

Evolution of erythrocyte morphology in amphibians (Amphibia: Anura)

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ABSTRACT. We compared the morphology of the erythrocytes of five anurans, two toad species – *Bufo gargarizans* (Cantor, 1842) and *Duttaphrynus melanostictus* (Schneider, 1799) and three frog species – *Fejervarya limnocharis* (Gravenhorst, 1829), *Microhyla ornata* (Duméril & Bibron, 1841), and *Rana zhenhaiensis* (Ye, Fei & Matsui, 1995). We then reconstructed the ancestral state of erythrocyte size (ES) and nuclear size (NS) in amphibians based on a molecular tree. Nine morphological traits of erythrocytes were all significantly different among the five species. The results of principal component analysis showed that the first component (49.1% of variance explained) had a high positive loading for erythrocyte length, nuclear length, NS and ratio of erythrocyte length/erythrocyte width; the second axis (28.5% of variance explained) mainly represented erythrocyte width and ES. Phylogenetic generalized least squares analysis showed that the relationship between NS and ES was not affected by phylogenetic relationships although there was a significant linear relationship between these two variables. These results suggested that (1) the nine morphological traits of erythrocytes in the five anuran species were species-specific; (2) in amphibians, larger erythrocytes generally had larger nuclei.

KEY WORDS. Amphibia; ancestral state reconstruction; erythrocyte size; morphological comparison; nucleus size.

Amphibians have evolved an array of adaptive structures and mechanisms to cope with environmental changes that result from their life histories, which involves a transition from water to land (FOXON 1964, WOJTAŚZEK & ADAMOWICZ 2003). One of these adaptations is unusually large erythrocytes, compared to other vertebrates (WOJTAŚZEK & ADAMOWICZ 2003). Most previous hematological studies on amphibians counted blood cells (ARSERIM & MERMER 2008, BARAQUET et al. 2013, DÖNMEZ et al. 2009) and measured their dimensions (DAS & MAHAPATRA 2012, MA et al. 2003, MAHAPATRA et al. 2012). Both intrinsic (e.g., species, sex, age and physiological state, ATTADEMO et al. 2014, HOTA et al. 2013, LAJMANOVICHA et al. 2014) and extrinsic factors (e.g., temperature and habitat, LOPEZ-OLIVERA et al. 2003) can affect blood parameters (e.g., the blood volume, hematocrit value, fragility and pH value; see ROUF 1969). For example, the number of erythrocytes differ not only among individuals within population and interspecies, but also with body mass, age and sex of individuals (ARIKAN et al. 2003, BANERJEE 1988, CHOUBEY et al. 1986, DAS & MAHAPATRA 2014), habitat conditions (ROMANOVA & EGORIKHINA 2006), and season (SAMANTARAY 1985, WOJTAŚZEK et al. 1997). Therefore, investigating blood parameters in amphibians can facilitate evaluations of the physiological and health levels of populations. These in turn may be used as bio-indicators of environmental conditions, since these parameters exhibit significant variability when individuals inhabit unstable environments (BARNI et al. 2007, DICKINSON et al. 2002).

Despite the fact that haematological profiles have been reported for many amphibians, reconstruction of the evolutionary history of traits of amphibian erythrocytes is rare. Here, we compare the morphology of the erythrocytes of five sympatric anuran species, including two toads – *Bufo gargarizans* (Cantor, 1842), *Duttaphrynus melanostictus* (Schneider, 1799) – and three frogs – *Fejervarya limnocharis* (Gravenhorst, 1829), *Microhyla ornata* (Duméril & Bibron, 1841), and *Rana zhenhaiensis* (Ye, Fei & Matsui, 1995) –, sampled from natural populations in Lishui, Zhejiang Province, China. These results were combined with recently published accounts on erythrocyte traits (erythrocyte size and nucleus sizes) from three Orders (Gymnophiona, Caudata and Anura) of Amphibia to allow reconstruction of ancestral states and to examine their phylogenetic relationships.

MATERIAL AND METHODS

From June to August, 2013, we captured 10 adults of each of the following species, *B. gargarizans*, *D. melanostictus*, *F. limnocharis*, *M. ornata* and *R. zhenhaiensis*, from field of Lishui, Zhejiang Province, China (28°27'N, 119°53'E). Their snout-vent length (SVL) was 57.5 ± 4.6 , 52.5 ± 2.4 , 39.2 ± 2.1 , 28.6 ± 0.6 and 41.4 ± 1.9 mm, respectively. All individuals were transported to the Herpetological Laboratory of the Lishui University (HLLSU), where they were identified and used for

preparation of blood smears. Vouchers of *B. gargarizans* are under accession numbers HLLSU-2013071001 to HLLSU-2013071010; *D. melanostictus* from HLLSU-2013072001 to HLLSU-2013072010; *F. limnocharis* from HLLSU-2013073001 to HLLSU-2013073010; *M. ornata* from HLLSU-2013074001 to HLLSU-2013074010; and *R. zhenhaiensis* from HLLSU-2013075001 to HLLSU-2013075010.

According to the methods of SALAMAT et al. (2013), blood smears were obtained by puncturing the heart of each individual. Blood smears were air-dried, fixed in methanol and stained with 10% Giemsa (diluted 1:10 in PBS, pH = 6.8) for 15 minutes and washed in running tap water for 2 minutes. Photos of 100 erythrocytes were taken randomly using a camera attached to a microscope. The morphological traits of erythrocytes, including erythrocyte length (EL) and width (EW), nuclear length (NL) and nuclear width (NW), were measured using ImageJ 1.43 software. Subsequently, erythrocyte size (ES) and nuclear sizes (NS) were calculated as $ES = [(NL \times NW \times \delta) / 4, \mu m^2]$ and $NS = [(NL \times NW \times \delta) / 4, \mu m^2]$, respectively. Erythrocyte and nuclear shape were compared with EL/EW and NL/NW ratios and nucleocytoplasmic ratio with NS/ES ratio (SALAMAT et al. 2013, SEVINÇ et al. 2004).

