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Phylogenetic signal and variation of visceral pigmentation in eight anuran families

Diogo B. Provete, Lilian Franco-Belussi, Lia R. de Souza Santos, Rodrigo Zieri, Rafaela M. Moresco, Itamar A. Martins, Silvio C. de Almeida & Classius de Oliveira

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Visceral pigmentation is found in several organs and structures of ectothermic animals, comprising the extracutaneous pigmentary system. Its function is not well defined, although it is known that melanin is produced and stored inside pigmented cells. Previous studies demonstrated that the distribution of visceral pigmentation is neither homogeneous among organs among anuran species. We described the diversity of visceral pigmentation in 12 organs/structures from 32 anuran species belonging to eight families in a phylogenetic context. We also determined in which node(s) of the phylogeny there is more variation in the pigmentation and whether this variation has phylogenetic signal. The visceral pigment cells in organs and structures of the abdominal cavity varied among genera. All species had pigmentation in the urogenital and cardiorespiratory systems, whereas the stomach lacks pigmentation in all species. We also found a phylogenetic signal for pigmentation in all organs and structures taken together, besides heart, testes, lumbar parietal peritoneum and lumbar nerve plexus when considered separately. Overall, considering all organs, the highest diversity of categories of pigmentation was found in the nodes corresponding to Cruciabatrachia and Athesphatanura. This study constitutes the first step towards understanding the evolution of visceral pigmentation in anurans.

Corresponding author: *Classius de Oliveira, Department of Biology, Laboratory of Comparative Anatomy, Institute of Biosciences, Letters, and Exact Sciences, São Paulo State University – UNESP, Rua Cristóvão Colombo, 2265, São José do Rio Preto, São Paulo 15054-000, Brazil. E-mails: classius@ibilce.unesp.br*

Diogo B. Provete, Graduate Program in Ecology and Evolution, Laboratory of Insect Ecology, Department of Ecology, Federal University of Goiás, Goiânia, 74001-970, Brazil. E-mail: dbprovete@gmail.com

Lilian Franco-Belussi, Lia R. de Souza Santos, Rodrigo Zieri, and Rafaela M. Moresco, Graduate Program in Animal Biology, Department of Biology, Laboratory of Comparative Anatomy, Institute of Biosciences, Letters, and Exact Sciences, São Paulo State University – UNESP, Rua Cristóvão Colombo, 2265, São José do Rio Preto, São Paulo 15054-000, Brazil. E-mails: lilian.belussi@gmail.com, rodrigozieri@yahoo.com.br, rafaelabiologia@yahoo.com.br.

Lia R. de Souza Santos, Current address: Federal Institute of Goiás – IFG, campus Rio Verde, Rio Verde, Goiás 75908-000, Brazil. E-mail: lirabio@yahoo.com.br

Itamar A. Martins, Department of Biology, Laboratory of Zoology, University of Taubaté – UNITAU, Taubaté, São Paulo 12030-180, Brazil. E-mail: istama@uol.com.br

Silvio C. de Almeida, Department of Zoology, Institute of Biosciences, São Paulo State University – UNESP, Botucatu, São Paulo 18618-970, Brazil. E-mail: scesar@ibb.unesp.br

Introduction

Invertebrates and vertebrates have specialized cells called chromatophores, whose pigments are related to several functions. There are five types of chromatophores in ectothermic vertebrates: melanophores (black or brown)-containing melanin; erythrophores-containing pteridine; xanthophores (yellow)-containing pteridine and carotenoids; and iridophores, with pale metallic coloration because of purine and pteridine cristals (Bagnara & Matsumoto 2006). These cells are found in fish, amphibians and reptiles. Leucophores are only reported in fish and have white coloration given by purine (Bagnara & Matsumoto 2006).

Ectothermic vertebrates have two distinct types of pigment cells containing melanin: melanocytes and melanophores (Agius & Roberts 2003). These dendritic cells are commonly found in the integument (Aspengren *et al.* 2009). Additionally, melanin-containing cells were also reported in some internal organs of fish (Agius 1980; Agius & Roberts 2003; Jordanova *et al.* 2008), salamanders (Pederzoli & Trevisan 1990; Prelovisek & Bulog 2003), anurans (Franco-Belussi *et al.* 2009, 2011; Moresco & Oliveira 2009) and turtles (Christiansen *et al.* 1996; Rund *et al.* 1998; Johnson *et al.* 1999). Visceral pigmentation was described in several organs and structures in these animals, such as the integument, heart, lungs, intestines, rectum, peritoneum, liver, spleen, kidneys, gonads, Bidder's organ, thymus, nerve plexus, meninges and blood vessels, constituting the extracutaneous pigmentary system (Zuasti *et al.* 1998; Gallone *et al.* 2002).

Previous studies (Zieri *et al.* 2007; Franco-Belussi *et al.* 2009, 2011; Moresco & Oliveira 2009) found that the pigmentation on the surface of testes varies among anuran species and genera. The testicular pigmentation has been also used as a character in systematic studies of Dendrobatoidea (Grant *et al.* 2006) and the genus *Hylodes* (Canedo 2008). For instance, members of the genera *Adelphobates*, *Colostethus*, *Dendropsophus* and *Leptodactylus* usually lack pigmentation on the testes (Grant *et al.* 2006; Franco-Belussi *et al.* 2009, 2011). On the other hand, members of the family Leiuperidae and other Dendrobatidae have pigmented testes (Grant *et al.* 2006; Franco-Belussi *et al.* 2009). In addition, the pattern of distribution and extent of iridiophores on the peritonea of glassfrogs (Centrolenidae) have been traditionally used as a character in the systematics of the group (Cisneros-Heredia & McDiarmid 2007). Taken together, these findings suggest that the visceral pigmentation shows a phylogenetic signal.

Contrarily, other studies reported that the pigmentation on testes varied during the breeding season in the bufonids *Rhinella schneideri* (Moresco & Oliveira 2009), and species of *Atelopus* (McDiarmid 1971), but not in *Physalaemus cuvieri* (Moresco & Oliveira 2009). Accordingly, the pigmentation on the dorsal surface of the kidneys increased from the beginning towards the end of the breeding season in *Dendropsophus nanus* (Moresco & Oliveira 2009). The pigmentation on the heart, testes and kidneys also appears to increase in *Eupemphix nattereri* following administration with lipopolysaccharide of *Escherichia coli* (Franco-Belusi & Oliveira 2011).

