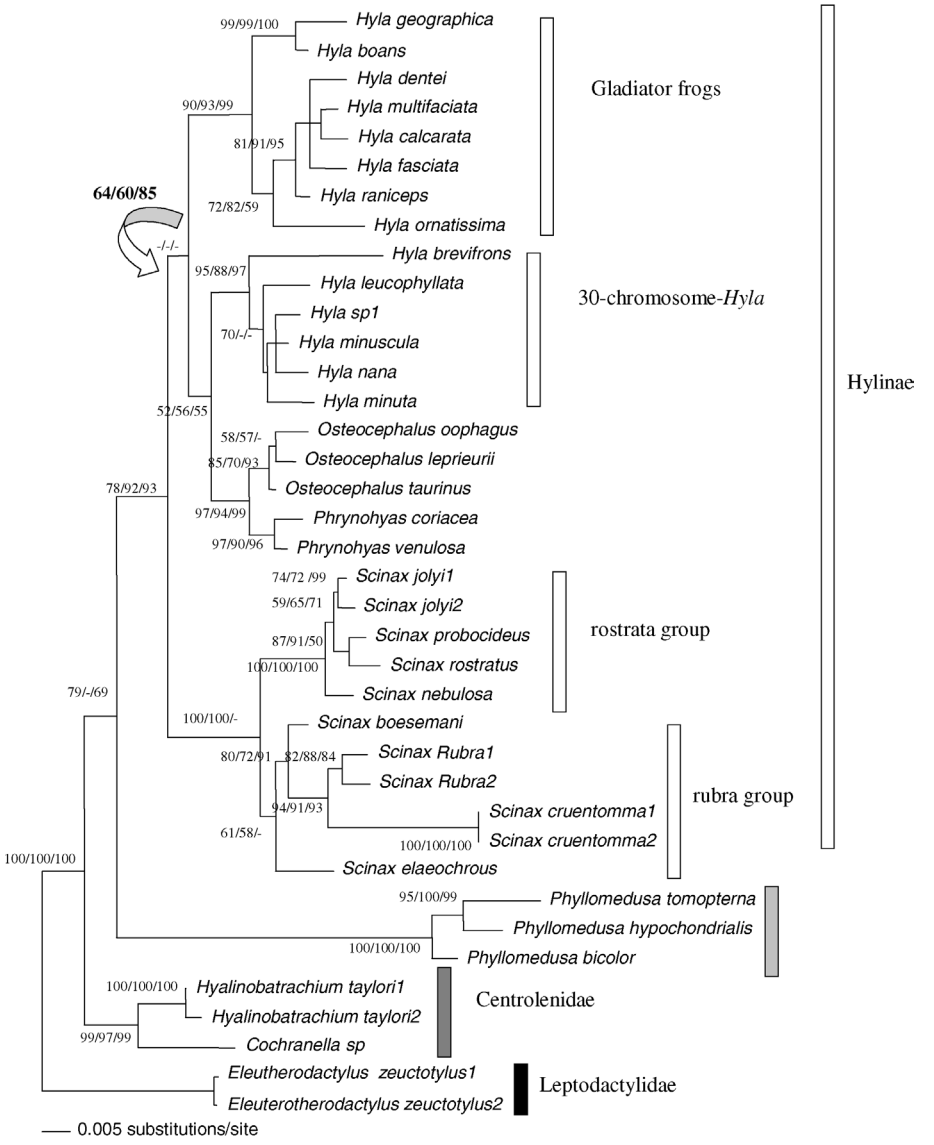

Fig. 4. Bootstrap analyses carried out with 1000 iterations using the 1590 pb of the tyrosinase and 18S rDNA among 38 'species' of *Hyla*, *Osteocephalus*, *Scinax*, *Phrynohyas*, *Phyllomedusa*, *Cochranella* and *Hyalinobatrachium* genera: Maximum-Likelihood using bootstrap value on the left, Maximum-Parsimony bootstrap value on the middle, Neighbour-Joining on a matrix of the Tamura–Nei (alpha = 0.21) model right bootstrap value. The Maximum-Likelihood tree is shown ($-\ln = 5853.9177$). The tree was rooted on *Eleutherodactylus* as outgroup. The arrow indicated the incongruence for one node between the Maximum-Likelihood tree and the three bootstrapped trees (ML, MP, NJ).

simonious tree (2674 steps). The g1 value was –0.79, the CI was equal to 0.430, and RI was equal to 0.662.