Prior to statistics, all variables were tested for normality and homogeneity. We used linear regression, one-way ANOVA, principal components analysis and Tukey's *post hoc* comparisons to analyze the data. Throughout this paper, values are presented as mean \pm SE, and the significance level is set at $\alpha = 0.05$. All statistical analyses were performed with the Statistica software (version 6.0 for PC, Tulsa, OK, USA).

The tests detailed previously were carried out using the topology including all collected amphibian species from Gymnophiona, Caudata and Anura. This topology of species was based on proximate phylogenetic correlation assembled from PYRON & WIENS (2011). We drew the tree and reconstructed the evolutionary history of ES and NS of amphibians by parsimony ancestral states in the program Mesquite 2.75 (MADDISON & MADDISON 2011). Because branch lengths lacked divergence time and genetic distance and any other metric proportional to the expected variance for the evolution of each analyzed trait were unavailable, we arbitrarily set the initial branch length to 1, which is appropriate for a speciation model of evolution (MARTINS & GARLAND 1991).

We used ordinary least squares (OLS) and phylogenetic general least squares (PGLS) regressions to estimate the slope for all conventional analyses. These two analyses were implemented in R 2.15.3 (R Development Core Team 2013), using the RMS (HARRELL 2012) and Caper (ORME et al. 2012) packages. We used PGLS regression to examine the relationship between NS and ES in amphibians. The PGLS analyses incorporate phylogenetic information into generalized linear models. They offer a powerful method for analyzing continuous data, and have been applied to estimate the evolutionary model and the relationships among the traits of interest

(BARROS et al. 2011, WARNE & CHARNOV 2008). In PGLS, the strength and type of the phylogenetic signal in the data matrix can be accounted for by adjusting branch length transformations, which show the degree of phylogenetic correlation in the data. In this study, we used λ from a maximum likelihood approach to evaluate the phylogenetic effects ($\lambda = 0$ indicates no phylogenetic effect, and $\lambda = 1$ indicates the strongest phylogenetic effect equivalent to that expected under the Brownian motion model). We used the Akaike Information Criterion (AIC) to estimate merits and drawbacks of the models tested. The best model has the lowest AIC. The model with better \hat{u} can be determined by a maximum-likelihood ratio test in which twice the difference in the natural log of the maximum likelihoods (LnL) of OLS and PGLS models will be distributed approximately as a χ^2 with degrees of freedom equal to the difference in the number of parameters estimated in the two models (WARNE & CHARNOV 2008).

RESULTS

Morphological traits of erythrocyte

The erythrocytes of the five anuran species are oval, and their morphological traits are depicted in Table 1. The results of One-way ANOVA indicate that the nine variables of erythrocyte morphology were all significantly different among the five species (Table 1). We found that (1) the mean values of EL and ratio of EL/EW and NL/NW were largest in *D. melanostictus* and smallest in *F. limnocharis*, the mean value of EW was larger in *B. gargarizans* than in the other species, the mean value of ES was larger in *B. gargarizans* and *D. melanostictus* than in the other species; (2) the mean values of NL and NS were largest in *D. melanostictus* and smallest in *F. limnocharis* and *M. ornata*, the mean value of NW was largest in *B. gargarizans* and smallest in *M. ornata*; (3) the mean value of nucleocytoplasmic ratio was largest in *D. melanostictus* and *R. zhenhaiensis* and smallest in *M. ornata* (Table 1). The variable coefficient was significantly different in NW ($F_{4,45} = 4.59$, $p < 0.01$, Fig. 1), but not in other erythrocyte morphological traits among the five species (all $p > 0.05$). The variable coefficient of NW was significantly larger in *D. melanostictus* and *R. zhenhaiensis* than in *B. gargarizans*, with *F. limnocharis* and *M. ornata* in between (Fig. 1).

A principal component analysis resolved two components (eigenvalues ≥ 1) from nine variables of erythrocyte morphology, accounting for 77.6% of the variation in the original data (Table 2). The first component (49.1% of variance explained) had high positive loading for EL, NL, NS and ratio of EL/EW. The second axis (28.5% of variance explained) mainly represented EW and ES. Erythrocyte morphology differed significantly among the five anuran species in their scores on the first axis ($F_{4,45} = 45.95$, $p < 0.0001$; BG^b , DM^a , FL^c , MO^c , RZ^b , Tukey's test; $a > b > c$) and the second axis ($F_{4,45} = 7.38$, $p < 0.001$; BG^a , DM^b , FL^b , MO^b , RZ^b , Tukey's test; $a > b$) (Fig. 2).

Table 1. Descriptive statistics, expressed as mean ± SE and range, for morphological traits of erythrocytes in five anuran species in Lishui, China, and results of one-way ANOVA for each variable of erythrocytes with species as the factor.

