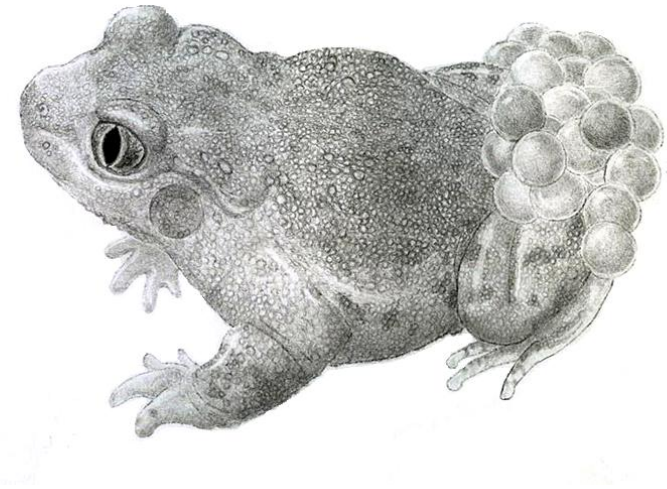

Phylogeny and molecular biology in amphibian ecotoxicology

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2013

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, ______/______/_________

Acknowledgements

I want to thank to my supervisor Ylenia Chiari for support at every single moment I needed, in teaching, guiding, by finding together the solution on several software dilemmas that we faced together, by helping to deal with stressful situations that we could not control or foreseen during the year, by cheering me up after some arising problems with the work, by giving me valuable advices that I will never forget during life, and finally, I want to thank her for seeing me as her student and friend, listening, accepting and sometimes hoping to hear more my opinions or doubts.

I also want to thank Miguel Carretero for all the confidence and support he gave me ever since I met him, for the discussion of the most interesting scientific topics related to our, his or other people's work, for all support he provided to my research, and for being such a good friend. Additional thanks to Enrique García-Muñoz (Kike) for his crucial work providing the sampling material needed for the experiments in very unusual and difficult climatic scenarios in Spain (very dry winter), and for all the laboratory ecotoxicological tests in the tadpoles, essential for our work, showing a great dedication and persistence towards the project and my thesis subject.

Concerning the article submission to Journal of Molecular Evolution with Miguel and Ylenia during the thesis progression, we want to give special thanks to Jean-Paul Doyon for his useful help with the reconciliation analysis and comments on and early version of the manuscript, and to Miguel Fonseca, João Lourenço, Benoit Nabholz, Diane Salvi and Ziheng Yang for feedback on this work.

Thanks also to Liliana Farelo for her incredible availability to help us at last-hour laboratory analyses in CIBIO, to Pedro Cardoso for his assistance in the preparation of early lab work (primer design, verify endogenous control genes, etc.) and sampling work in Aveiro, and to Bárbara Santos and Isabel Lopes for providing the assistance needed by the time of the sampling of Aveiro mesocosms and also collection of some samples for analysis; to Eduardo Lopes of Bio-RAD for helping setting up the RT-qPCR experiments and feedback on the cDNA and gene characterization, to Scott Glaberman for all the help obtaining the LC50 data for the study on phylogenetic signal in amphibian sensitivity to copper sulfate, to Isabella Capellini for helping with the analyses for this study, and to Inês Cunha for the wonderful drawing of the front page. To FCT, thanks for partial financial support to the study.

Finally I want to express my deep thanks to my family and friends which gave me a lot of support, and to my dearest friend of all and boyfriend Pedro Andrade, the one person capable of brighten my thoughts, and life.