Biological traits may have different evolutionary histories. As a consequence, they can be either labile and possibly converge or be conserved (Harvey & Pagel 1991). Phylogenetic signal is the tendency of related species to resemble each other more than they resemble species drawn at random from a phylogeny (Harvey & Pagel 1991; Blomberg & Garland 2002), that is, when there is a correlation between traits and phylogeny. This is equivalent to say that trait diversity is skewed to the root of a phylogenetic tree (Pavoine *et al.* 2010). On the other hand, traits can be labile and the highest diversity can be found at the tips of the tree. This often occurs when traits converge.

In this paper, we extend the scope of previous studies and record the occurrence of pigmentation on the surface not only of testes but also on 16 organs and structures of 35 anuran species belonging to 13 genera from eight families nested within Meridianura (*sensu* Frost *et al.* 2006). Additionally, for the first time, the visceral pigmentation of several organs in anurans is analysed using a phylogenetic comparative method. The test of phylogenetic signal is a key prerequisite for studies of character evolution. The presence of a phylogenetic signal has important implications to trait evolution and how comparative data are analysed. Conversely, the absence of phylogenetic signal may justify the use of standard statistics in comparative studies (Blomberg & Garland 2002).

Methods

Data collection

We collected calling adult anurans at night near breeding sites and with pitfall traps in several locations in the state of São Paulo, southeastern Brazil (see Appendix S1). These specimens are housed at the collection of the Laboratory of Comparative Anatomy, Department of Biology, UNESP, São José do Rio Preto (see Appendix S2). We used at least five adult specimens of each species for the analysis of pigmentation. The specimens were anesthetized with 1 g/L of benzocaine and dissected to expose the organs. We used a stereoscopic microscope (Leica MZ16; Leica Microsystems GmbH, Wetzlar, Germany), coupled with an image capture system for photographic recordings. All procedures followed the recommendations of the COBEA (Brazilian College of Animal Experimentation) and the Ethics Committee of our university (Protocol #70/07 CEEA). We also analysed additional specimens from the amphibian collections of the Department of Zoology and Botany, UNESP (DZSJRP), Laboratory of Zoology, University of Taubaté (CCLZU), and the Jorge Jim collection (JJ; now incorporated into the herpetological collection of the National Museum, MN-RJ). The material examined is listed in Appendix S2. We also obtained data from the literature (Franco-Belussi *et al.* 2011, 2012) for species of the family Hylidae.

We recorded the distribution of visceral melanocytes in 16 organs or structures, namely pericardium, cardiac blood vessels, heart, lungs, stomach, intestine, rectum, visceral

peritoneum, kidneys, renal veins, urinary bladder, testes, fatty bodies, lumbar plexus nerves (lumbar portion), parietal peritoneum, and intestinal mesenterium. We recorded the pigmentation on these organs/structures based on coloration intensity, following the protocol of Franco-Belussi *et al.* (2009), which is similar to those used by Grant *et al.* (2006). The intensity of pigmentation on organs was divided into four categories, ranging from absence of pigmentation to entirely pigmented, as follows: category (0) lack of pigment cells on the surface of organs, in which the usual colour of the organ is evident; category (1) a few scattered pigment cells, giving the organs a faint pigmentation; category (2) presence of a large amount of pigment cells; category (3) presence of a massive amount of pigment cells, rendering an intense pigmentation to the structure, changing its usual colour and superficial vascularization (Franco-Belussi *et al.* 2009). We assigned a certain category of pigmentation to an organ/structure considering the pigmentation of the majority of individuals. For example, when three specimens out of five had pigmentation category 0, and the remaining two had category 1, we assigned category 0 to that organ in that species, since the method used (Pavoine *et al.* 2010; see below also) does not incorporate intraspecific trait variability and only uses a single value.

Taxon sampling and statistical analysis

Species selection for this study took into account availability of phylogenetic information, published data about visceral pigmentation, and its availability in scientific collections. We included species from the following families in the analysis: Brachycephalidae, Craugastoridae, Cycloramphidae, Hylidae, Hylodidae, Leiuperidae, Leptodactylidae and Strabomantidae (see Appendix S2). To conduct the analysis, we assembled by hand a supertree for the species sampled using the software MESQUITE 2.74 (Maddison & Maddison 2010), considering the topological relationships proposed by the following studies: Ponssa (2008) for species of *Leptodactylus*; Wiens *et al.* (2010) for species of *Scinax* and *Dendropsophus*; Hedges *et al.* (2008) for species of *Ischnocnema*, *Holoaden* and *Haddadus*; Tárano & Ryan (2002) for species of *Physalaemus*. We followed the phylogeny of amphibians proposed by Frost *et al.* (2006), as modified by Grant *et al.* (2006) and Hedges *et al.* (2008), for