Variables	<i>B. gargarizans</i>	<i>D. melanostictus</i>	<i>F. limnocharis</i>	<i>M. ornata</i>	<i>R. zhenhaiensis</i>	Results of statistical analyses
Erythrocyte length (EL, μm)	28.17 ± 0.46	30.02 ± 0.90	23.92 ± 0.22	25.20 ± 0.22	26.96 ± 0.37	F4, 45 = 23.02, p < 0.0001
	25.20 – 30.27	26.79 – 36.38	22.56 – 24.82	24.20 – 26.19	25.31 – 29.16	BG ^{ab} , DM ^a , FL ^d , MO ^{cd} , RZ ^{bc}
Erythrocyte width (EW, μm)	20.18 ± 0.50	18.30 ± 0.48	17.71 ± 0.28	18.06 ± 0.21	18.02 ± 0.22	F4, 45 = 7.50, p < 0.001
	18.30 – 22.28	15.40 – 21.16	16.65 – 19.70	17.40 – 19.37	17.34 – 19.61	BG ^a , DM ^b , FL ^b , MO ^b , RZ ^b
Ratio of EL/EW	1.41 ± 0.03	1.66 ± 0.05	1.37 ± 0.03	1.40 ± 0.02	1.51 ± 0.04	F4, 45 = 13.23, p < 0.0001
	1.26 – 1.56	1.53 – 1.96	1.25 – 1.50	1.31 – 1.50	1.35 – 1.70	BG ^{bc} , DM ^a , FL ^c , MO ^{bc} , RZ ^b
Erythrocyte size (ES, μm ²)	447.56 ± 14.87	433.97 ± 21.79	333.18 ± 6.70	358.10 ± 5.99	382.29 ± 4.24	F4, 45 = 15.01, p < 0.0001
	363.21 – 516.81	338.67 – 560.78	305.69 – 379.34	333.67 – 387.55	363.56 – 404.21	BG ^a , DM ^a , FL ^b , MO ^b , RZ ^b
Nucleus length (NL, μm)	10.49 ± 0.28	12.54 ± 0.32	9.20 ± 0.15	9.57 ± 0.15	11.32 ± 0.20	F4, 45 = 34.31, p < 0.0001
	8.70 – 11.71	11.00 – 14.74	8.62 – 9.89	8.90 – 10.38	10.28 – 12.47	BG ^b , DM ^a , FL ^c , MO ^c , RZ ^b
Nucleus width (NW, μm)	6.46 ± 0.19	6.30 ± 0.16	5.88 ± 0.112	5.36 ± 0.09	6.18 ± 0.12	F4, 45 = 9.67, p < 0.0001
	5.90 – 7.92	5.67 – 7.50	5.36 – 6.51	4.89 – 5.74	5.71 – 6.82	BG ^a , DM ^{ab} , FL ^{bc} , MO ^c , RZ ^{ab}
Ratio of NL/NW	1.65 ± 0.04	2.04 ± 0.05	1.60 ± 0.03	1.82 ± 0.04	1.88 ± 0.05	F4, 45 = 16.07, p < 0.0001
	1.44 – 1.81	1.80 – 2.37	1.49 – 1.85	1.57 – 2.02	1.53 – 2.19	BG ^{cd} , DM ^a , FL ^d , MO ^{bc} , RZ ^{ab}
Nucleus size (NS, μm ²)	53.59 ± 2.74	62.36 ± 2.70	42.62 ± 1.32	40.41 ± 0.94	55.01 ± 1.24	F4, 45 = 22.02, p < 0.0001
	40.34 – 69.99	50.57 – 77.05	38.23 – 50.74	36.33 – 44.34	49.30 – 60.38	BG ^b , DM ^a , FL ^c , MO ^c , RZ ^{ab}
Ratio of NS/ES	0.12 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.11 ± 0.00	0.15 ± 0.00	F4, 45 = 5.03, p < 0.01
	0.09 – 0.15	0.12 – 0.22	0.11 – 0.17	0.09 – 0.13	0.13 – 0.17	BG ^{ab} , DM ^a , FL ^{ab} , MO ^b , RZ ^a

BG: *B. gargarizans*, DM: *D. melanostictus*, FL: *F. limnocharis*, MO: *M. ornata*, RZ: *R. zhenhaiensis*. Means with different superscripts differ significantly (Tukey's post hoc test α = 0.05, a > b > c).

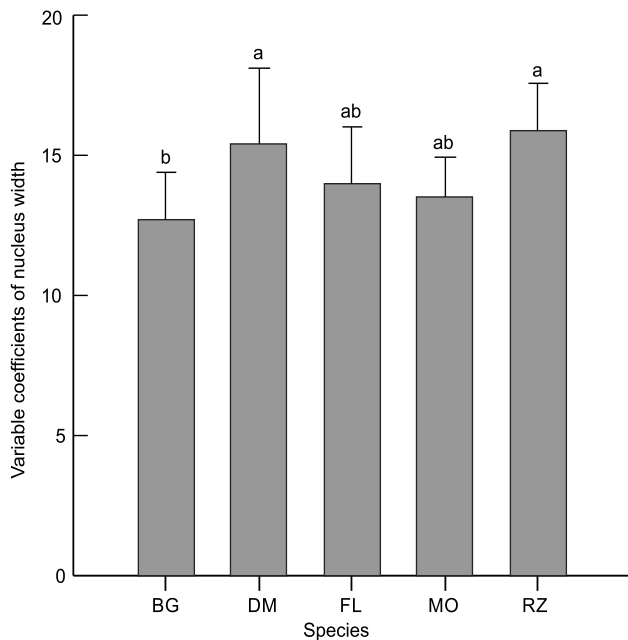


Figure 1 The variable coefficients of nucleus width of five species. BG: *B. gargarizans*, DM: *D. melanostictus*, FL: *F. Limnocharis*, MO: *M. Ornata*, RZ: *R. zhenhaiensis*. Different superscripts indicate significant difference (Tukey's post hoc test, α = 0.05, a > b).