Abstract

Amphibian ecotoxicology is one of the crucial scientific fields necessary to understand the underlying mechanisms behind the rapid decline of this animal group and to what extent anthropogenic actions are responsible for it. Pollution and contaminants in natural environments are seriously threatening biodiversity. The molecular response of organisms at cellular level (e.g. gene expression) is the first sign of defense to react to harsh polluted conditions. This may therefore serve to illustrate the type of genetic or chemical responses that evolved to prevent species decline. In this context, to gather a better understanding on how amphibians may respond to contaminants, we focused our study on the metallothionein superfamily. In vertebrates, these metal binding proteins are involved in various metal-detoxification mechanisms and can therefore be considered as candidate genes involved in the organisms reaction of detoxification from heavy metal contamination in the environment. As a first task of our study, we carried out a phylogenetic and functional diverge analysis to study the evolution of this multigene family in vertebrates. The aim of our study was to estimate gene gain and loss events as well as to uncover gene sites correlated with functional divergence among taxa and paralog genes. This approach will also permit to design cost-effective metallothionein protein functional studies in non-model vertebrates for which little is known. We focused our work on the effects of pollution due to the heavy metal copper (used as a component of commonly used pesticides in agriculture, such as copper sulfate). The second goal of this thesis was to assess the phylogenetic signal (if any) in the sensitivity of amphibians to copper sulfate (measured as LC50) and to temperature variation. We wanted to estimate the influence of temperature on amphibian LC50 to copper sulfate to gain further understanding on how future raising in environmental temperature could affect this parameter, and therefore organism survival. Our results show a strong influence of temperature on amphibian LC50 to copper sulfate. Once this influence is taken into account, our data show a phylogenetic signal in LC50. This result, if confirmed on a larger taxon sampling, can be used as a predictive tool in amphibian conservation and ecotoxicology. We then concentrated on developing methods to study the molecular response to copper sulfate on a target European amphibian species, the Natterjack toad *Epidalea calamita* (Laurenti, 1768). We choose this species since the symptoms, (developmental and behavioral effects) due to exposure to copper sulfate are well known. For this study, we collected genetically similar individuals belonging to a single clutch (obtained by one couple mating in a controlled environment). Eggs from the clutch were raised separately in different aquaria (laboratory conditions) and mesocosms (semi-natural environment) and treated either with no contaminant or with a range of copper sulfate concentrations that are commonly found in the environment with known effects (see above). The treatment includes also two different temperature to test. The samples were collected at the tadpole stage and flash frozen in liquid nitrogen to preserve optimal gene expression. RNA was extracted from these samples and retro - transcripted to cDNA. The goal of this part of our work was to isolate the metallothionein gene (one gene was expected based on previous and our works) in the study species and to develop samples and tools to study metallothionein gene expression following treatment with copper sulfate and different temperature in *E. calamita*. One of the cDNA samples treated with copper sulfate was used to isolate the single metallothionein gene by a pair of non-specific primers. Finally, as one of the outcomes of this work is to develop tools to study metallothionein gene expression following treatment with copper sulfate, the last goal of the project was to develop and test optimal endogenous controls (constitutive genes) to be used for future gene expression experiments using qRT-PCR. The qRT-PCR permits a quantification of the genetic expression changes. This technique requires a proper selection of reference genes to be used as endogenous control, fulfilling the characteristic of showing a constant expression for the tested situation and species. Therefore, from an initial list of 28 known reference genes, we selected six candidate genes for which we could design degenerate primers to characterize them from cDNA and test their stability of expression in *E. calamita* and in the experimental settings.

Keywords: Ecotoxicology, Amphibian, Molecular phylogenetics, Metallothionein, Endogenous control

