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Review

Metallothioneins in reptiles: Gene expression, function and evolution

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ABSTRACT

Metallothioneins are metal-containing proteins that bind zinc, copper and other metallic ligands thanks to the cysteine residues of their polypeptide chain. In addition to supporting homeostatic buffering of metallic micronutrients into the cells by providing Zn and Cu supply, metallothioneins fulfil a broad range of other functions, including detoxification of toxic metals such as cadmium and mercury, scavenging of harmful reactive oxygen species, and others. Thus it is not surprising that the versatile metallothioneins are found from bacteria to fungi, protists, plants, and most animal groups. In spite of many studies on metallothioneins, little is known about their origin, evolution and diversification. This can be attributed to the lack of detailed comparative studies. Among vertebrates, a great deal of knowledge on metallothionein structure and function relies on studies carried out on mammalian metallothionein isoforms, followed by studies on piscine and avian metallothioneins, whereas very few data are available on reptilian and amphibian metallothioneins. To gain further insights into the history of this fascinating protein in vertebrates, we undertook a study about the expression, the function and the evolution of metallothionein gene(s) in reptiles. In this review, we summarize the results obtained studying the gene expression and the detoxification function of the metallothionein in embryonic and adult tissues of the lacertid *Podarcis sicula* and the evolution of the metallothionein gene in squamate reptiles.

KEYWORDS: cadmium, embryonic development, gene expression, metallothionein, molecular evolution, reptiles

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INTRODUCTION

Metallothioneins (MTs) are a large class of low molecular weight, cysteine-rich, metal-binding proteins found in all eukaryotes so far investigated [1].

These proteins are involved in: 1) the homeostatic control of essential metals such as zinc and copper [2, 3]; 2) the defence of organisms against metal poisoning and oxidative stress [4, 5].

Studies initially focused on mammalian metallothionein, where four major isoforms of MT (termed MT1 to MT4) have been identified. Although all genes encode for conserved peptide chains that retain 20 invariant metal-binding cysteines, MT1 and MT2 are widely expressed in most tissues/organs, whereas MT3 and MT4 are mostly detected in neural tissues and stratified squamous epithelia, respectively [6, 7]. In addition, studies on structural-functional features of mammalian MTs led to the identification of minor isoforms (13 in humans) originated by MT1 gene duplication events [8].

Compared with the multiplicity of mammalian MTs, a different situation is observed in lower vertebrates, in which only one or two distinct MTs

have been isolated in each species examined [9, 10, 11], thus suggesting that the evolution of MT genes may have followed different pathways in different groups of taxa. This observation might be explained, at least in part, by the scarcity of data on non-mammalian MTs, but it also may indicate a complex evolutionary history of MTs in the vertebrates.

Also regarding the MT functions, the huge amount of data on mammalian MT does not clarify the main function of this protein and the reason of the massive MT gene duplications.

Comparative studies of MTs focusing on the different vertebrates taxa might shed some light on function and evolution of MTs in vertebrates. In consideration of the few data currently available, we decided to perform a research on MTs in reptiles. This review summarizes data obtained from our studies.

Basal expression of metallothionein gene in lizard tissues

Metallothionein content was investigated in liver and ovary of the Italian wall lizard *Podarcis sicula*. Metallothionein protein in the liver was isolated by gel filtration chromatography; the protein was detected by determining the metals bound to the proteins by atomic absorption spectrophotometry [12]. In the *P. sicula* ovary the total metal content present was associated with high molecular mass metal-binding proteins, whereas no metal was bound to proteins with the apparent molecular weight of MT [12].

By exploiting a Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) strategy, a cDNA encoding the lizard MT was obtained from the liver. The lizard MT was made of 63 amino acids with 20 cysteines arranged in a fashion typical of vertebrate MTs.

Gene expression analyses showed that MT transcripts were present in all tissues and organs examined; densitometric analysis demonstrated that the level of MT mRNA was brain>liver> kidney>ovary>gut. No different transcripts for the MT were found in the different tissues, thus allowing us to assume the presence of a single MT isoform in this animal [13].