Combined nuclear and mitochondrial analysis confirmed the previous results: paraphyly of the genus *Hyla* and the relationship between Phrynohyas and Osteocephalus with the *Hyla* 30 chromosomes group (Fig. 5). The genus *Scinax* was the sister group to *Hyla* plus *Osteocephalus* plus *Phrynohyas*; however, this topology was not supported. The Phyllomedusinae was the sister group to the Hylinae (55/90/88).

4. Discussion

4.1. *The genus* Hyla

Our results confirmed that two major groups constitute the paraphyletic genus *Hyla* in French Guiana. The first group is constituted by *H. brevifrons*, *H. leucophyllata*, *H. nana*, *H. minuta*, *H. sp1* and *H. minuscula* and the second group by *H. boans*, *H. fasciata*, *H. calcarata*, *H. dentei*, *H. geographica*, *H. raniceps*, *H. mul-*

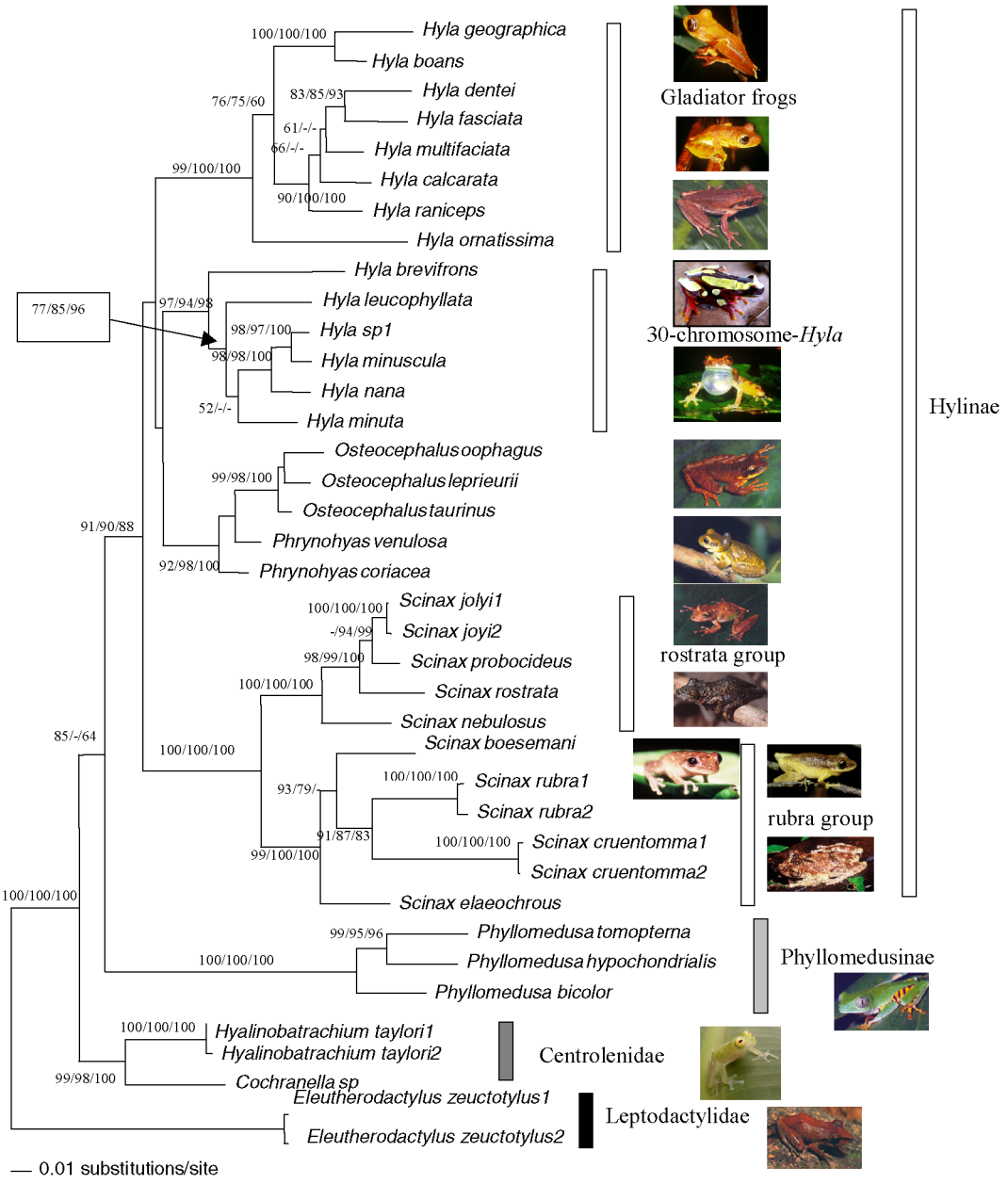

Fig. 5. Bootstrap analyses carried out with 1000 iterations using the 2403 pb of the mitochondrial and nuclear genes among 38 'species' of *Hyla, Osteocephalus, Scinax, Phrynohyas, Phyllomedusa, Cochranella* and *Hyalinobatrachium* genera: Maximum-Likelihood using bootstrap value on the left, Maximum-Parsimony bootstrap value on the middle, Neighbour-Joining on a matrix of the Tamura–Nei (alpha = 0.25) model right bootstrap value. The Maximum-Likelihood tree is shown ($-\ln = 15\,785.73299$). The tree was rooted on *Eleutherodactylus* as outgroup.

tifaciata and *H. ornatissima*. These species represent the 30-chromosome *Hyla* group [9] and the second one the Gladiator frogs, respectively groups [26]. The paraphyly of the genus *Hyla* confirmed herein underlines the necessity to reconsider the taxonomy of one of these group.

We detected incongruence between the mitochondrial and the nuclear gene trees. Indeed, the genera *Osteocephalus* and *Phrynohyas* clustered with the genus *Hyla* on the nuclear gene tree. This pattern was different considering the mitochondrial gene tree but the bootstrap values are too low and thus might be due to saturation and homoplasy on mitochondrial data. These nuclear gene results were in agreement with the tree constructed on the basis of morphology by Da Silva [27], which revealed two groups in the paraphyletic

genus *Hyla*. The first group ($2n = 30$) was polyphyletic according to the analysis by Da Silva (*Hyla* + *Phyllodytes* + *Xenohyla*) and the node for the second group ($2n = 24$) was not supported by the synapomorphy of this group, i.e., shape of the first distal prepollex nearly cylindrical (CI = 0.033); four elements anterior to the distal prehallux (CI = 0.088). However, our results add information on the phylogenetic relationships between 'Gladiator frogs', *Scinax*, *Hyla* 30 chromosomes and *Osteocephalus* + *Phrynohyas*.

