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DNA methylation in reptiles

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Abstract

Very recent investigations have provided evidence for a higher DNA methylation level in polar and sub-antarctic fishes compared to temperate/tropical fishes, the latter being in turn higher than the DNA methylation level of warm-blooded vertebrates. These results confirm and extend the finding [Jabbari, K., Cacciò, S., Pais de Barros, J.P., Desgres, J., Bernardi G., 1997. Evolutionary changes in CpG and methylation levels in the genome of vertebrates. Gene 205, 109–118] that DNA methylation level of vertebrates is inversely related to body temperature. Here we studied the methylation level of reptilian genomes. The species previously analyzed exhibited methylation levels closer to those of mammals and birds rather than to those of fishes and amphibians. The sample was, however, too small to reach a final conclusion. Here we used Reversed-Phase-High-Performance Liquid Chromatography (RP-HPLC) to analyze the DNA methylation levels of 43 reptiles representing three out of four orders and 20 families. Such analysis has shown that snakes and lizards exhibit methylation levels covering the whole range comprised between those of temperate/tropical fish and mammals, while turtles, and, more so, crocodiles are close to mammals. We discuss some ecological and physiological data that explain these results.

Keywords: Body temperature; Homeotherms; Heterotherms; Life style

1. Introduction

Results presented in the preceding paper (Varriale and Bernardi, 2006-this issue) made clear that an inverse correlation holds between body temperature and DNA methylation in fishes. Here we approached the problem of DNA methylation in the case of reptiles. The reptiles previously studied (Jabbari et al., 1997) comprised one crocodile, two turtles, one varan and three snakes. This sample was small, heterogeneous (since they comprised three old data from Vanyushin et al. (1970, 1973)) and not representative from a phylogenetic point of view since the two turtles and two of the snakes belonged to the same genera. These reptiles showed DNA methylation levels that covered a two-fold range, a majority of values being closer to those of warm-blooded rather than to those of cold-blooded vertebrates. The results available so far suggested therefore that reptiles might be characterized by a lower methylation level compared to fishes and amphibians, possibly indicating a hypothetical methylation transition at the emergence of the common ancestor of reptiles and mammals, namely at the appearance of amniotes.

We warned, however, that the number of reptilian DNAs analyzed was small, that rather different levels were found in the few reptiles investigated and that no estimate was available for the CpG levels (Jabbari et al., 1997). Under these circumstances, two possibilities should be considered, either that a reptilian ancestor had undergone a decrease in methylation, which was then transmitted by descent to extant reptiles, mammals and birds; or that the methylation changes occurred independently in reptiles (possibly, only in some of them), in mammals and in birds. Here we have expanded the sample to 43 reptiles representing three out of four orders and 18 families and have reached the conclusion that the second hypothesis is correct.

2. Materials and methods

For Materials and methods see preceding paper (Varriale and Bernardi, 2006-this issue). Modal buoyant densities of reptilian

Abbreviations: RP-HPLC, Reversed-Phase-High-Performance Liquid Chromatography; GC, molar ratio of guanosine+cytidine; 5mC, 5-methylcytosine.

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DNAs are reported in Supplementary Table S2 and analytical ultracentrifuge profiles of reptilian DNAs in Supplementary Fig. S1.

3. Results

3.1. Analysis of 5mC level in reptiles

Table 1 presents the taxonomy and the sources of the genomes analyzed as well as the percent values of GC and 5mC obtained from the RP-HPLC analyses for each of the reptilian species, and the ratios 5mC/GC. The geographic distribution of the species is presented in Supplementary Table S1. Our dataset comprises 43 reptilian species covering three out of the four extant orders of reptiles, *Testudines* (turtles), *Squamata* (snakes and lizards), and *Crocodylia* (crocodiles). The fourth order, *Rhynchocephalia*, only comprises two living species belonging to the same family, *Sphenodontidae*. The only value from literature that we used was *Testudo graeca* because it was analyzed by our group with RP-HPLC (Jabbari et al., 1997). We did not use the other values from the literature because either they had been obtained with techniques different from RP-HPLC or they were reanalyzed in the present work.