The presence of a detectable amount of MT mRNA in the ovary without a concomitant presence of the MT protein is very intriguing and deserves particular attention, because it shows that in this organ the MT synthesis can be regulated both at genomic and transcriptomic level. We found that in the ovary the MT mRNA was present in ovaries in all the periods of the ovarian cycle; however, the amount of MT transcripts dramatically increased in the pre-reproductive and reproductive period. Likewise MT mRNA accumulated throughout follicles maturation, reaching the highest level in vitellogenic follicles [12, 14].

In situ hybridization demonstrated that in the liver MT was expressed by monocytes and Kupffer cells, whereas no MT transcripts were detected in hepatocytes [15]. As regards ovaries, we found that MT mRNA localization changed according to the maturation of ovarian follicles. In early previtellogenic follicles, MT transcripts were localized in the cytoplasm and, in a lesser extent, in the nucleus of oocytes, whereas the small stem cells forming the follicular epithelium were unlabelled. In mid previtellogenesis follicles, an intense hybridization signal was present in the epithelium, in particular in the cytoplasm of both small stem cells and differentiated pyriforms. In larger previtellogenic and in vitellogenic follicles the MT mRNA was significantly reduced in pyriforms, but present in the cytoplasm of all small cells [16].

Cadmium-induced expression of metallothionein gene in lizard tissues

Cadmium (Cd) is a persistent contaminant accumulated in the environment from both anthropogenic and natural sources. Every year, large quantities of this metal are released in the different environmental compartments and may pose a significant threat to the biota exposed. Intracellular damage caused by cadmium exposure includes protein denaturation, lipid peroxidation, generation of reactive oxygen species and DNA strand breaks [17]. Many studies have also demonstrated that this ion has a teratogenic or lethal effect on embryos, related to the dose and exposure time [18, 19, 20, 21].

In spite of the wide number of studies carried out in laboratory mammals, data on Cd effects on

wild terrestrial vertebrates are still limited. In particular, information on the consequences of environmental Cd exposure on reptiles' survival and biodiversity is particularly scanty. Reptiles are presently considered highly susceptible to a number of environmental pollutants and this has contributed to the global decline of several wild populations of turtles, crocodilians and lizards.

One of the roles commonly attributed to MT is defence against the harmful effects produced by toxic heavy metals [4]. Because of its metal sequestering capability, MT is able to render cells, tissues and organisms resistant to otherwise toxic levels of metals such as Cd. The first measurable effect of Cd in the cell is the increase of MT mRNA levels and the *de novo* synthesis of MT protein [12, 13, 22].

Although the detoxifying role of MT is definitely ascertained, it is still not known whether MT plays this role successfully in all the tissues/ organisms.

To answer this question, we tested short and long-term Cd effects on the tissues of the lizard *P. sicula* via a single intraperitoneal or multiple oral doses of Cd ions.

The first measurable effect of Cd uptake in *P. sicula* tissues was a dramatic increase of MT content. Table 1 gives an overview of the expression and synthesis of MT in *P. sicula* tissues under normal conditions and after cadmium exposure.

Liver

After two days from an intraperitoneal Cd administration (2 µg/g body mass) a twofold increase in both MT proteins and MT transcripts were determined in liver [13, 15]. *In situ* hybridization demonstrated that Cd did not alter MT expression: MT transcripts were detected exclusively in the large Kupffer cells and in monocytes in vessels, whereas the hepatocytes remained completely unstained. The increase in MT mRNA content in liver was due to the increased number of Kupffer cells/monocytes expressing the metalloprotein: in the intraperitoneally injected animals, the number of stained cells shifted from 24 ± 7.6 to 51.0 ± 7.3 per arbitrary unit of surface [15].