We found that the clustering of *H. sp1* with *H. minuscula* (with 3% divergence) is in agreement with their acoustic signals [19]. A more accurate phylogenetic analysis coupling systematics and phylogeography of these particular groups needs to be done, as for the *H. leucophyllata* complex [9].

4.2. *The genus* Scinax

The molecular homophyly [64] of *Scinax* was supported by high bootstrap proportions, whatever the molecular marker used. The molecular results were in agreement with the morphological results based on a single shared derived trait, i.e., the absence of, or reduced webbing between toes I and II in adults [65] and on ten synapomorphies [29].

The phylogenetic reconstruction indicated that the *rubra* group (*S. rubra*, *S. cruentomma*, *S. boesemani*, *S. elaeochroa*) and the *rostrata* group (*S. jolyi*, *S. proboscideus*, *S. rostrata* and *S. nebulosa*) are respectively homophyletic. This result is in contradiction with the morphological cladistic analysis made by Faivovich [29] in which the author stated the nesting of the *rostrata* group within the *rubra* species group. Hierarchical positions of the species of the *rubra* group are also incongruent with the topology proposed by Faivovich. However this could be due to taxa sampling. According to the Tamura–Nei distance, genetic variation within *Scinax* was 0.417 ± 0.067 for the total mitochondrial DNA alignment. A previous study based on the partial 16S [28] displayed a genetic variation within *Scinax* to 0.171 ± 0.016. Our results indicate a higher degree of genetic diversity (whatever the distance and the marker used) within a restricted sample of *Scinax* than other studies [66]. Indeed, Kosuch et al. [67] found 8 to 10% of genetic divergence in the 16S rDNA gene among the different species (African + Asian species) belonging to the genus *Hoplobatrachus* and 11% between the Asian species *Rana gracilis* and the African species *R. lepus*. This analysis of the genetic divergence should be considered in light of:

(1) the varied pattern of substitutions along the 16S rDNA molecule that could result in over or underestimation of genetic distances depending on the portion of the gene studied, and
(2) the homogeneity/heterogeneity of this pattern of substitutions between the lineages.