The values of 5mC and GC are plotted in Fig. 1. It is immediately noticeable that the 5mC levels of reptiles show a scatter covering the whole range of values from mammals to tropical/temperate fishes if we consider both species (Fig. 1a) and families (Fig. 1b). In fact, the two groups of crocodilians and turtles are the GC-richest and the least methylated genomes, whereas squamates represent, in terms of DNA methylation, the most heterogeneous order among reptiles.

The different behavior of DNA methylation of reptiles as plotted in Fig. 1, compared to all other vertebrate classes and groups (including polar, temperate and tropical fishes; see Varriale and Bernardi, 2006-this issue), is stressed by the fact that no significant correlation ($R=0.04$) is obtained in this case. The correlation found when plotting 5mC vs. genome size (Fig. 2) is remarkably weaker ($R=0.12$) than those found for fishes (see Varriale and Bernardi, 2006-this issue). Moreover the slope is very slightly positive whereas it is negative for fishes. These findings indicate that in reptiles methylation is so variable that no effect of genome size can be detected.

3.2. Analysis of CpG doublets in reptiles

A comparison of the frequency of CpG doublets of coding sequences from reptiles with those of orthologous sequences from mammalian (mainly human) genes was attempted, but results were not meaningful for two reasons: scarcity of available data, and the contribution of CpG islands which are more abundant in mammalian compared to reptilian genomes (Aïssani and Bernardi, 1991a,b; and our unpublished observations).

3.3. Correlation between GC levels as derived from nucleoside analysis and from CsCl density gradient centrifugation

As in the preceding paper, GC levels of reptilian DNAs were calculated from both modal buoyant densities and from nucleoside analysis (Fig. 3). As in the case of fish DNAs (see preceding paper), the former values were lower than the latter ones by 2–3% GC. This difference is due, at least in part, to DNA methylation. Indeed when GC is derived from buoyant density, the calculation is based on the correlation between GC and buoyant density of bacterial DNAs, which are not at all or barely methylated. The effect of DNA methylation on buoyant density is to lower it (Kirk, 1967). This is not, however, the full explanation because the ~3% difference in the GC estimate would be accounted for by a ~4% difference in 5mC, whereas it is about ~2% in fish DNAs and ~1.5% in reptilian DNAs. An additional contribution may come from the frequency of di- and tri-nucleotides that are different compared to the reference bacterial DNAs. An interesting observation in this connection is the similarity of the plots obtained for fishes and reptiles which will be discussed elsewhere. In any case, the correlation of Fig. 3 is useful because it allows correcting GC values directly derived from ρ_0 using the equation of Schildkraut et al. (1962).

4. Discussion

4.1. DNA methylation levels of reptiles: inter-ordinal differences

Reptiles tend to be tropical and a progressive decrease in species number occurs with increasing latitude (Heatwole and Taylor, 1987), *Sphenodon* having the lowest temperature (6–16 °C) when active (Halliday and Adler, 2002). Reptiles have evolved different forms of thermoregulation (see Huey, 1982, for a review). Behavioral modulation of the thermal flux is the main thermoregulator that allows reptiles to maintain body temperature within a certain range: strategies involve basking, hibernating, changing body posture, shading, or selectively exploiting the thermal variation in the environment. Thermal physiology also plays a role in the modulation of body temperature, but analyses of body temperatures demonstrated limited variation within most genera or species. Behavioral as well as physiological adjustments might contribute to thermal homeostasis, but in changing environmental conditions reptiles can undergo large variations in body temperature. This means that the correlation between body and environmental temperatures is not straightforward.