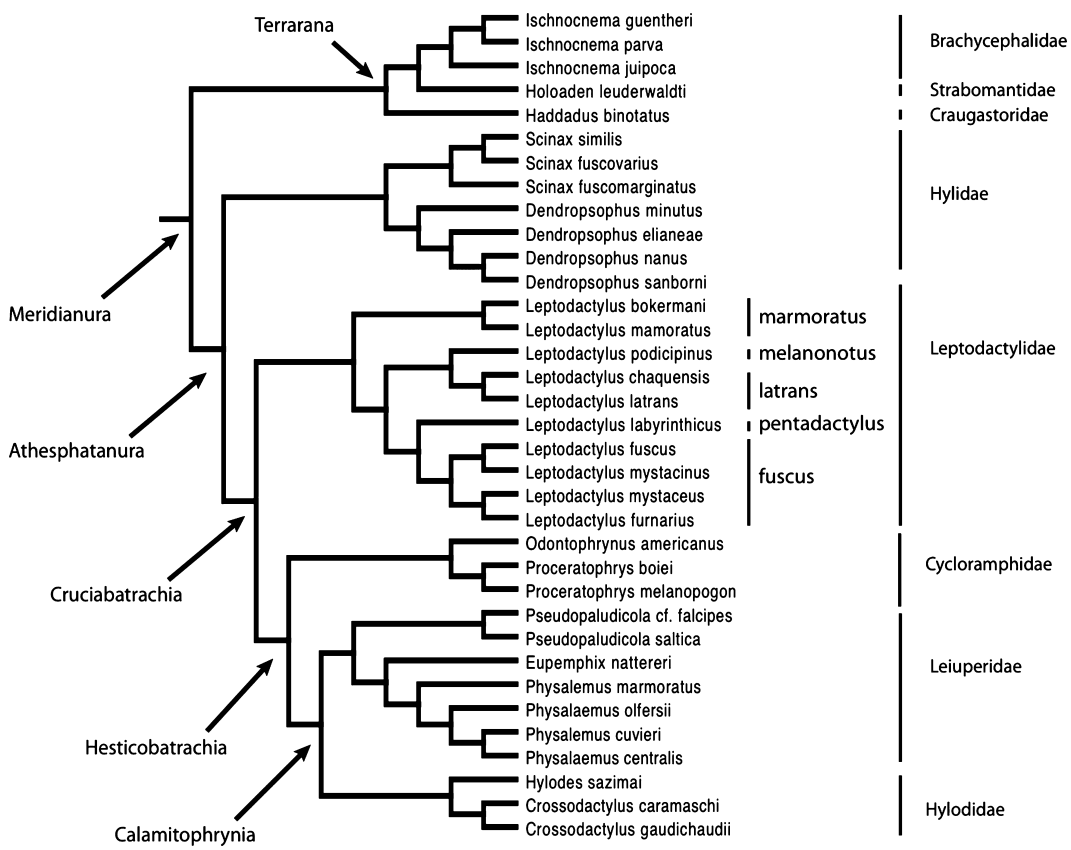

Fig. 1 Phylogenetic tree constructed for the analysis of trait diversity showing the higher-order groups (left), families (right) and species groups within *Leptodactylus*.

the placement of families and higher-order groups (Fig. 1).

We used the analysis proposed by Pavoine *et al.* (2010) to study how the categories of pigmentation vary along the nodes of the phylogeny and also to test for a phylogenetic signal in the pigmentation found in 12 of the 16 organs/structures. We excluded from the following analysis the organs that did not have pigmentation in all species, such as urinary bladder, fatty bodies, intestine, and stomach. After constructing the phylogenetic tree for the species sampled, we calculated the distance among species based on the categories of pigmentation using the Gower index of similarity, as modified by Pavoine *et al.* (2009) to deal with ordinal traits, as was the case in our study. Posteriorly, we tested whether traits are distributed in a way that only one node in the tree expresses the whole trait diversity, whether trait diversity is evenly distributed across nodes, or whether trait values are skewed to the root or the tips of the phylogeny

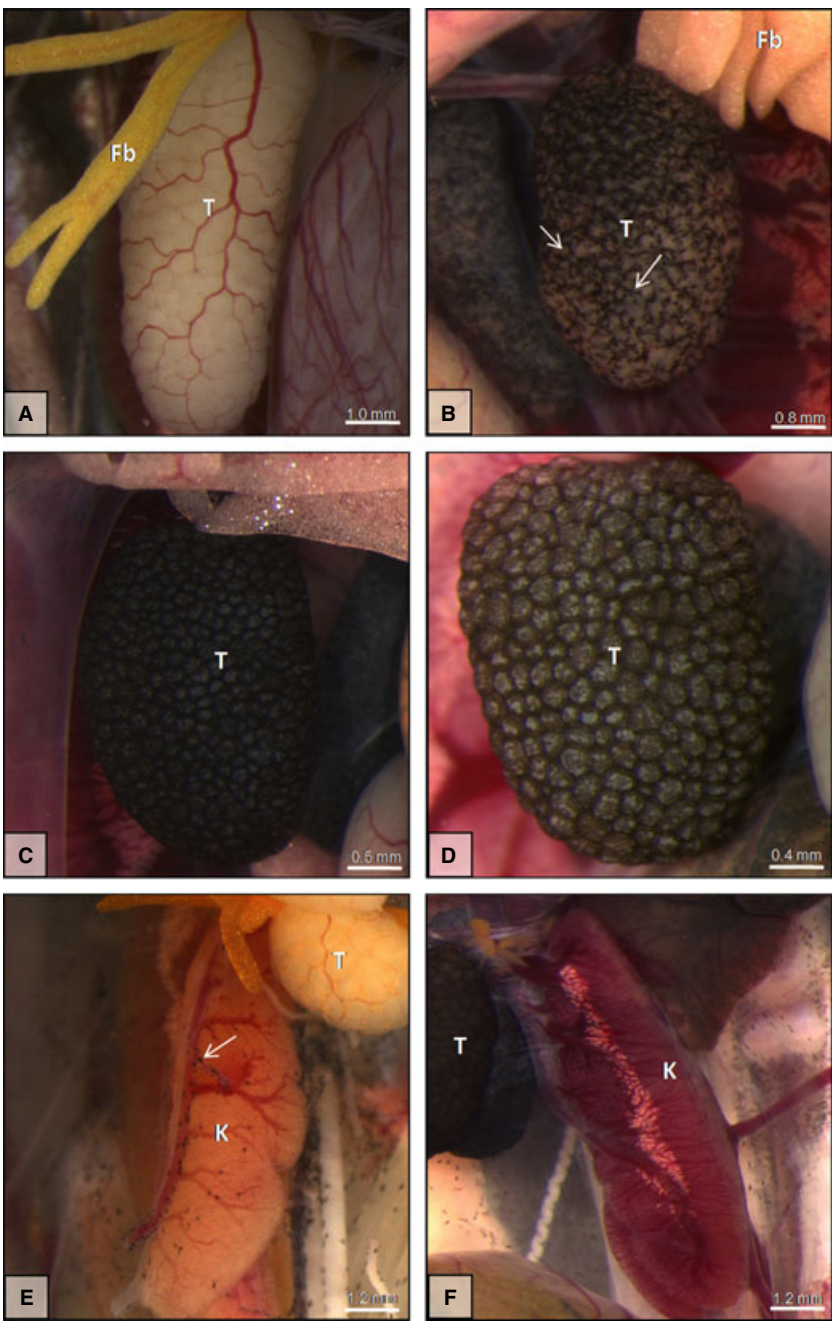

Fig. 2 Organs of the urogenital system. —A. Testis without pigmentation in *Odontophrynus americanus*, showing an intense vascularization. —B. Testis with moderate pigmentation in *Eupemphix nattereri*. —C. Intense pigmentation in *Physalaemus centralis*. —D. Testis of *Physalaemus cuvieri* with an intense pigmentation, in which the typical vascularity of the gonads is masked. —E. Dorsal view of the kidneys of *Leptodactylus bokermanni* with scattered melanocytes. —F. Ventral view of the kidneys of *Proceratophrys boiei* without pigmentation. Fb, fat bodies; K, Kidney; T, Testis. Arrows indicate melanocytes.