Resumo

A ecotoxicologia de anfíbios é um dos ramos científicos fulcrais para compreender quais os mecanismos subjacentes ao rápido declínio deste grupo taxonómico, e em que medida a acção antropogénica é responsável por isso. A poluição e contaminantes em meios naturais estão a ameaçar seriamente a biodiversidade. A resposta molecular a nível celular (p.e. expressão génica) é o primeiro sinal de reação de defesa à poluição. Esta reação pode portanto ilustrar o tipo de resposta genética ou química que pode ter evoluído de forma a prevenir o desaparecimento da espécie. Neste contexto, para melhor compreender de que forma os anfíbios podem reagir a poluentes, focamos este estudo na superfamília das metalotioninas. Estas proteínas de ligação a metais estão envolvidas nos mais diversos mecanismos de detoxificação em vertebrados, podendo então os genes estar implicados na resposta de defesa a uma contaminação do meio ambiente por metais pesados. Como primeiro passo neste estudo, efetuámos análises filogenéticas e de divergência funcional para estudar a evolução desta família de genes, em vertebrados. O objetivo deste projeto foi estimar o número de eventos de ganho ou perda de genes assim como revelar posições do gene correlacionadas com a divergência funcional entre taxa e genes parálogos. Esta abordagem vai de igual forma permitir projetar estudos sobre a funcionalidade das metalotioninas em vertebrados não-modelo, ainda debilmente estudados. Centramos este estudo nos efeitos da poluição devido ao cobre (metal pesado constituinte de pesticidas comumente usados na agricultura, tais como o sulfato de cobre). O segundo objetivo trata da avaliação do sinal filogenético (se existente) à sensibilidade dos anfíbios ao sulfato de cobre (medida como LC50) e à variação de temperatura. Pretendemos estimar a influência da temperatura na LC50 de anfíbios ao sulfato de cobre para obter maior conhecimento sobre como o futuro aumento de temperatura no meio ambiente pode afetar este parâmetro e, desta forma, comprometer a sobrevivência dos organismos. Os nossos resultados mostram uma forte influência da temperatura na LC50 de anfíbios em relação ao sulfato de cobre. Assim que esta influência é considerada, os resultados evidenciam um sinal filogenético para a LC50. Este resultado, se confirmado numa maior amostragem de taxa, pode ser utilizado como uma ferramenta de previsão em conservação e ecotoxicologia de anfíbios. Focamos também o desenvolvimento de métodos para poder estudar a resposta molecular da espécie-alvo europeia, o Sapo-corredor (*Epidalea calamita*), ao sulfato de cobre. Escolhemos esta espécie dado que os sintomas devido à contaminação de sulfato de cobre (efeitos no desenvolvimento e comportamento) são já bem conhecidos. Para este estudo adquirimos indivíduos geneticamente semelhantes pertencentes a uma única massa de ovos, obtida após acasalamento do casal em ambiente controlado. Os ovos desenvolveram-se separadamente em aquários distintos (condições laboratoriais) e mesocosmos (condições semi-naturais) e foram tratados na ausência de contaminante ou com um gradiente de concentrações de sulfato de cobre frequentemente encontradas no meio ambiente e com efeitos já conhecidos. O tratamento incluiu também duas temperaturas diferentes a testar. As amostras foram obtidas em estado de desenvolvimento de girino e vitrificadas em azoto líquido para preservar a expressão génica. O RNA foi extraído destas amostras e retro-transcrito para cDNA. O objetivo deste passo foi isolar o gene da metalotionina (apenas um gene era esperado, baseado no nosso estudo e anteriores) na espécie-alvo e desenvolver amostras e ferramentas para estudar a expressão génica deste gene seguida do tratamento com sulfato de cobre a diferentes temperaturas. Uma das amostras de cDNA proveniente de tratamento com o contaminante foi utilizada para isolar a única metalotionina por intermédio de primers não-específicos. Finalmente, como um dos objetivos desta investigação é desenvolver ferramentas para estudar a expressão génica seguida do tratamento com sulfato de cobre, o último ponto a atingir neste projeto é desenvolver e testar genes de referência para controlos endógenos optimizados para uso futuro em experiências de expressão génica usando qRT-PCR. O qRT-PCR permite a quantificação das variações de expressão génica. Esta técnica requer a selecção cuidada de genes de referência a usar no contolo endógeno, refletindo uma expressão constante para a situação e espécie testadas. Portanto, de uma lista inicial de 28 genes de referência conhecidos, seleccionamos seis genes candidatos para os quais pudemos desenhar primers degenerados para caracterizá-los a partir do cDNA e testar a sua estabilidade em *E. calamita* e nas configurações experimentais.