On the basis of the above complex situation and of the correlation between DNA methylation and body temperature over extended periods of time, one should expect quite a variability in the levels of DNA methylation of reptiles. This is what the results of Fig. 1 show. Indeed, as far as genome methylation level is concerned, reptiles are very heterogeneous in comparison with other vertebrates, in agreement with other findings on several features of reptilian genomes. These concern nucleotide composition, band asymmetry in cesium chloride, compositional heterogeneity (Aïssani and Bernardi, 1991a,b;

Table 1
List of the reptilian species analyzed [1]

Order	Family	Species	Source[2]	c-value[3]	Species			Families/genera[5]	
					GC	5mC	R[4]	GC	5mC
Testudines	*Chelydridae*	*Chelydra serpentina*	a	2.63	47.68	1.33	2.79		
		Macrochelys temminckii	a		48.98	1.11	2.26	48.33	1.22
	Emydidae	*Trachemys scripta elegans*		2.62	46.98	1.14	2.43		
		Chrysemis picta	b	2.59	47.44	0.96	2.02	47.21	1.05
	Testudinidae	*Testudo graeca*			45.70	0.77	1.68		
		Testudo hermanni		3.52					
	Cheloniidae	*Chelonia mydas*	c	2.64	47.38	1.00	2.10		
		Caretta caretta	c		46.74	0.96	2.05	47.06	0.98
Squamata	*Agamidae*	*Chlamydosaurus kingii*	a		44.67	0.93	2.08		
	Chamaeleonidae	*Furcifer oustaleti*	d		44.49	1.14	2.57		
	Iguanidae	*Iguana iguana*	a	2.89	44.33	1.36	3.08		
	Phrynosomatidae	*Sceloporus magister*	e		45.84	0.85	1.85		
	Gekkonidae	*Tarentola mauritanica*	f	2.65	46.59	0.94	2.01		
		Gekko gecko		2.84	46.05	1.09	2.37	46.32	1.01
	Gerrhosauridae	*Zonosaurus madagascariensis*	d		47.11	1.19	2.53		
	Teiidae	*Tupinambis teguixin*	g	2.70	45.60	0.86	1.88		
	Lacertidae	*Podarcis sicula*	d	2.20	47.02	1.47	3.13		
		Podarcis muralis	g	2.36	48.18	1.34	2.79	47.60	1.41
	Scincidae	*Mabuya gravenhorstii*	d		45.94	0.83	1.81		
		Mabuya sp.		1.72					
	Anguidae	*Anguis fragilis*	f	2.23	47.60	1.01	2.11		
	Boidae	*Acrantophis dumerili*	a		44.41	0.94	2.12		
		Boa constrictor	a	1.90	41.95	0.68	1.62		
		Python molurus	a		43.18	0.81	1.88		
		Python reticulatus	a	1.60	42.38	0.65	1.54		
	Colubridae	*Hierophis viridiflavus*	f		44.16	1.32	3.00		
		Elaphe lineata	f		43.90	1.40	3.19		
		Elaphe obsoleta		2.14	43.31	1.19	2.75		
		Elaphe guttata	h		41.83	1.29	3.09		
		Elaphe mandarina	a		43.05	1.23	2.86		
		Sibon longifrenis	g		47.82	1.36	2.84		
		Natrix tessellata	g	1.83	44.12	1.03	2.32		
		Liopholidophis lateralis	d		44.36	1.41	3.19		
		Geodipsas sp	d		43.28	1.38	3.19		
		Madagascarophis sp	d		42.71	1.10	2.58		
		Clelia rustica	g		42.84	1.31	3.06		
	Elapidae	*Walterinnesia aegyptia*	g		42.77	0.80	1.86		
		Notechis scutatus	g		42.44	1.05	2.46	42.61	0.92
	Viperidae	*Bothrops jararaca*		2.25	42.99	1.16	2.70		
		Bothrops neuwiedi			43.33	1.17	2.71	43.16	1.17
		Viper aspis francisredi	f	2.91	43.40	1.20	2.76		
		Daboia palestinae	g		43.94	1.17	2.66		
		Daboia russelii		2.07					
		Echis coloratus	g		42.53	0.92	2.16		
		Echis carinatus		1.30					
Crocodylia	*Crocodylidae*	*Crocodylus niloticus*		3.40	48.44	0.85	1.75		
	Alligatoridae	*Alligator mississippiensis*	h	2.58	48.56	0.96	1.97	48.50	0.90

[1] Taxonomy is from www.embl-heidelberg.de/~uetz/LivingReptiles.html. The underlined samples refer to reptiles only used for *c*-values.