(Pavoine *et al.* 2010). In this context, phylogenetic signal is detected when higher trait diversity is skewed to the root of the phylogeny. Our supertree did not include branch lengths, because the analysis uses the quadratic entropy index to measure trait diversity and only considers tree topology (Pavoine *et al.* 2010), that is, it assumes a punctuated model of evolutionary change (Garland *et al.* 1992). The analysis was conducted in self-written codes ('decdiv' and 'dist.ktab') provided by S. Pavoine and the packages *ape* (Paradis *et al.* 2004) and *ade4* (Dray & Dufour 2007) in the R software v. 2.12.2 (R Development Core Team. 2011).

Results

Visceral pigment cells occurred differently in organs and structures of the abdominal cavity among the 35 species analysed. Below, we include descriptions of each organ/structure grouped by systems (see also Table S1). Additional colour pictures are available from MorphoBank (http://morphobank.org/permalink/?P701).

Variation in pigmentation among organs/structures

Species from all groups had pigmentation on the testes, except for Terrarana, Leptodactylidae and Cycloramphidae (Fig. 2A), whose testes were white-yellow. We found an intermediate amount of pigmentation (category 2) on the testes of the genus *Eupemphix*, whereas *Physalaemus*, *Pseudopaludicola*, and Hylodidae had an intense pigmentation (category 3) on that organ (Fig. 2B–D). The majority of species of Terrarana (except for *Ischnocnema juipoca*) and Leptodactylidae (except for *Leptodactylus bokermanni* and *Leptodactylus labyrinthicus*) lack pigmentation on the kidneys, whereas all species (except for *Proceratophrys boiei*) within Hesticobatrachia had small amounts (category 1) of pigmentation (Fig. 2E,F). There were no differences between the kidney and testicular antimeres.

The pigmentation varied in the cardio-respiratory system. The species from Terrarana, Leptodactylidae, Hylodidae and *Proceratophrys* had no pigmentation in the pericardium, whereas *Odontophrynus* and Leiuperidae had a small amount (category 1) of pigmentation. The species from Terrarana and Leptodactylidae lack pigmentation in the heart and cardiac blood vessels, whereas species of *Odontophrynus* and Calamitophrynia had only a small amount of pigmentation (category 1) on cardiac blood vessels. The genera *Eupemphix* and *Proceratophrys* had little pigmentation (category 1) on the heart. All genera had

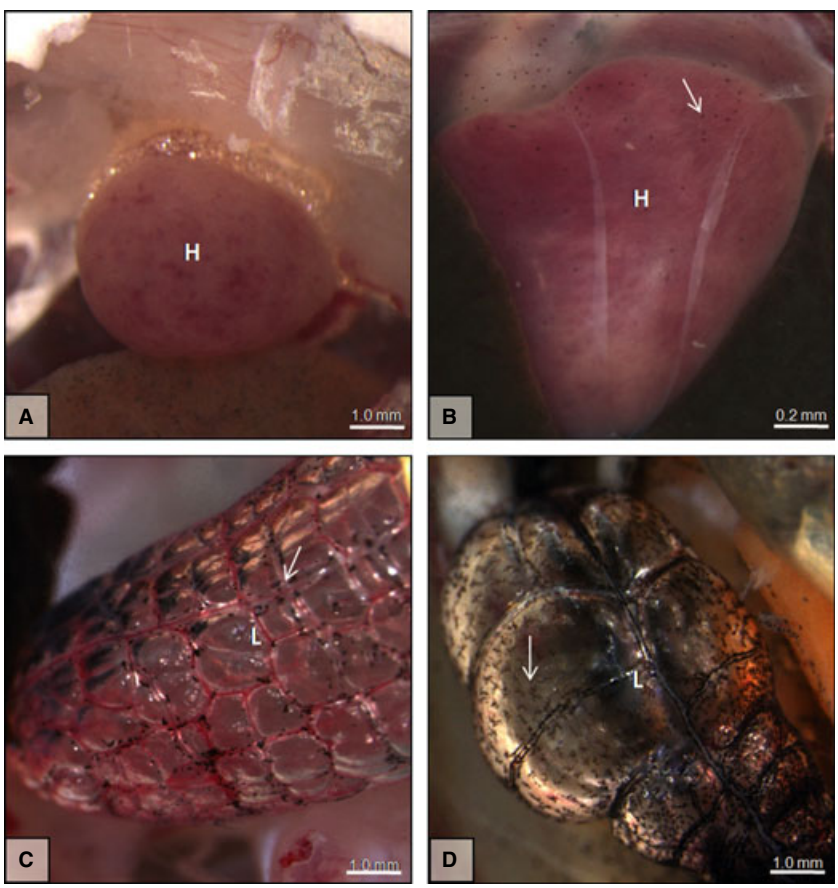

Fig. 3 Organs of the cardio-respiratory system. —A. Heart of *Pseudopaludicola falcipes* without pigmentation. —B. Heart of *Leptodactylus marmoratus* showing a few pigment cells in the pericardium. —C. Lung of *Leptodactylus furnarius* with moderate pigmentation. —D. Lung of *Leptodactylus bokermanni* with moderate amount of pigment. H, Heart; L, Lung. Arrows indicate melanocytes.

some degree of pigmentation (category 1 and 2) on the lungs, except *Haddadus*. We found little pigmentation on the lungs in species of *Ischnocnema*, Leptodactylidae (except for *Leptodactylus furnarius*), Cycloramphidae and Leiuperidae, whereas *Holoaden* and all Hylodidae had a moderate amount (category 2) of pigmentation (except for *Crossodactylus gaudichaudii* with category 1; Fig. 3).