On the contrary, a chronic dietary treatment (1 µg $CdCl_2$ per g of body mass, every second day for 60 days) failed to alter the MT expression in liver. Our results showed that orally administered cadmium ions accumulated slowly in the livers, reaching the maximal concentration after 60 days of treatment [13]. It is conceivable that the slow increase in Cd ions observed in the liver, accompanied with a constitutively high amount of MT transcript, was unable to bring about a measurable induction of MT expression in this organ. However, both types of treatments were not lethal to animals, and did not result in gross morphological alterations in hepatic tissues; livers from Cd contaminated lizards showed morpho-functional alterations such as oedema, hydropic swelling, changes in sugar and lipid metabolism [15]. These effects were similar regardless of single intraperitoneal or multiple oral administration; whether this indicates an apparent independence from Cd concentration or is due to the protective role of MT proteins induced after a massive Cd uptake is still unknown.

Ovary

Similar results were obtained analysing Cd effects in lizard ovaries. Both types of Cd administration

Table 1. Metallothionein in adult lizard tissues.

Tissue	Basal expression		Cd-induced expression	
	MT transcript	*MT protein*	*MT transcript*	*MT protein*
Brain	+	**n. d.**	+	**n. d.**
Gut	+	**n. d.**	++	**n. d.**
Kidney	+	**n. d.**	++	**n. d.**
Liver	+	+	++	++
Ovary	+	-	++	+

n. d. = non determined

(intraperitoneally and orally) were able to change follicular organization, increase follicular athresia and embryo mortality, reduce clutch size and cause alteration in sugar metabolism [23, 24]. The induction of MT mRNA transcript following the long-term (60 days) dietary or the short term (single) intraperitoneal Cd treatment was assessed by dot blot analysis. In the first case, a massive (fourfold) increase in MT mRNA was observed only at the end of the treatment; 30 days of treatment were ineffective to induce MT mRNA expression. The single intraperitoneal Cd-treatment gave rise to a similar fourfold increase in MT transcript content after two days from administration [13, 14]. Noteworthy, the latter treatment also gave rise to the *de novo* synthesis of MT protein [12, 14]. If the synthesis is only due to the newly induced MT transcripts or also stems from the messengers already present in the ovary is still a matter of debate.

Kidney

Cadmium-mediated induction of MT gene expression was found also in the lizard kidney. Dot blot analyses performed on total RNA from chronic dietary and acute intraperitoneally Cd-treated lizards showed an induction pattern similar to that observed in ovaries: a sudden increase in response to acute toxicity, a delayed increase in response to chronic toxicity [13].

Gut

As expected, dietary cadmium administration elicited an early response in gut cells: MT mRNA amount increased dramatically once the contamination began and the increase continued throughout the treatment [13].

Brain

Cadmium uptake in lizard brain deserves particular attention. We found that both intraperitoneally and dietary administered cadmium was able to cross the blood-brain barrier and to accumulate in cells, persisting in the tissue even after the end of the administration [13]. From these results it can be assumed that even an occasional, accidental contamination could have a long-term effect on the nervous system of these animals. With respect to MT gene expression, lizard brain did not seem to be sensitive to cadmium exposure, independently of the way the metal was administered, possibly because of the high level of MT physiologically present in this organ, as indicated by the high amount of MT transcript detected in control animals, that exceeded the amount found in the detoxifying organs, such as liver and kidney [13].

Basal expression of metallothionein gene in lizard embryonic tissues

MTs play critical functions during embryonic development, as demonstrated by their expression in early embryogenesis of plant and animal species [25]. In mammalian embryos MT mRNAs are present as maternal messengers stored in eggs; the MT-I and MT-II genes are among the first to be transcribed from the embryonic genome and are coordinately expressed throughout the preimplantation period of embryonic development [26]. In mouse foetus MT expression is tissue-specific: MT-mRNA is present in great amount in the liver and at lesser extent in the kidney, but is absent in the brain [27]. In Xenopus embryos, MT transcript is first detected in brain and in tissues implicated in detoxification processes, such as liver hepatocytes and kidney tubules [28].