[2] Sources: (a) Romano Desensi, Italy; (b) Jon Costanzo, Department of Zoology, Miami University, Oxford OH 45056, USA (c) Fulvio Maffucci and Flegra Bentivegna, Aquariology Laboratory, SZN, Naples, Italy; (d) Rosaria Scudiero and Elio Parisi, IBP-CNR, Naples Italy; (e) Giorgio Bernardi, Laboratory of Molecular Evolution, SZN, Naples, Italy; (f) Gaetano Odierna, Università degli Studi di Napoli Federico II, Naples, Italy; (g) Dusan Kordis, Department of Biochemistry and Molecular Biology, Jozef Stefan Institute, Ljubljana, Slovenia; (h) Axel Janke, Department of Genetics, Division of Evolutionary Molecular Systematics, University of Lund, Lund, Sweden. *Testudo graeca* was analyzed by Jabbari et al. (1997). Blank spaces indicate unknown source.

[3] *c*-values expressed in picograms (pg) are from the database Animal Genome Size Database (Release 2.0; Gregory, T.R. 2005), available at URL http://www.genomesize.com. We reported the average for some samples which had multiple annotations: *Anguis fragilis, Boa constrictor, Natrix tessellata, Podarcis muralis, Chrysemis picta, Trachemys scripta elegans, Testudo hermanni.*

[4] *R* is the 5mC/GC ratio multiplied by 10^2.

[5] Averages were calculated for species belonging to the same family/genus (indicated by thick vertical bars). This was not done when GC and/or 5mC values for species within a family/genus diverged too much each other (indicated by thin vertical bars).

Hughes et al., 2002; see also Bernardi, 2004) and karyotypic evolutionary rates (Olmo et al., 2002; Olmo, 2005). A close look at the data of Fig. 1 reveals a number of interesting features. In terms of both high GC and low methylation level, the extreme case is that of crocodiles. Turtles are characterized by rather high GC levels and low methylation. Finally the squamates show the widest spread in both GC and methylation.

4.2. Crocodiles

Concerning body temperature, it has been observed that *Alligator mississippiensis* are mostly active in the 32–35 °C range, which is the most frequently recorded temperature. Indeed, physiologists demonstrated that crocodilians possess a high degree of thermoregulation (Smith, 1979; Seebacher et al., 1999) and are able to produce sufficient metabolic heat to elevate their body temperature above water temperature. A recent paper also stresses the similarities between the heart of crocodilians and endotherms, showing the possibility of an endothermic ancestor of modern alligators and crocodiles (Summers, 2005).