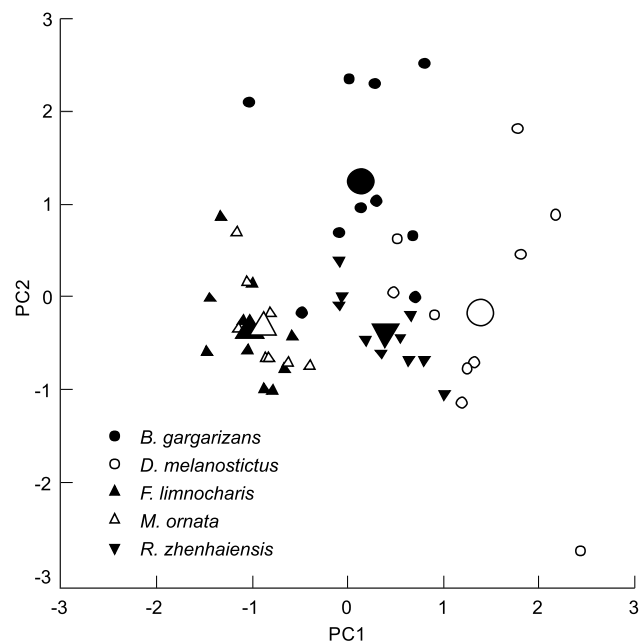


Figure 2 Positions of five anuran species in the space defined by the first two axes of a principal component analysis based on nine variables of erythrocyte morphology. Enlarged symbols show the mean values of scores on the two axes.

Table 2. Loading of the first two axes of a principal component analysis on nine variables of erythrocyte morphology.

	Factor loading	
	PC 1	PC 2
Erythrocyte length (EL)	0.789*	0.403
Erythrocyte width (EW)	0.052	0.974*
Ratio of EL/EW	0.776*	-0.354
Erythrocyte size (ES)	0.549	0.789*
Nucleus length (NL)	0.967*	-0.184
Nucleus width (NW)	0.592	0.377
Ratio of NL/NW	0.632	-0.490
Nucleus size (NS)	0.924*	0.059
Ratio of NS/ES	0.592	-0.535
Variance explained	49.1%	28.5%

* Variables with the main contribution to each factor.

Variability of erythrocyte morphology in amphibians

We assembled published data with our own data on ES, NS for amphibians (Appendix 1). Data from 109 species of amphibians show that mean ES ranged from 119.4 μm^2 to 2649 μm^2 (N = 108) and the mean NS ranged from 18.1 μm^2 to 517 μm^2 (N = 71). Our reconstruction of evolutionary changes in these variables shows strong positive correlations between NS and ES in amphibians (Fig. 3). The ES and the NS were both significantly different among the three orders of Amphibia (Both $p < 0.01$). Both traits were greater in Caudata than in Gymnophiona and Anura (Fig. 4). Table 3 summarizes the relationships between NS and ES in amphibians according to OLS and PGLS analyses. Mean NS was positively correlated with mean ES in both the OLS and PGLS model (Fig. 5, Table 3). PGLS analysis showed that phylogenetic relationships did not affect NS and ES ($\lambda = 0$) although there were significant linear relationship between NS and ES (Fig. 5, Table 3).

DISCUSSION

Hematological parameters vary significantly among amphibian species (ARIKAN et al. 2010, BARAQUET et al. 2013). For example, OLMO & MORESCALCH (1975) documented that interspecific variation is significant in the volume of erythrocytes and nuclei of seven Plethodontidae (Amphibia: Urodela) species. In our study, we found species-specificity in nine morphological traits of erythrocytes in the five anuran species. In general, variation in the morphological traits of erythrocytes in toads (*B. gargarizans* and *D. melanostictus*) was larger than in frogs (*F. limnocharis*, *M. ornata*, and *R. zhenhaiensis*). Furthermore, GÜL et al. (2011) found that the number of erythrocytes is also different in toads and frogs. The mean value of erythrocyte counts was greater in toads (*Pseudepidalea viridis* and *Pelobates syriacus*; $n = 850530/\mu\text{l}$; GÜL et al. 2011) than in frogs

(*Hyla arborea*, *Rana dalmatina* and *Pelophylax ridibundus*; $n = 741332/\mu\text{l}$; GÜL et al. 2011). The morphological traits of erythrocytes were different between toads and frogs and this difference may be attributed to the following three reasons. First, the different habitats of toads and frogs may affect the variability of erythrocyte morphology (ROMANOVA & EGORIKHINA 2006). Toads mainly inhabit terrestrial environments, whereas frogs inhabit semi-aquatic or aquatic environments (GÜL et al. 2011). The terrestrial habitat has selected a series of adaptive structures and mechanisms in frogs that have enabled them to function under conditions of changeable humidity and partial oxygen pressure in terrestrial environments (BARAQUET et al. 2013, FOXON 1964, WOJTASZEK & ADAMOWICZ 2003). Second, erythrocyte size may be dependent on the level of metabolism in vertebrates (WOJTASZEK & ADAMOWICZ 2003). Through our field investigation, we found that two toad species (*B. gargarizans* and *D. melanostictus*) that crawl slowly and have lower metabolic rate consume less energy than the other three species that are agile in their jumping and swimming activity. Therefore, erythrocyte morphology may have evolved to adapt to various levels of activity in vertebrates. Finally, the body size of animals influences erythrocyte size (FRÝDLOVÁ et al. 2012). In our study, the means obtained for the snout-vent length of two toad species (*B. gargarizans* and *D. melanostictus*) were greater than the means of the other three frog species (*F. limnocharis*, *M. ornata*, and *R. zhenhaiensis*); this distinction was consistent with erythrocyte size. This finding is logical from a physiological point of view, since smaller erythrocytes have relatively larger surface areas, and therefore, exchange oxygen more efficiently. It is reasonable to expect that erythrocyte size is adjusted to the actual mass-specific metabolic rate that gradually decreases during ontogenetic growth (CLEMENTE et al. 2009, SMITH et al. 2008).