Few data are available on MT expression in amniotic oviparous species, in which the embryos develop from eggs containing all the nutritive resources needed for the embryonic development [29]. For this reason, embryos of oviparous vertebrates are considered as closed systems independent of the environment as a source of nutrition [30].

As described above, in mature oocytes of the oviparous lizard *Podarcis sicula* MT mRNA accumulates abundantly, suggesting storage of MT transcripts possibly to meet the future needs of the growing embryo [12, 14]. Therefore, we decided to investigate the spatial and temporal expression of the MT gene and the localization of MT transcripts throughout *P. sicula* development to determine the expression pattern of this gene in amniotic oviparous vertebrates.

P. sicula freshly laid eggs were incubated in a terrarium maintained at natural conditions of humidity and temperature [16]. *In situ* hybridization analyses to localize the MT transcripts were performed on embryos collected at regular time

intervals (0, 5, 10, 20, 40 and 50 days) from deposition until hatching [16]. Table 2 summarizes the results obtained by such analyses.

At discoblastula stage MT transcripts were present in the cytoplasm of both embryonic and extra embryonic blastomeres. This could be ascribed to a uniform distribution of previously accumulated maternal mRNAs or to the expression of MT embryonic gene. Further investigations are needed to respond to this matter of debate.

At the beginning of organogenesis (5-10 days post deposition, embryo length 8-16 mm), MT transcripts were uniformly distributed in ventricular zones of the developing central nervous system and in the undifferentiated retina. MT mRNA was also present in mesodermal derivates such as somites and mesenchyme but not in kidney tubules or in the main endodermal derivates such as gut mucosa and the anlages of liver and lung.

Around 20 days post deposition (embryo length 19 mm), the MT-mRNA distribution in embryonic tissues slightly changed. In the telencephalon and diencephalon, labelling remained exclusively on the ventricular zones, while in the mesencephalon and in the medulla oblongata, labelling also appeared on the developing optic cortex and on several nuclei. Significant labelling also appeared on the ependymal cells of all vesicles and in the horns of the spinal cord. In the eye, the retinal differentiation resulted in a redistribution of MT-mRNA that concentrated in the ganglion cells layer and in the inner and outer nuclear layers. In the trunk, for the first time an intense hybridization signal appeared in renal glomeruli, whereas the tubules, the lung, the liver and the gut mucosa remained unstained.

Expression of the MT gene in *Podarcis* embryos underwent shifts in both regional and cellular localization during subsequent stages of development. In embryos at 40 days post deposition (length 26 mm) MT transcripts simultaneously disappeared from the telencephalon, diencephalon, mesencephalon and from the ganglion cells layer of stratified retina. In the medulla oblongata and in the cerebellum MT mRNAs were concentrated in the nuclei and in the granular and molecular layers respectively; in the spinal cord messengers were present in the white matter, in both fibres and nuclei of glial cells, whereas the grey matter was completely unlabelled. These events suggested either a consumption of the pool of maternal

Table 2. Detection of MT transcripts in lizard embryo during development.

	Days from deposition				
	5	**10**	**20**	**40**	**50**
Head					
Telencephalon	+	+	+	-	+
Diencephalon	+	+	+	-	+
Mesencephalon	+	+	+	-	+
Medulla oblongata	+	+	+	+	+
Eye	+	+	+	+	+
Trunk					
Spinal cord	+	+	+	+	+
Kidney					
Tubules	-	-	-	-	-
Glomeruli	-	-	+	+	+
Lung	-	-	-	+	+
Liver	-	-	-	-	+
Gut	-	-	-	-	+

mRNAs or alternatively, a temporary silencing of the MT embryonic gene. What really happens and the nature of the triggering factors remains to be clarified. In visceral organs, the MT-mRNA appeared for the first time in the lung parenchyma, whereas was still absent in the liver, gut mucosa and kidney tubules.