All genera lack pigmentation in the stomach and middle portion of intestine. The species from Terrarana, Leptodactylidae, Cycloramphidae, and Hylodidae lack pigmentation in the rectum (final portion of intestine), whereas *Physalaemus* and *Eupemphix* had little pigmentation (category 1) and *Pseudopaludicola* showed an intense amount (category 3) of pigmentation (Fig. 4). The intestinal mesentery of *Haddadus*, *Ischnocnema*, *Proceratophrys*, and *Hylodes* lack pigmentation, whereas the remaining species have some degree (categories 1 and 2) of pigmentation (Fig. 5A,B). We found a great variation in the pigmentation categories in the parietal peritoneum and associated lumbar nerves of all species (Fig. 5C,D).

Phylogenetic signal and diversity of visceral pigmentation

The diversity of pigmentation categories in each organ/structure is significantly biased towards the root in the heart, testicle, lumbar parietal peritoneum, and lumbar nerve plexus (Fig. 6). The diversity of pigmentation categories is biased towards a single node if distances among species are calculated separately on the heart, testicle, lumbar parietal peritoneum, lumbar nerve plexus, cardiac blood vessels, and kidneys (Fig. 6). The diversity of pigmentation categories is biased towards a few nodes if distances among species are calculated separately on the heart, testes, lumbar parietal peritoneum, renal vessels, and kidneys (Fig. 6). The phylogenetic signal is thus significant in the heart, testes, lumbar parietal peritoneum, and lumbar nerve plexus. The diversity measured on all combined traits (Fig. 6L) had phylogenetic signal biased towards a few close-to-root nodes, with a high contribution of differences in pigmentation categories between Athesphatanura and Cruciabatrachia. The diversity of pigmentation categories seems to be independent from the phylogeny in the pericardium, intestinal mesenterium, and vertebral column (Fig. 6). Additionally, the variation in pigmentation categories is significantly higher close to the tips of the tree in the rectum and lungs of Cruciabatrachia, Hesticobatrachia, and Terrarana, respectively.

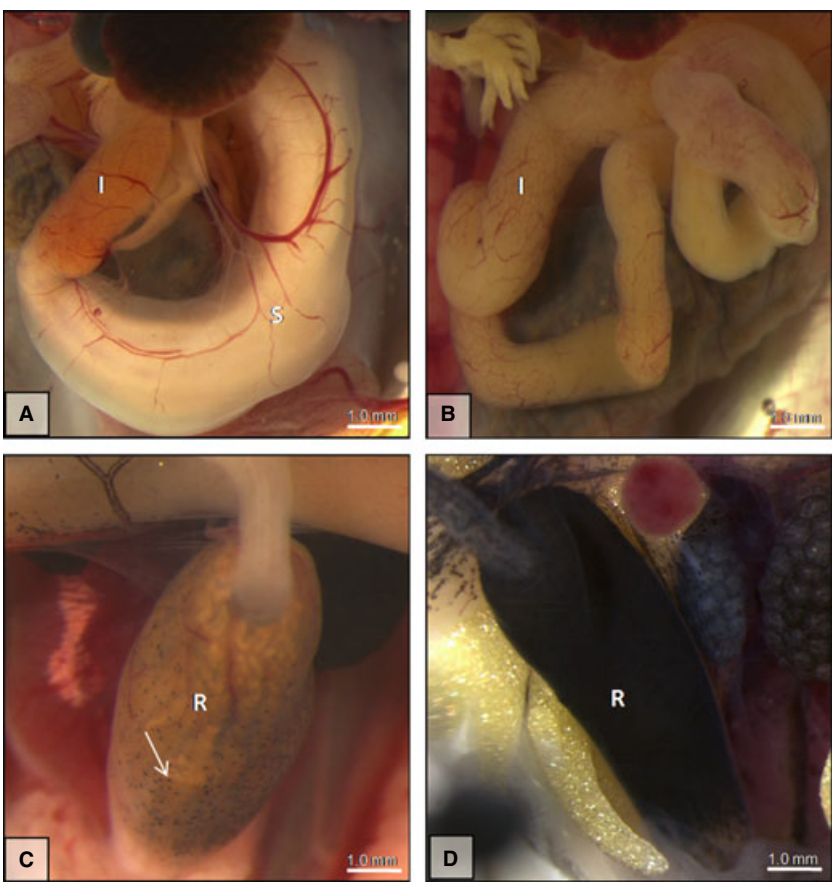

Fig. 4 Digestive system. —A. Stomach of *Leptodactylus furnarius* without pigment. This pattern is observed in all species. —B. Intestine of *Leptodactylus furnarius* without pigmentation. —C. Rectum of *Physalaemus cuvieri* with little pigmentation. —D. Rectum of *Pseudopaludicola falcipes* with an intense pigmentation. I, Intestine; R, Rectum; S, Stomach. Arrow indicates melanocytes.