The morphological traits of erythrocytes are variable among individuals of a species. HOTA et al. (2013) found that the erythrocyte profile of *M. ornata* is variable during the larval and adult periods. The coefficient of variation (CV) indicated that the level of difference varied among individuals in the same species. Our results showed that the mean values of CV of NW in *D. melanostictus* and *R. zhenhaiensis* were greater than in *B. gargarizans* (Fig. 1). These differences may be attributed to the different habitats (RUIZ et al. 1983, SALAMAT et al. 2013) and/or variable activity levels (ALLANDER & FRY 2008, SYKES & KLAPHAKE 2008). Moreover, erythrocyte morphology varies with geography in amphibian species. We pooled erythrocyte size data on *B. gargarizans* from previous studies and our current study, and found that the erythrocyte profile (EL and EW) differed among three populations from different sampling locations (GUO et al. 2002, ZHOU et al. 2011). The EL and EW of *B. gargarizans* in Lishui (28°27'N, 119°53'E) were greater than in Chongqing (29°81'N, 106°39'E, GUO et al. 2002), which were greater than in Shuicheng (26.58°N, 104°82'E, ZHOU et al. 2011). However, erythrocyte shape (ratio of EL/EW) showed an op-

Table 3. Regressions of nuclear sizes (NS) on erythrocyte size (ES) in amphibians based on ordinary least squares (OLS) regression and phylogenetic generalized least squares (PGLS) regression. Significant associations between variables are shown in bold.

Models	N	Slope	Elevation	r ²	ln likelihood	AIC	λ	F	df	p
OLS NS vs. ES	70	0.183 ± 0.009	-1.932 ± 8.638	0.857	-367.534	741.068	-	407.44	1, 68	< 0.0001
PGLS NS vs. ES	70	0.185 ± 0.009	-2.100 ± 9.148	0.850	-371.570	751.140	0	390.9	1, 68	< 0.0001