Immediately before hatching (50 days post deposition, length 35-40 mm), the distribution of MT transcripts underwent further relevant changes. Significant labelling was present on the cortical areas of the telencephalon and mesencephalon, on the ependymal cells of the tele- die- and mesencephalic vesicles and on several basal nuclei. MT-mRNA distribution in the medulla oblongata and in the spinal cord did not change with respect to the previous stage. In the differentiated retina the MT-mRNA was present in the outer and inner nuclear layers and in the ganglion cells layer. The two plexiform layers and the optic fibres layer were unstained. A significant hybridization signal was observed for the first time also in the gut mucosal cells and in the liver, in particular on the cytoplasm of the Kupffer cells and monocytes. No changes were observed in the kidney tubules that remained unstained.

In summary, the results demonstrate that the absence of MT transcripts in gut, liver and kidney persisted throughout the development until hatching. This may be due to peculiarities of this closed system where embryos grow using the internal egg nutrient content and detoxifying organs are not yet required. Immediately before hatching newly synthesized MT transcripts were detected and this may be related to the fact that the embryo prepares to become an open system obtaining food from the environment.

Hence, the results show the complexity of the pattern of expression of MT genes during vertebrate development, highlighting differences in lizard (and probably in other oviparous terrestrial vertebrates) embryonic spatial and temporal MT expression compared with mammalian embryos, in which the expression of MT gene is activated early and abundantly in the visceral organs with detoxifying functions, whereas the gene is activated late, immediately before of the birth or at birth, in the brain [31].

Cadmium-induced expression of metallothionein gene in lizard embryonic tissues

Several studies indicate that MTs protect embryos from intracellular damage caused by cadmium. In mammals, MTs favour the maintenance of placental integrity, protect against hepatic poisoning and protect trophoblast cells from morphological alteration and apoptosis [32, 33, 34].

The intake of cadmium in vertebrates occurs mainly by ingestion of contaminated food and water. Generally, embryonic exposure and uptake varies greatly and is species dependent. In mammals, the placenta is the primary target for this metal [35, 36]; however, placental transport during embryogenesis has been demonstrated [37, 38]. Embryos of terrestrial oviparous vertebrates have been considered well protected from the external environment and the presence of environmental contaminants in eggs or developing embryos has been attributed to maternal transfer during vitellogenesis [39]. But recently it has been demonstrated that metals and organic contaminants may cross the parchment-like shell of reptilian eggs, affecting embryos morphology and gene expression [40, 41, 42, 43, 44].

In our studies, no mortality was observed in embryos incubated in Cd contaminated soil at levels compatible with environmental pollution (50 mg Cd/Kg soil). However embryos at different developmental stages showed severe malformations, which would be incompatible with adult survival [21, 45]. Cd-induced morphological alterations, mostly concentrated in encephalic and optic areas, are listed in Table 3. Severe malformations affected the cranial bone structure (including the lack of the cranial vault, the deformation of the skull base, the palate, the optic capsule and jaws), the encephalic areas of telencephalon, diencephalon and mesencephalon and the eye. No alterations are observed in medulla oblongata, spinal cord and visceral organs, whose structures are always comparable to those of controls throughout development.

Densitometric analyses of Northern blots showed an appreciable increase in the amount of MT mRNA in embryos developed in Cd contaminated soil with respect to untreated embryos, suggesting the induction of the embryonic MT gene by heavy

Table 3. Main morphological abnormalities in *Podarcis sicula* embryos incubated in Cd contaminated soil.

Morphological alterations	% of affected embryos
Anencephaly	5
Exencephaly, ventricle swelling	65
Microphalmia, retinal malformations	50
Facial abnormalities	20
Limb abnormalities	1

metals. However, *in situ* hybridization experiments did not reveal significant changes in spatio-temporal localization of MT mRNA in developing brain and retina of control and Cd-treated embryos. Encephalic areas and optic cups carrying severe alterations showed considerable amount of MT transcripts whose localization was the same as found in intact structures [21, 45].