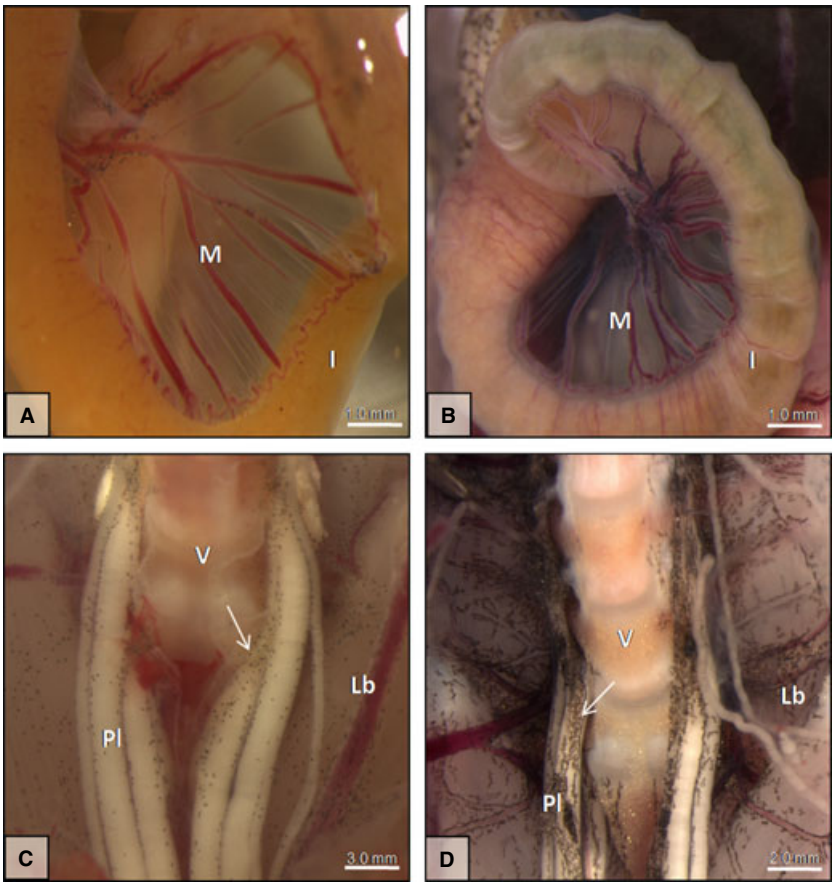

Fig. 5 Peritoneum and nerve plexus. —A. Mesentery of *Proceratophrys boiei* with a few melanocytes. —B. Mesentery of *Eupemphix nattereri* with moderate pigmentation. —C. Lumbar plexus nerve of *Leptodactylus marmoratus* with a few pigment cells. —D. Lumbar nerve plexus and parietal peritoneum of *Physalaemus marmoratus* with moderate pigmentation. I, Intestine; Lb, Lombar Peritoneum; M, Mesentery; Pl, lumbar nerve plexus; V, Vertebral Spine. Arrows indicate melanocytes.

Discussion

We found that the visceral pigmentation shows phylogenetic signal when considering all organs in the 35 species analysed, besides the heart, testes, lumbar parietal peritoneum, and lumbar nerve plexus, when analysed separately. In addition, the pigmentation in the pericardium, intestinal mesenterium, vertebral column, cardiac blood vessels, kidneys, and renal veins seems to vary independently from the phylogeny. Furthermore, the highest diversity of pigmentation in the rectum and lungs was significantly skewed towards the tips of the phylogenetic tree.

Pigment cells are not found in the gonads of the majority of anuran species (e.g., Franco-Belussi *et al.* 2011). In our analysis, pigments were only found on the gonads of species of Calamitophrynia (Leiuperidae + Hylodidae). When present, visceral melanocytes are closely related with the vascular system and associated connective membranes (Oliveira & Zieri 2005). The function of pigmentation in the gonads of anurans is not defined yet, but a recent study demonstrated that the pigmentation increased quickly after administration of LPS from *Escherichia coli* in a leiuperid (Franco-Belusi & Oliveira 2011). Melanic pigments can absorb and neutralize free radicals, cations, and other potentially toxic substances derived from the degradation of cellular material (Zuasti *et al.* 1989). But interestingly, testicular pigmentation only occurred in Calamitophrynia. Other studies reported that some Dendrobatidae also have pigmented testes (Grant *et al.* 2006). We did not include any dendrobatid in our analysis, but it is the sister taxon of Hylodidae (Grant *et al.* 2006) and also included in Calamitophrynia. Therefore, the pigmentation in the testes seems to increase the fitness of individuals by providing protection against bacterial infections, which may explain why it is phylogenetically constrained. Phylogenetic signal may be the result of natural selection forces acting randomly in direction and magnitude or genetic drift (Blomberg & Garland 2002). Similarly, conservative phenotype-dependent responses to selection and the occupation of similar habitats also contribute to the tendency of related species to resemble each other (Harvey & Pagel 1991; Garland *et al.* 2005). Leiuperids and hylodids differ in many life history traits. For example, they occur in different habitats: generally ponds in open areas and along forest streams in eastern South America, respectively, and have also contrasting activity periods: nocturnal and diurnal, respectively. These factors could potentially