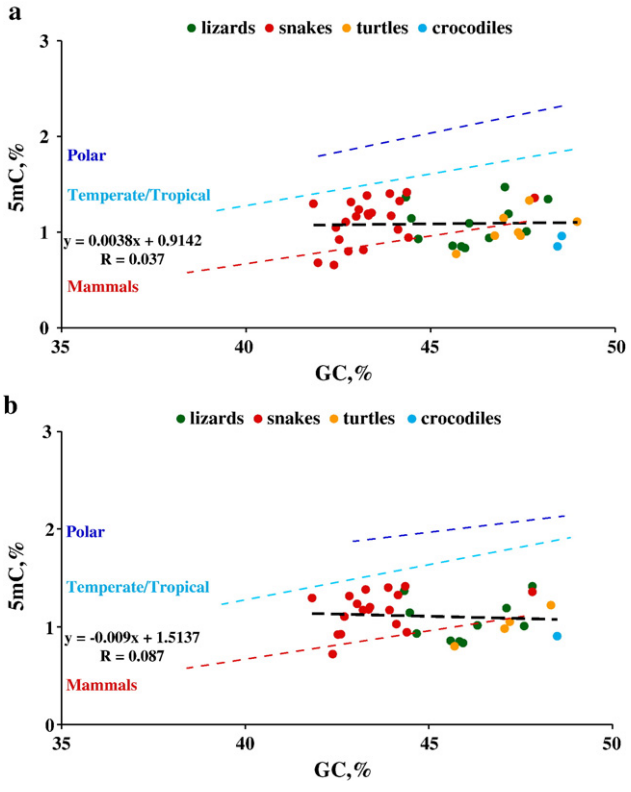

Fig. 1. a. Plot of 5mC levels against GC levels for the genomes of lizards (green circles), snakes (red circles), turtles (yellow circles) and crocodiles (blue circles). Values are listed in Table 1, column Species. The regression line, correlation coefficient (*R*) and equation for reptiles are shown (black line). Regression lines for polar fishes (dark blue line), temperate/tropical fishes (light blue line; Varriale and Bernardi, 2006-this issue), and mammals (red line; data from Jabbari et al., 1997, and from Varriale and Bernardi, in preparation) are also given as references. 1b. Plot of average 5mC levels against average GC levels for families/genera (listed in Table 1, column Families/genera). Families represented by single species (listed in Table 1, column Species) were also included in the figure, whereas families whose values could not be averaged (see Table 1) were not. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

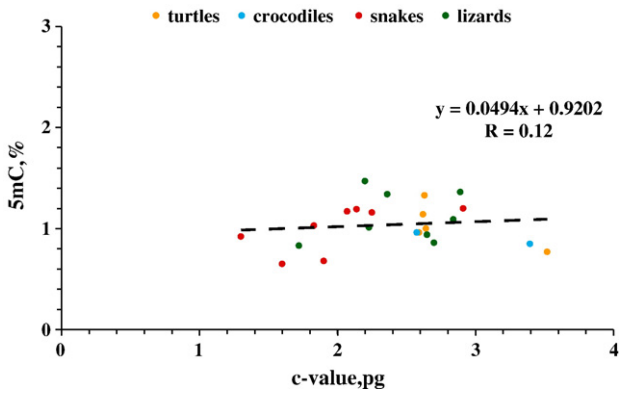

Fig. 2. Plot of 5mC against genome size for lizards (green circles), snakes (red circles), turtles (yellow circles) and crocodiles (blue circles). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.3. Testudines

The case of a terrestrial turtle, *T. graeca*, was previously investigated (Thiery et al., 1976; Bernardi and Bernardi, 1990; Aïssani and Bernardi, 1991a). Its genome, already shown to be characterized by a remarkable compositional heterogeneity, exhibited one of the two lowest 5mC levels among all reptiles studied here. This may be explained by the rather high body temperature reached by some terrestrial turtles. Aquatic turtles also display a very low 5mC level which could possibly be explained by the high body temperature that they can reach. Examples are reported for a number of species and families. Freshwater turtles of the family *Emydidae* (such as *Trachemys scripta elegans* and *Chrysemys picta* analyzed here) achieve body temperatures significantly above both air and water temperatures when basking (Boyer, 1965; and references therein) This is a frequent behavioral way of heating up, found also for *Chelydra serpentina*, often observed in warm shallow water (Brattstrom, 1965). The two marine turtles that

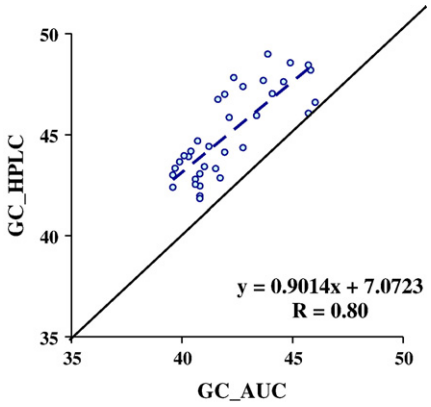

Fig. 3. Correlation between genomic GC levels as obtained by nucleoside analysis (HPLC) and by analytical ultracentrifugation (AUC) in reptiles.

we have analyzed in this study (*Caretta caretta* and *Chelonia mydas*) live in relatively warm waters. Moreover, body temperatures are often 1–3 °C above water temperatures and it can be assumed this is most likely the result of the accumulation of metabolically produced heat (Mrosovsky and Pritchard, 1971).