In contrast, a Cd-induced spatio-temporal shift in embryonic MT gene expression was observed in some developing trunk organs. For kidney and lung, the timing and localization of MT transcripts in Cd-treated embryos did not differ from control embryos, whereas in gut and liver an early expression of MT gene was seen in Cd-treated embryos [21]. In intestinal mucosa and liver sinusoids particularly, the hybridization signal appeared in 10-day-old embryos but in control embryos MT mRNA was detectable only immediately before hatching [16, 21].

The abundance of MT transcripts observed in the early stages of embryonic development in the brain and in the eyes did not seem to provide for protection against the toxic effects of cadmium. Possibly the MT mRNA was translated in gut and liver but not in brain; alternatively, proteins translated from these transcripts in brain were engaged in functions other than heavy metal detoxification. On the contrary, the earlier Cd induced MT gene expression in liver and gut seems to act by preventing morphological alterations of the developing visceral organs.

Together, this data suggests a possible correlation between Cd-inability to induce MT expression and the morphological alterations observed in lizard embryos.

Evolution of metallothionein gene(s) in squamate reptiles

In order to establish the presence of more than a single MT isoform in reptiles and, more in general, to fill the gap of information about MT gene(s) evolution in reptiles, we cloned and sequenced the cDNA encoding the MT from 11 distinct species belonging to the Squamata order. Samples for *Podarcis sicula*, *Anguis fragilis*, *Chalcides chalcides*, *Elaphe quatorlineata* and *Zootoca vivipara* were caught in the outskirt of Naples, under the permission of the Italian Health Ministry [46]; liver samples from the Malagasy squamate species (*Calumma brevicornis*, *Furcifer pardalis*, *Oplurus quadrimaculatus*, *Phelsuma barbouri*, *Paroedura masobe*, *Pygomeles braconnieri*) were kindly provided by Dr. Gennaro Aprea [46]. All reptilian MT identified in this study have a coding sequence of 189 bp corresponding to a protein of 63 amino acids, 20 of them being cysteines, homologous to other vertebrate MTs. The alignment of the deduced amino acid sequences reveals a high degree (more than 80%) of overall similarity among squamates (Fig. 1). Noteworthy, some of them are characterized by the presence of residues such as arginine and histidine, typical of avian MTs. The presence of the histidine at the C-terminal site of the MT sequence of *A. fragilis*, *O. quadrimaculatus*, *C. brevicornis*, *F. pardalis*, *P. braconnieri* and *C. chalcides* could affect the metal coordination and the dimerization capability of proteins [8, 47, 48, 49]. Hence, it is possible that some of squamate MTs have a different metal buffering capacity that could modify the metal ion release.

Our attempts to ascertain the presence of expressed MT isoforms in squamate reptiles failed, since we

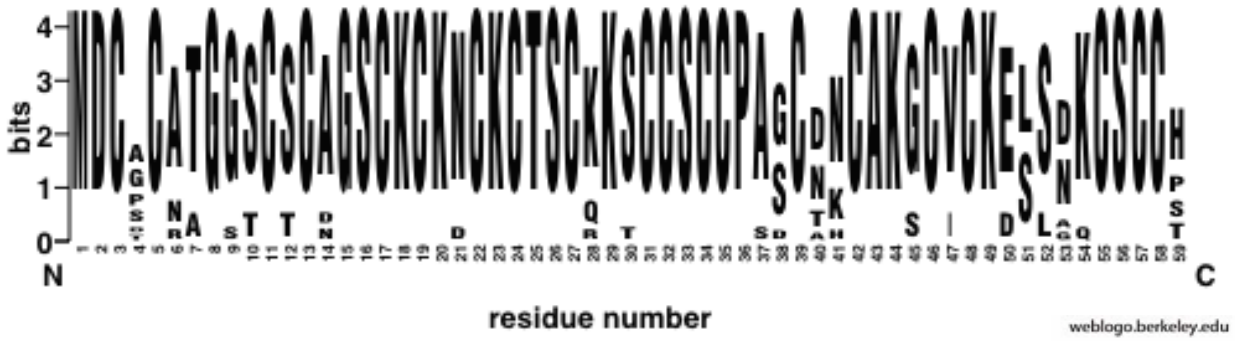

Fig. 1. Logo representation of aligned metallothionein amino acid sequences from 11 distinct species belonging to the Squamata order.