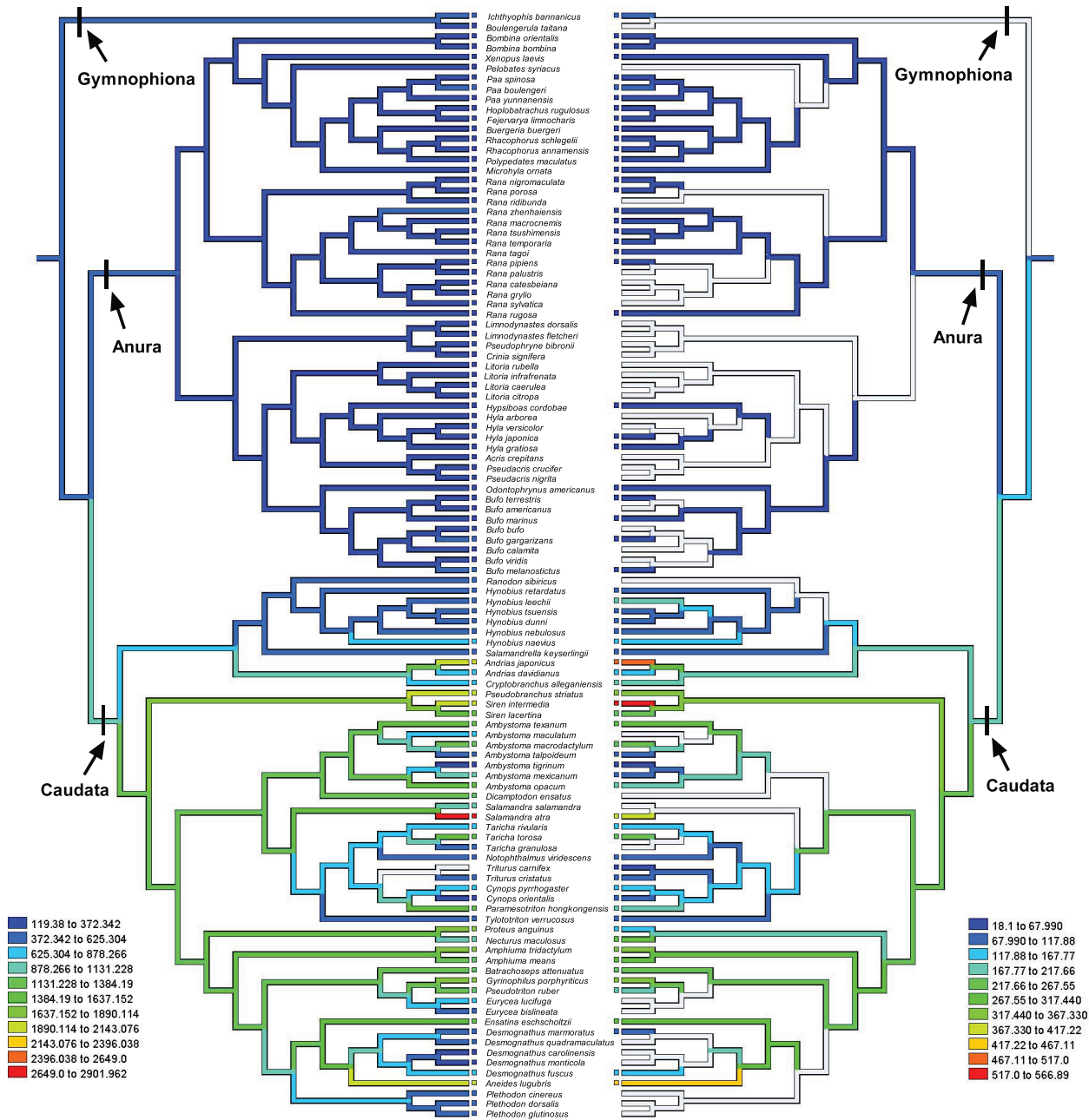


Figure 3 Mirror trees of the evolutionary history reconstructions of erythrocyte size (left side) and nucleus size (right side) of amphibians (blank branch is lack of data), according to phylogenetic hypotheses of PYRON & WIENS (2011).

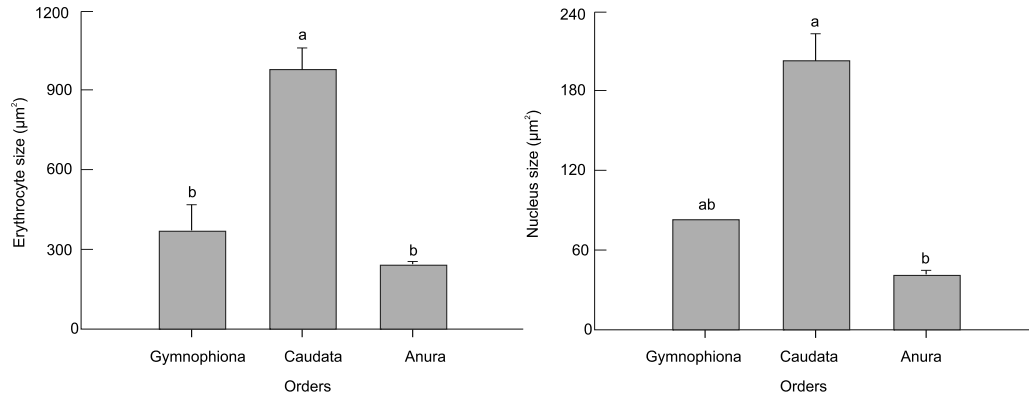


Figure 4 The erythrocyte size and nucleus size of different orders in Amphibia. Different superscripts indicate significant difference (Tukey's *post hoc* test, $\alpha = 0.05$, $a > b$).

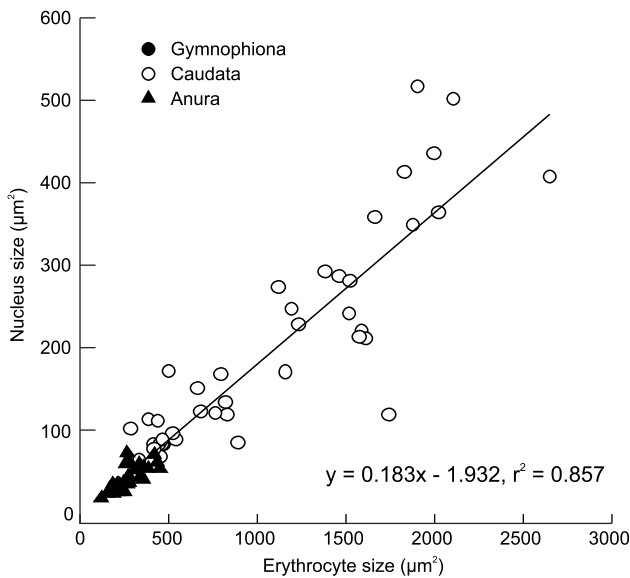


Figure 5 Ordinary least squares (OLS) regression of nucleus size on erythrocyte size in amphibians. Regression equation and coefficient are given in the figure.

posite trend in the three populations (Lishui: 1.41; Chongqing: 1.50; Shuicheng: 1.57). These geographic variations in erythrocyte morphological traits may be associated with differences in latitude, elevation, or environmental and climatic variables in different sampling locations (GOODMAN et al. 2013). Previous studies have found that morphological variation in the erythrocyte traits of amphibians was greater than that in mammals, birds and reptiles (DUELLMAN & TRUEB 1994, GREGORY 2001a, LI et al. 1989, SEVINÇ et al. 2004, WU et al. 1998). Erythrocyte size in animals is generally negatively correlated with the place where the species appears in an evolutionary tree (whether more basal or more apical, indicating a more recent divergence in time).

However, within Amphibia, species of Gymnophiona have larger erythrocytes than the other species of Caudata and Anura (SZARSKI & CZOPEK 1966). Similar results were found in our study, indicating that the ES and NS in Anura were the smallest among the three orders, but the ES and NS in Caudata were larger than in Gymnophiona (Fig. 4). This may be the result of insufficient data from a limited number of species (only two species in Gymnophiona) collected from previous reports. Likewise, we still could predict that erythrocyte size in Caudata and Gymnophiona evolved to be larger than that in Anura.

PGLS analysis to recover phylogenetic relationships, showed that these did not affect NS and ES, although there were significant linear relationships between NS and ES (Fig. 5, Table 3). Similar results were found in 24 species of salamanders, which indicate that the more standard relationships between cell size and NS are similarly significant whether phylogenetically-corrected or not (GREGORY 2003). The increase in erythrocyte size may occur adaptively (e.g., to provide more efficient metabolism), and is correlated with an increase in genome size (GREGORY 2001b). MUELLER et al (2008) demonstrated that positive direct correlations between genome size and NS are significant in the salamander family Plethodontidae. Moreover, the “nucleoskeletal” theory emphasizes the need for a balanced ratio of nuclear and cytoplasmic volumes for the maintenance of cell growth and division, and the key importance of cell size to organismal fitness (GREGORY 2003).