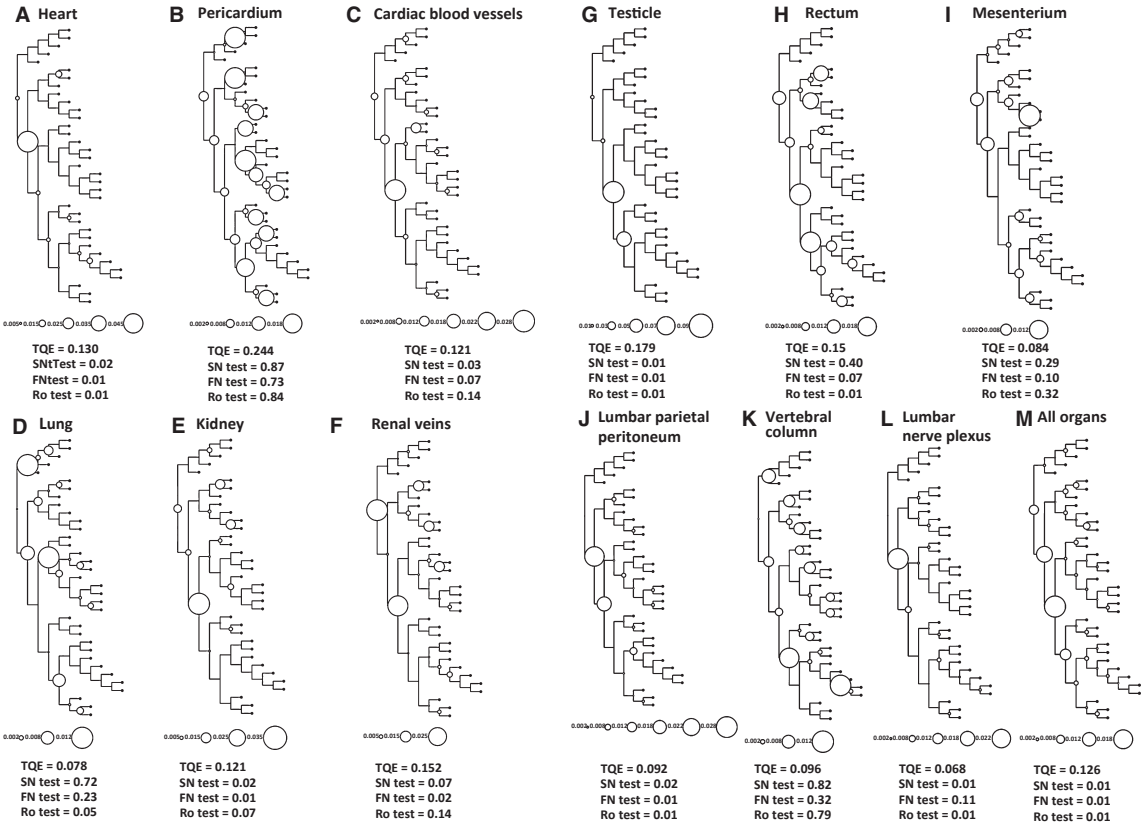

Fig. 6 Decomposition of the diversity of categories of pigmentation among the nodes of the anuran phylogenetic tree. (A–L) Variation of pigmentation categories as measured in a single specified organ. —M. Variation of pigmentation categories as measured considering the pigmentation categories of all organs among the 32 species. Circles at nodes provide the contribution of nodes to trait diversity, scale are given below each tree. The larger the circle, the larger the trait diversity in that node. Results of the permutation tests are given at the bottom of each tree: SN, single-node skewness test; FN, few-nodes skewness test; Ro, root/tips skewness test (two sided). Total quadratic entropy (TQE) represents the overall value of trait diversity, the higher the TQE, the higher the diversity of pigmentation categories in a given organ. See Fig. 1 for node labels. [Correction added on 8 October 2012, after first online publication: Fig. 6 was replaced with the correct test values for section K].

influence melanocyte dynamics and the occurrence of pigmentation on testes. However, experimental studies on the function of the pigmentation on testes are still scarce, and further information is needed to properly infer the underlying process producing this phylogenetic signal.

Conversely, pigmentation on the pericardium, mesenterium, vertebral column, rectum, lungs, cardiac blood vessels, kidneys, and renal veins seems to be more labile. Indeed, the pigmentation in those organs was convergent in our phylogeny. Specifically, the diversity of pigmentation categories was skewed towards the tips of the phylogeny in the rectum and lungs. This can occur when distantly related species evolve towards similar phenotypic traits, because of similar environmental conditions (Pavoine *et al.* 2010). In fact, the pigmentation in these organs seems to have a strong phenotypic plasticity and could change according to the local environment. The visceral pigmentation may vary according to physiological (e.g. age, nutritional status and diseases; Agius & Agbede 1984) and/or environmental factors (e.g. temperature). Temperature is an environmental factor that varies geographically and may change pigmentation and metabolism of the liver (Barni *et al.* 2002). However, in our analysis, pigmentation seems to vary around a pattern that is species specific.

We did not find pigmentation in fatty bodies, urinary bladder, intestine and stomach. A similar pattern is also reported for *Dendropsophus* (Franco-Belussi *et al.* 2011) and *Scinax* (Franco-Belussi *et al.* 2012). The absence of pigmentation may be due to tissue types and embryonic origins, or even cannot be visible on organ's surface.

In the analysis that took into account all organs, the nodes corresponding to Athesphatanura and Cruciabatrachia had the highest diversity of pigmentation categories. The pattern of visceral pigmentation in anurans found in

this study shows that the overall pigmentation of organs is a very conserved trait. From this perspective, our data open a new field of inquiry. Histological analyses of liver, testes and spleen, along with experiments that manipulate factors that influence melanin production and melanocyte migration, should be conducted to explain the possible functions and differential occurrence of these pigment cells.

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References

Agius, C. (1980). Phylogenetic development of melano-macrophage centers in fish. *Journal of Zoology*, *191*, 11–31.

Agius, C. & Agbede, S. A. (1984). An electron microscopical study on the genesis of lipofuscin, melanin and haemosiderin in the haemopoietic tissues of fish. *Journal of Fish Biology*, *24*, 471–488.

Agius, C. & Roberts, R. J. (2003). Melano-macrophage centres and their role in fish pathology. *Journal of Fish Biology*, *26*, 499–509.

Aspengren, S., Hedberg, D., Sköld, H. N. & Wallin, M. (2009). New insights into melanosome transport in vertebrate pigment cells. *International Review of Cell and Molecular Biology*, *272*, 245–302.

Bagnara, J. T. & Matsumoto, J. (2006). Comparative anatomy and physiology of pigment cells in nonmammalian tissues. In J. J. Nordlund, R. E. Boissy, V. J. Hearing, R. A. King & J.-P. Ortonne (Eds) *The Pigmentary System: Physiology and Pathophysiology*. (pp. 9–40). New York: Oxford University Press.

Barni, S., Vaccarone, R., Bertone, V., Fraschini, A., Bernini, F. & Fenoglio, C. (2002). Mechanisms of changes to the liver pigmentary component during the annual cycle (activity and hibernation) of *Rana esculenta* L. *Journal of Anatomy*, *200*, 185–194.

Blomberg, S. P. & Garland, T. (2002). Tempo and mode in evolution: phylogenetic inertia, adaptation and comparative methods. *Journal of Evolutionary Biology*, *15*, 899–910.

Canedo, C. (2008). Revisão taxonômica do gênero *Hylodes* Fitzinger, 1826 (Anura, Hylodidae) [Taxonomic review of the genus *Hylodes* Fitzinger, 1826 (Anura, Hylodidae)]. Unpublished PhD. Diss., Universidade Federal do Rio de Janeiro. [in Portuguese].

Christiansen, J. L., Grzybowski, J. M. & Kodama, R. M. (1996). Melanomacrophage aggregations and their age relationships in the yellow mud turtle, *Kinosternon flavescens* (Kinosternidae). *Pigment Cell Research*, *9*, 185–190.

Cisneros-Heredia, D. F. & McDiarmid, R. W. (2007). Revision of the characters of centrolenidae (Amphibia: Anura: Athesphatanura), with comments on its taxonomy and the description of new taxa of glassfrogs. *Zootaxa*, *1572*, 1–82.

Dray, S. & Dufour, A. B. (2007). The ade4 package: implementing the duality diagram for ecologists. *Journal of Statistical Software*, *22*, 1–20.

Franco-Belusi, L. & Oliveira, C. (2011). Lipopolysaccharides induce changes in the visceral pigmentation of *Eupemphix nattereri* (Anura: Leiuperidae). *Zoology*, *114*, 298–305.

Franco-Belussi, L., Zieri, R., Santos, L. R. S., Moresco, R. M. & Oliveira, C. (2009). Pigmentation in anuran testes: anatomical pattern and variation. *Anatomical Records*, *292*, 178–182.

Franco-Belussi, L., Santos, L. R. S., Zieri, R. & Oliveira, C. (2011). Visceral pigmentation in *Dendropsophus* (Anura: Hylidae): occurrence and comparison. *Zoologische Anzeiger*, *250*, 102–110.