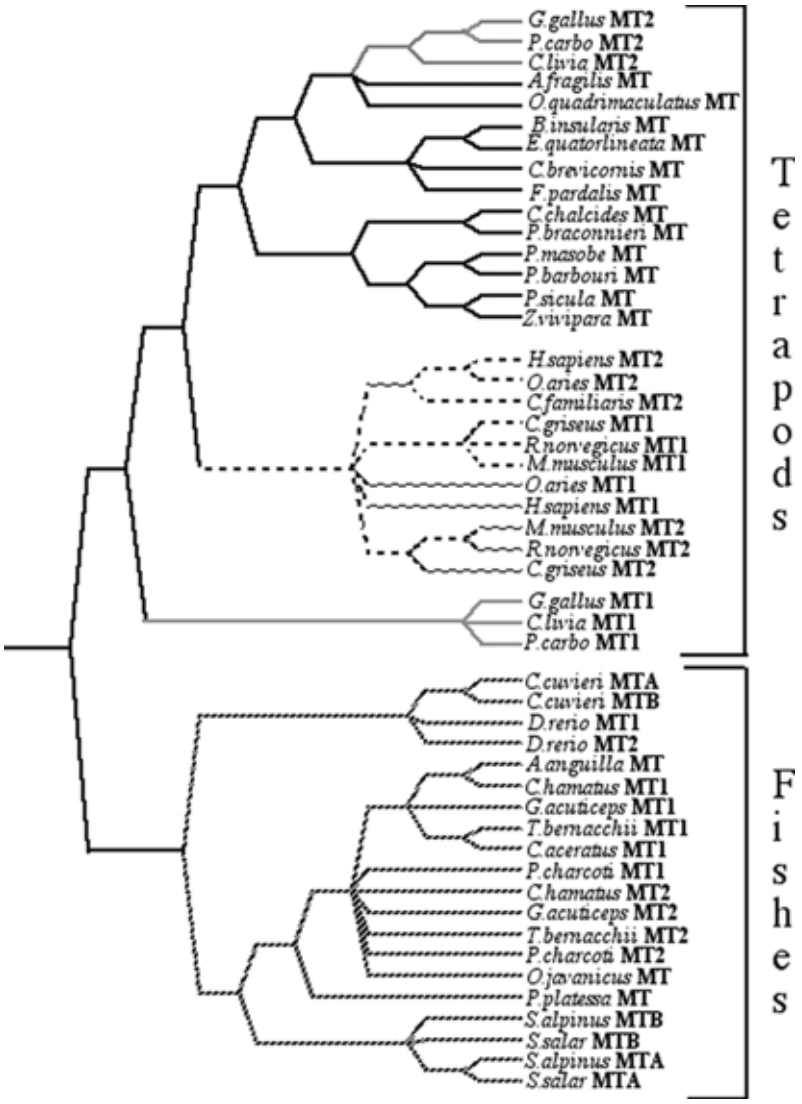

Fig. 2. Phylogenetic tree of the amino acid sequences of the vertebrates MT inferred by the Bayesian method implemented in the software Mr. Bayes v. 3.1.

obtained always a unique MT form from lots of sequenced clones [46]. Likewise, the same isoform was sequenced from different organs [13]. Currently, we are not able to establish if other reptilian genes encoding MT isoforms have been lost or are simply not sequenced.