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Appendix 1. Erythrocyte size and erythrocyte nuclei size in Amphibia (μm^2).

	Erythrocyte size	Nucleus size	Reference
Gymnophiona			
Caeciliidae			
<i>Boulengerula taitana</i>	270.77	–	GREGORY (2003)
Ichthyophiidae			
<i>Ichthyophis bannanicus</i>	467.72	83.41	Li et al. (2009)
Caudata			
Ambystomatidae			
<i>Ambystoma macrodactylum</i>	1192.00	247.00	OLMO & MORESCALCH (1975)
<i>Ambystoma maculatum</i>	711.42	–	GREGORY (2003)
<i>Ambystoma mexicanum</i>	887.74	84.78	GREGORY (2003)
<i>Ambystoma opacum</i>	1611.00	212.00	OLMO & MORESCALCH (1975)
<i>Ambystoma talpoideum</i>	538	90.00	OLMO & MORESCALCH (1975)
<i>Ambystoma texanum</i>	1462.00	287.00	OLMO & MORESCALCH (1975)
<i>Ambystoma tigrinum</i>	333.25	64.32	GREGORY (2003)
Amphiumidae			
<i>Amphiuma means</i>	1383.48	292.00	MONNICKENDAM & BALLS (1973)
<i>Amphiuma tridactylum</i>	1877.09	348.34	HARTMAN & LESSLER (1964)
Cryptobranchidae			
<i>Andrias davidianus</i>	821.92	133.52	Li et al. (1989)
<i>Andrias japonicus</i>	2105.00	502.00	OLMO & MORESCALCH (1975)
<i>Cryptobranchus alleganiensis</i>	791.98	168.08	GREGORY (2003)
Dicamptodontidae			
<i>Dicamptodon ensatus</i>	1182.83	–	GREGORY (2003)
Hynobiidae			
<i>Hynobius dunni</i>	437.00	111.00	OLMO & MORESCALCH (1975)
<i>Hynobius leechii</i>	501.38	172.56	WANG 1996, MA et al. (2003)
<i>Hynobius naevius</i>	681.00	123.00	OLMO & MORESCALCH (1975)
<i>Hynobius nebulosus</i>	445.79	83.32	GREGORY (2003)
<i>Hynobius retardatus</i>	413.00	83.00	OLMO & MORESCALCH (1975)
<i>Hynobius tsuensis</i>	464.85	84.19	GREGORY (2003)
<i>Ranodon sibiricus</i>	409.98		YE et al. (2012)
<i>Salamandrella keyserlingii</i>	386.75	113.22	MA (2005)

Continues

Appendix 1. Continued.

	Erythrocyte size	Nucleus size	Reference
Plethodontidae			
<i>Aneides lugubris</i>	1995.00	435.00	OLMO & MORESCALCH (1975)
<i>Batrachoseps attenuatus</i>	1233.00	228.00	OLMO & MORESCALCH (1975)
<i>Desmognathus carolinensis</i>	306.22	–	GREGORY (2003)
<i>Desmognathus fuscus</i>	765.00	122.00	OLMO & MORESCALCH (1975)
<i>Desmognathus marmoratus</i>	417.00	77.00	OLMO & MORESCALCH (1975)
<i>Desmognathus monticola</i>	344.05	–	GREGORY (2003)
<i>Desmognathus quadramaculatus</i>	585.51	–	GREGORY (2003)
<i>Ensatina eschscholtzii</i>	1523.00	281.00	OLMO & MORESCALCH (1975)
<i>Eurycea bislineata</i>	445.95	–	GREGORY (2003)
<i>Eurycea lucifuga</i>	628.95	–	GREGORY (2003)
<i>Gyrinophilus porphyriticus</i>	1664.00	359.00	OLMO & MORESCALCH (1975)
<i>Plethodon cinereus</i>	431.26	–	GREGORY (2003)
<i>Plethodon dorsalis</i>	440.04	–	GREGORY (2003)
<i>Plethodon glutinosus</i>	529.89	–	GREGORY (2003)
<i>Pseudotriton ruber</i>	1157.00	171.00	OLMO & MORESCALCH (1975)
Proteidae			
<i>Necturus maculosus</i>	1119.55	273.13	HARTMAN & LESSLER (1964)
<i>Proteus anguinus</i>	1740.56	118.63	GREGORY (2003)
Salamandridae			
<i>Cynops orientalis</i>	286.76	102.54	MA et al. (2003)
<i>Cynops pyrrhogaster</i>	660.05	150.82	GREGORY (2003)
<i>Notophthalmus viridescens</i>	454.58	68.82	GREGORY (2003)
<i>Paramesotriton hongkongensis</i>	1575.00	213.00	OLMO & MORESCALCH (1975)
<i>Salamandra atra</i>	2649.00	407.00	OLMO & MORESCALCH (1975)
<i>Salamandra salamandra</i>	878.89	–	GONIAKOWSKA-WITALINSKA (1978)
<i>Taricha granulosa</i>	603.77	–	GREGORY (2003)
<i>Taricha rivularis</i>	828.00	119.00	OLMO & MORESCALCH (1975)
<i>Taricha torosa</i>	1518.00	241.00	OLMO & MORESCALCH (1975)
<i>Triturus carnifex</i>	–	62.49	GREGORY (2003)
<i>Triturus cristatus</i>	466.53	89.46	GREGORY (2003)
<i>Tylotriton verrucosus</i>	524.00	97.00	OLMO & MORESCALCH (1975)
Sirenidae			
<i>Pseudobranchius striatus</i>	2021.00	364.00	OLMO & MORESCALCH (1975)
<i>Siren intermedia</i>	1902.00	517.00	OLMO & MORESCALCH (1975)
<i>Siren lacertina</i>	1587.05	221.44	GREGORY (2003)
Anura			
Alytidae			
<i>Bombina bombina</i>	256.67	60.14	ATATÜR et al. (1999), WOJTASZEK & ADAMOWICZ (2003)
Bombinatoridae			
<i>Bombina bombina</i>	201.01	–	MISEK & SZARSKI (1978)
<i>Bombina orientalis</i>	306.31	52.22	GREGORY (2003)
Bufonidae			
<i>Bufo americanus</i>	183.59	–	GREGORY (2003)
<i>Bufo bufo</i>	220.25	–	ATATÜR et al. (1999)
<i>Bufo calamita</i>	201.01	–	GREGORY (2003)
<i>Bufo gargarizans</i>	447.56	53.59	Our study
<i>Bufo marinus</i>	192.97	31.81	HARTMAN & LESSLER (1964)
<i>Bufo melanostictus</i>	433.97	62.36	Our study
<i>Bufo terrestris</i>	153.70	23.59	GREGORY (2003)
<i>Bufo viridis</i>	178.29	–	ATATÜR et al. (1999)