Franco-Belussi, L., Santos, L. R. S., Zieri, R. & Oliveira, C. (2012). Visceral pigmentation in three species of the genus *Scinax* (Anura: Hylidae): distinct morphological pattern. *Anatomical Records*, *295*, 298–306.

Frost, D. R., Grant, T., Faivovich, J., Bain, R. H., Haas, A., Haddad, C. F. B., De Sá, R. O., Channing, A., Wilkinson, M., Donnelan, S. C., Raxworthy, C. J., Campbell, J. A., Blotto, B. L., Moler, P., Drewes, R. C., Nussbaum, R. A., Lynch, J. D., Green, D. M. & Wheeler, W. C. (2006). The amphibian tree of life. *Bulletin of the American Museum of Natural History*, *297*, 1–370.

Gallone, A., Guida, G., Maida, I. & Cícero, R. (2002). Spleen and liver pigmented macrophages of *Rana esculenta* L. A new melanogenic system? *Pigment Cell Research*, *15*, 32–40.

Garland, T., Harvey, P. H. & Ives, A. R. (1992). Procedures for the analysis of comparative data using phylogenetically independent contrasts. *Systematic Biology*, *41*, 18–32.

Garland, T., Bennett, A. F. & Rezende, E. L. (2005). Phylogenetic approaches in comparative physiology. *Journal of Experimental Biology*, *208*, 3015–3035.

Grant, T., Frost, D. R., Caldwell, J. P., Gagliardo, R., Haddad, C. F. B., Kok, P. J. R., Means, D. B., Noonan, B. P., Schargel, W. E. & Wheeler, W. C. (2006). Phylogenetic systematics of dart-poison frogs and their relatives (Amphibia: Athesphatanura: Dendrobatidae). *Bulletin of the American Museum of Natural History*, *299*, 1–262.

Harvey, P. H. & Pagel, M. D. (1991). *The Comparative Method in Evolutionary Biology*. Oxford: Oxford University Press.

Hedges, S. B., Duellman, W. E. & Heinicke, M. P. (2008). New World direct-developing frogs (Anura: Terrarana): Molecular phylogeny, classification, biogeography, and conservation. *Zootaxa*, *1737*, 1–182.

Johnson, J. C., Schwiesow, T., Ekwall, A. K. & Christiansen, J. L. (1999). Reptilian melanomacrophages function under conditions of hypothermia: observations on phagocytic behavior. *Pigment Cell Research*, *12*, 376–382.

Maddison, W. P. & Maddison, D. R. 2010. Mesquite: a modular system for evolutionary analysis. Ver. 2.74. Available via http://mesquiteproject.org.

McDiarmid, R. W. (1971). Comparative morphology and evolution of frogs of the Neotropical genera *Atelopus*, *Dendrophryniscus*, *Melanophryniscus* and *Oreophrynella*. *Bulletin of the Los Angeles County Museum of Science*, *12*, 1–66.

Moresco, R. M. & Oliveira, C. (2009). A comparative study of the extracutaneous pigmentary system in three anuran amphibian species evaluated during the breeding season. *South American Journal of Herpetology*, *4*, 1–8.

Oliveira, C. & Zieri, R. (2005). Pigmentação testicular em *Physalaemus nattereri* (Steindachner) (Amphibia, Anura) com observações anatômicas sobre o sistema pigmentar extracutâneo. *Revista Brasileira de Zoologia*, *22*, 454–460.

Paradis, E., Claude, J. & Strimmer, K. (2004). APE: analyses of phylogenetics and evolution in R language. *Bioinformatics*, *20*, 289–290.

Pavoine, S., Vallet, J., Dufour, A.-B., Gachet, S. & Daniel, H. (2009). On the challenge of treating various types of variables: application for improving the measurement of functional diversity. *Oikos*, *118*, 391–402.

Pavoine, S., Baguette, M. & Bonsal, M. B. (2010). Decomposition of trait diversity among the nodes of a phylogenetic tree. *Ecological Monographs*, *80*, 485–507.

Pederzoli, A. & Trevisan, P. (1990). Pigmentary system of the adult alpine salamander *Salamandra atra aurorae*. *Pigment Cell Research*, *3*, 80–89.

Ponssa, M. L. (2008). Cladistic analysis and osteological descriptions of the frog species in the *Leptodactylus fuscus* species group (Anura, Leptodactylidae). *Journal of Zoological Systematics and Evolutionary Research*, *46*, 249–266.

Prelovisek, P. & Bulog, B. (2003). Biogenesis of melanosomes in Kupffer cells of *Proteus anguinus* (Urodela, Amphibia). *Pigment Cell Research*, *16*, 345–350.

R Development Core Team. (2011). R: A language and environment for statistical computing. *Ver. 2.12.2*. Vienna, Austria: R Foundation for Statistical Computing. Available via http://www.R-project.org/.

Rund, C. R., Christiansen, J. L. & Johnson, J. C. (1998). In vitro culture of melanomacrophages from the spleen and liver of turtles: comments on melanomacrophage morphology. *Pigment Cell Research*, *11*, 114–119.

Tárano, Z. & Ryan, M. (2002). No pre-existing biases for heterospecific call traits in the frog. *Animal Behaviour*, *64*, 599–607.

Wiens, J. J., Kuczynski, C. A., Hua, X. & Moen, D. S. (2010). An expanded phylogeny of treefrogs (Hylidae) based on nuclear and mitochondrial sequence data. *Molecular Phylogenetics and Evolution*, *55*, 871–882.

Zieri, R., Taboga, S. R. & Oliveira, C. (2007). Melanocytes in the testes of *Eupemphix nattereri* (Anura, Leiuperidae): histological, stereological and ultrastructural aspects. *Anatomical Records*, *290*, 795–800.

Zuasti, A., Jará, J. R., Ferrer, C. & Solano, F. (1989). Occurrence of melanin granules and melanosynthesis in the kidney of *Sparus auratus*. *Pigment Cell Research*, *2*, 93–99.

Zuasti, A., Jiménez-Cervantes, C., García-Borrón, J. C. & Ferrer, C. (1998). The melanogenic system of *Xenopus laevis*. *Archives of Histology and Cytology*, *61*, 305–316.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Geographic coordinates of site collection.

Appendix S2. Material examined.

Table S1. Categories of pigmentation recorded in 12 organs of anuran species.

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