A phylogenetic analysis was carried out according to the Bayesian method [46], using MT amino acid sequences from the major vertebrates groups. In the bayesian tree shown in Fig. 2 it was possible to identify two major clades, one of piscine MTs and another comprising MTs of tetrapods. In the latter, the avian MT-I isoforms produced a well-separated clade merging basal to the other groups of tetrapod MTs. On the contrary, avian MT-2 isoforms are included in the squamate MTs clade, forming a "Diapsida cluster" with good bootstrap support. Hence, MT gene duplication in avian species was an ancestral event that occurred before their speciation; avian MT1 gene represents an ancestral form of MT, retained in birds and lost in the other tetrapods.

Phylogenetic position of the investigated reptilian taxa in the MT gene tree is in contrast with the one expected from the species phylogeny, inferred using multiple line of evidence [50, 51, 52]. This suggests that reptilian clade might contain paralogous genes, thus implying that MT genes in squamates might have experienced more than a single duplication event. Therefore we decided to infer a reconciled tree [46, 53], starting from the assumption that the discord between species tree and MT gene tree was due to duplication of gene loci and subsequent extinction of (or failure to sample) some loci in some lineages. The phylogeny inferred in such a way suggested the occurrence of 3 duplication events and 10 losses along different lineages (Fig. 3). As stated above, gene loss may simply mean that certain genes have never been sequenced, but in a number of cases the genes may be really missing or functionally inactivated.

These data, that represent the first survey on the molecular evolution of squamate MTs, unravel the complex evolutionary pattern of these MTs, characterized by a number of gene duplication events and, possibly, by instances of gene loss. Taking into account reptilian MTs, the phylogeny of vertebrate MT genes demonstrates the presence, in birds, of an ancestral form of MT,

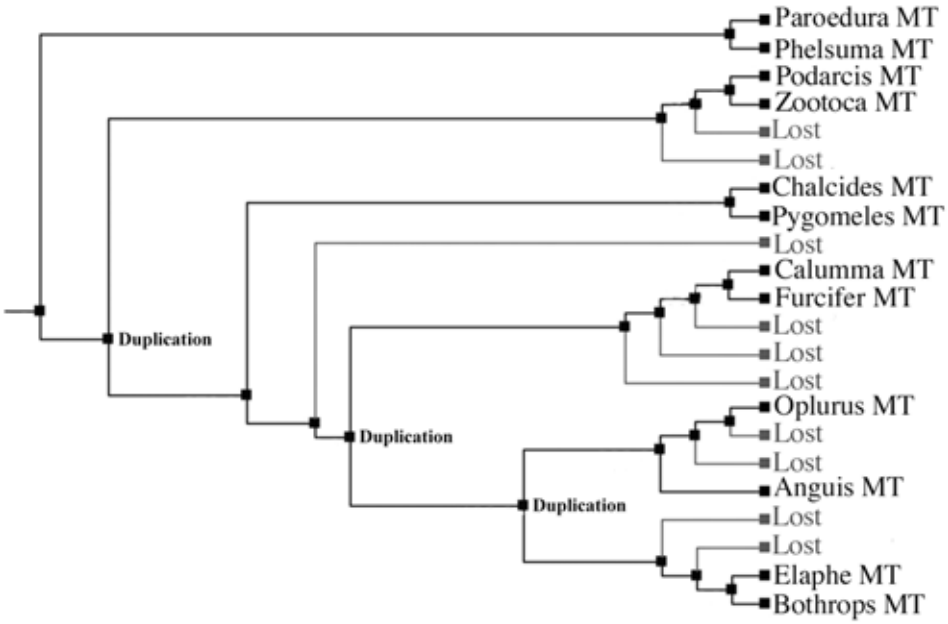

Fig. 3. Representation of the reconciled tree for the squamates MT gene. The inferred tree supports the occurrence of 3 duplication events and 10 losses along different lineages; hypothetical branches leading to lost or missing sequences are shown as grey lines.

basal to all the other tetrapod MTs, and also discloses for the vertebrate MTs an unexpected complexity in the primary structure that might suppose some functional differences in proteins.

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