4.4. Squamata

Iguanidae represent a family in which many species, such as *Iguana iguana*, are diurnal baskers which are able to maintain relatively high and constant activity temperatures, within the range of 30–35 °C (Avery, 1982). A similar situation can be observed for many species belonging to the family *Agamidae* and *Lacertidae*, which are also diurnal baskers, but not for all species of *Anguidae* and *Chamaeleonidae*.

In snakes, the thermal relationship with the environment tends to be less precise than those of diurnal lizards because of their larger surface-to-volume ratios, conduction of heat and passive thermoregulation are relatively more important than basking in solar radiation. The elongate shape also creates problems of definition and measurement because temperature may vary considerably in different parts of the body.

4.5. DNA methylation levels of reptiles: intra-ordinal differences

The reason why the values of Table 1 for some species of reptiles were not averaged per families, as done in several cases for fishes (Varriale and Bernardi, 2006-this issue), is that significant differences were found not only within orders, but also within families and even within genera. Indeed, both GC and 5mC values were significantly different for the two *Python* and the four *Elaphe* species. While this may be due, at least in part, to the presence of satellite DNAs, the possible role of differences in body temperature cannot be ruled out.

5. Conclusion

This report, like the preceding one (Varriale and Bernardi, 2006-this issue), tried to connect biological and molecular data, highlighting the importance of temperature as an environmental factor able to act on genomes from ectotherms through natural selection. We focused here on the characteristics of life style of reptiles studied by ecologists and physiologists to find biological explanations for the results that we obtained at the genomic level. Physiological studies show that different reptiles follow different ways for increasing or decreasing body temperature, and can tolerate different ranges of temperatures. This biological diversity is reflected in genomic diversity. The results obtained in the present work provide a proof of such diversity. If the compositional transitions that took place in the genomes of vertebrates at the emergence of mammals and birds had appeared in the common ancestor of tetrapods, as proposed by Hughes et al. (1999), or in the common ancestor of reptiles and mammals, as proposed by Duret et al. (2002), we should have found identical or similar 5mC levels among reptiles (a situation found in the genomes of mammals and birds), whereas clearly this is not the case.

The new data support a strong influence of environment on reptilian genomes and are in full contrast with the conclusion of Duret et al. (2002) who proposed an "amniote hypothesis" about the GC increase transition in vertebrate genomes. This hypothesis might still have been compatible with the data obtained for reptiles by Jabbari et al. (1997), because the reptilian points were closer to points from warm-blooded vertebrates than to those from cold blooded. However, at that time only a small sample had been investigated, and it was not possible to reach any final conclusion. We found, now, that while DNA methylation is low in the case of turtles and crocodilians (which are also characterized by a relatively large degree of DNA heterogeneity; Aïssani and Bernardi, 1991a,b), namely in the case in which body temperature is relatively high, lizards and, more so, snakes, clearly show a strong variability in DNA methylation covering the whole 5mC range comprised between fish/amphibians and mammals/birds. This variability could be due to the different "average" body temperatures of these reptiles. One should not forget that reptiles represent a group different in many aspects both from thermoconformer ectotherms, such as fishes and amphibians, and from endotherms, such as mammals and birds. Reflecting their physiological peculiarity, their genomes display different DNA methylation levels that are intermediate between these two groups.

Interestingly, a positive correlation between body temperatures and GC level in some desert lizards as compared with some snakes was reported by Olmo (2003), who hypothesized that a higher GC level could help DNA to resist the destabilizing effect of temperature and genetic damage, which could derive from an increased metabolic rate correlated with an increased production of free radicals.

A final remark is that 5mC and CpG levels appear to integrate the effects of temperature and time of exposure to temperature. If this is true, more complex changes in the genome structure, such as the formation of GC-rich isochores, should be better correlated with DNA methylation rather than with body temperature. In the case of large-scale comparisons, such as that of fishes/amphibians with mammals/birds, temperature is a good reference because the exposure times under considerations are very large. In the case of reptiles, however, the formation of GC-rich isochores, the compositional heterogeneity and the compositional asymmetry should be related to 5mC and CpG levels, body temperature having a low significance in the absence of information of time of exposure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2006.05.034.

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