Continues

Appendix 1. Continued.

	Erythrocyte size	Nucleus size	Reference
Dicroglossidae			
<i>Fejervarya limnocharis</i>	333.18	42.63	Our study
<i>Hoplobatrachus rugulosus</i>	169.25	29.37	Hu et al. (2005)
<i>Paa boulengeri</i>	418.85	71.71	ZHOU et al. (2011)
<i>Paa spinosa</i>	188.02	35.28	Hu et al. (2005)
<i>Paa yunnanensis</i>	356.79	56.03	ZHOU et al. (2010)
Hylidae			
<i>Acris crepitans</i>	330.46	–	GREGORY (2003)
<i>Hyla arborea</i>	200.45	–	ATATÜR et al. (1999)
<i>Hyla gratiosa</i>	213.64	26.15	HARTMAN & LESSLER (1964)
<i>Hyla japonica</i>	222.17	34.85	GREGORY (2003)
<i>Hyla versicolor</i>	195.09	–	GREGORY (2003)
<i>Hypsiboas cordobae</i>	265.40	40.86	BARAQUET et al. (2013)
<i>Litoria caerulea</i>	197.50	–	GREGORY (2003)
<i>Litoria citropa</i>	253.40	–	GREGORY (2003)
<i>Litoria infrafrenata</i>	155.43	–	S. Young (unpubl. data)
<i>Litoria rubella</i>	144.20	–	GREGORY (2003)
<i>Pseudacris crucifer</i>	183.59	–	GREGORY (2003)
<i>Pseudacris nigrita</i>	141.37	–	GREGORY (2003)
Limnodynastinae			
<i>Limnodynastes dorsalis</i>	184.57	–	GREGORY (2003)
<i>Limnodynastes fletcheri</i>	138.26	–	GREGORY (2003)
Microhylidae			
<i>Microhyla ornata</i>	358.10	40.41	Our study
Myobatrachinae			
<i>Crinia signifera</i>	215.24	–	GREGORY (2003)
<i>Pseudophryne bibronii</i>	230.91	–	GREGORY (2003)
Odontophrynidae			
<i>Odontophrynus americanus</i>	187.69	23.84	GRENAT et al. (2009)
Pelobatidae			
<i>Pelobates syriacus</i>	161.36	–	ATATÜR et al. (1999)
Pipidae			
<i>Xenopus laevis</i>	119.38	18.10	GREGORY (2003)
Ranidae			
<i>Rana catesbeiana</i>	307.91	–	COPPO et al. (2005)
<i>Rana grylio</i>	314.84	–	ZHANG et al. (1999)
<i>Rana zhenhaiensis</i>	382.29	55.01	Our study
<i>Rana macrocnemis</i>	278.31	36.45	ARSERIM & MERMER (2008)
<i>Rana nigromaculata</i>	275.83	63.38	Hu et al. (2005)
<i>Rana palustris</i>	203.48	–	GREGORY (2003)
<i>Rana pipiens</i>	206.01	35.94	GREGORY (2003)
<i>Rana porosa</i>	267.99	36.05	GREGORY (2003)
<i>Rana ridibunda</i>	276.65	–	ATATÜR et al. (1999)
<i>Rana rugosa</i>	336.35	60.32	GREGORY (2003)
<i>Rana sylvatica</i>	330.46	–	GREGORY (2003)
<i>Rana tagoi</i>	273.24	44.77	GREGORY (2003)
<i>Rana temporaria</i>	250.00	25.76	GREGORY (2003)
<i>Rana tsushimensis</i>	247.59	38.05	GREGORY (2003)
Rhacophoridae			
<i>Buergeria buergeri</i>	202.16	30.04	GREGORY (2003)
<i>Polypedates maculatus</i>	176.34	32.04	MAHAPATRA et al. (2012)
<i>Rhacophorus annamensis</i>	199.49	31.02	GREGORY (2003)
<i>Rhacophorus schlegelii</i>	225.63	33.55	GREGORY (2003)