We eliminated the first artefact by using a homologous region of the 16S rDNA gene. To account for the second, we constructed a NJ tree rooted on the *Xenopus laevis* sequence and found no significant difference in the rate of substitution between the different lineages (data not shown). These results demonstrate the long evolutionary history of the genus *Scinax*. This supports the opinion of Pombal et al. [25] (with respect to morphology, and ecology) who suggested that the genera *Scinax* and *Hyla* need to be separated into several genera.

4.3. *Molecular vs morphological taxonomic levels*

In the case of congruence between morphological and molecular data, it is clear that the genetic diversity within lower taxonomic levels will be lower than that within higher taxonomic levels. Nevertheless, Hylidae and Hylinae are supported as homophyletic with respect to the positions of Centrolenidae and Pyllomedusinae. These families and sub families level are thus corroborated. The mitochondrial unresolved pattern is certainly biased because of strong saturation and homoplasy. The heterogenity in branch length and distances between Centrolenidae, Hylinae and Phyllomedusinae for mitochondrial and nuclear data (no significant differences between the genetic distances of the two families Hylidae and Centrolenidae and the genus *Scinax*) is certainly due to heterogeneous rate of molecular evolution.

Our results (Table 5) clearly show that the phylogenetic signals based on mitochondrial genes and nuclear genes are not congruent. This incongruence could be due to the saturation effect. The mitochondrial genes displayed two patterns:

(1) the first was observed at the family level, as some *Hylinae* species (*Osteocephalus* and *Phrynohyas*) clustered with the subfamily Phyllomedusinae (with no support).
(2) The second pattern was due to the homophyletic genus *Scinax* (considering the two species groups present in French Guiana), which displayed similar degree of genetic diversity than the genus *Hyla*, which was paraphyletic according to total evidence analysis.

This extreme difference in mitochondrial and nuclear diversity may be the result of numerous phenomena, as listed by Avise [68]. It seems therefore obvious that the genus *Scinax* represents a genus of a long evolutionary history, given that the sampling effort was identical for the two genera in this region.

5. Conclusion

Strategies for the protection of biological diversity require the identification of areas that shelter the highest species and genetic diversity, and maximal protection of contiguous environmental gradients within these areas [69]. It will be very difficult to protect neotropical frogs efficiently, if only a few clades have been validated using molecular markers. A recent study using molecular markers [42] revealed evidence of striking convergences in larval and adult traits for the Madagascan and Asian Ranidae. The level of homoplasy in morphological, ecological and physiological data for the Neobatrachia is still underestimated and amphibian systematic is largely still based on morphological traits. These lead to an underestimation of the biodiversity within amphibians. Moreover, it is very important to use both mitochondrial and nuclear markers to avoid incongruence between gene trees and species trees in molecular phylogenetic reconstructions.

Indeed, the level of genetic diversity between genera within the same subfamily can vary considerably. For example, the genera *Scinax* and *Hyla* displayed a mitochondrial genetic diversity of 0.417 ± 0.067 and 0.392 ± 0.050, whereas *Osteocephalus* and *Phrynohyas* yielded a value of 0.129 ± 0.019. This can be interpreted in two ways: the genera *Scinax* and *Hyla* must be split into more than one genus (due to the high genetic distance), or the genera *Osteocephalus* and *Phrynohyas* must be merged into only one genus (due to the small genetic distance). Considering the phylogenetic relationships within the Hylidae, i.e., homophyly of *Scinax*, paraphyly of *Hyla*, and the nuclear genetic diversity, the first hypothesis seems more likely.

The species identified on the basis of morphology could be an indicator of species richness but certainly underestimate the real number of specific entity especially in the tropics [11]. Another approach, DNA barcoding, was recently proposed by Hebert [70]. This methodology use large scale screening of one or a few reference genes in order to assign unknown individuals to species and thereby could enhance discovery of new species. However, some authors raise doubts about the contribution of this approach [71]. For example, problems may arise in groups of frequent hybridisation and recent radiations. The real challenge lies with tropical taxa and those with limited dispersal and thus substantial phylogeographic structure [71].

Considering our results, the fact that a complete inventory of amphibians in French Guiana is currently impractical and the fact that populations are declining in many parts of the world, we recommend that species richness should be initially assessed on the basis of morphology, systematically coupled with a study of molecular genetic diversity (nuclear and mitochondrial gene sequences). The higher taxonomic diversity must be coupled to phylogenetic diversity for the conservation: "*all genera* (*based on morphology*) *are equal but some genera are more equal than others* (*based on molecular*)." This approach will avoid major biases in the estimation of the biodiversity in neotropical regions, therefore providing an important conservation tool.

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