Palavras-chave: Ecotoxicologia, Anfíbios, Filogenética Molecular, Metalotionina, Controlo endógeno

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Abbreviations

AICc – Akaike's information criterion corrected for finite sample size

CDS - Coding sequence

CCDS - Consensus coding sequence

dN - Non-synonymous substitution rate

dS - Synonymous substitution rate

ERA – Ecological risk assessment

fd - degrees of freedom

GRAVY - Grand average of hydropathicity

ISS - index of substitution saturation

ISS.c - index of substitution saturation critical value

K - Bayes factor value

LC50 – Lethal Concentration 50

LR - Likelihood Ratio

LRT - Likelihood Ratio Test

ML - Maximum Likelihood

MT - Metallothionein

nst - number of substitutions

pinvar - proportion of invariable sites

PGLS – Phylogenetic least-squares models

pp - posterior probability

qRT-PCR – quantitative Real-Time PCR

t - time

T - temperature

Ta - annealing temperature

ω - ratio between dS and dN

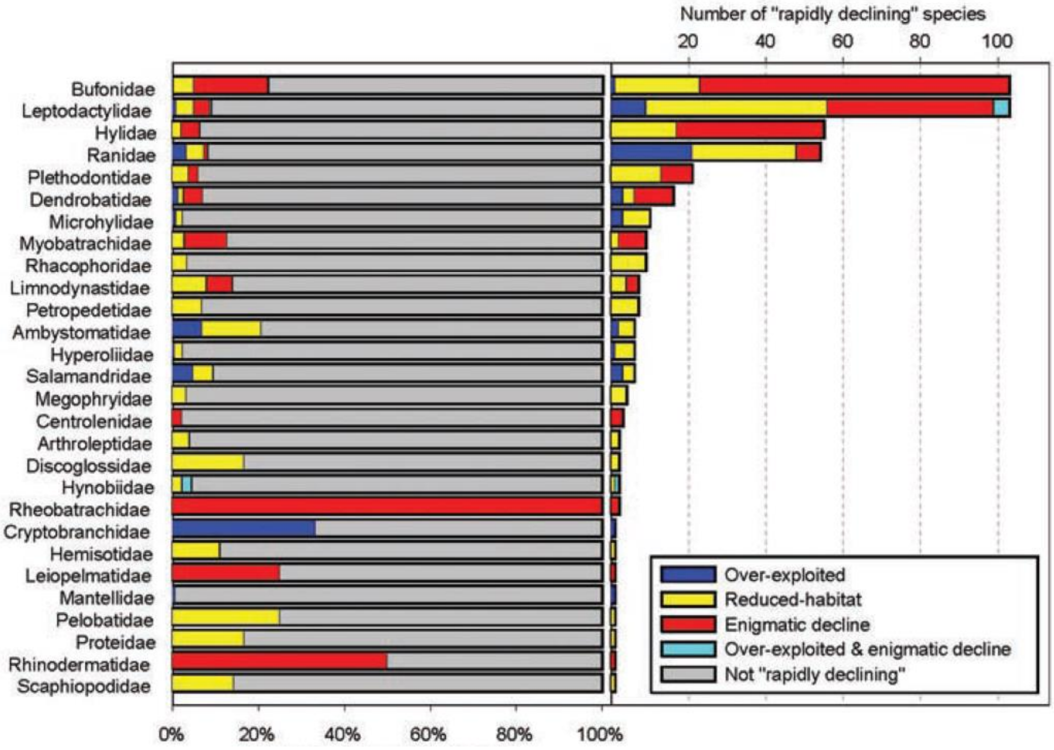
Introduction

Ecotoxicology

Ecotoxicology is a scientific field studying the effects that toxic compounds have on organisms, their community or the ecosystem in which they live in. Ecotoxicological approaches are increasingly relying on the integration of distinct scientific fields in an attempt to improve the understanding of the mechanisms underlying the impacts of contaminants across all levels of biological organization. The multidisciplinary essence of Ecotoxicology, combining Toxicology and Ecology, is essential to characterize, comprehend, and predict the effects of contaminants on biological systems (Moriarty 1983). This methodology line is believed to be relatively accurate tracing environmental demands, which is highly important in an accelerating “humanized world”. In fact, the scientific community accepts that anthropogenic effects are increasingly endangering biodiversity (reviewed in Barnosky *et al.* 2011). Among the human-driven impacts on environment, pollution and climate change are of great concern (Vitousek 1994; Botkin *et al.* 2007).

Classical amphibian ecotoxicology

Amphibians are highly susceptible to the impact of human activities that cause environmental changes (Beebee and Griffiths 2005; Hopkins 2007; Blaustein *et al.* 2010, 2011) in ecosystems. In fact, amphibians have been facing a global declining in the last decades at unprecedented rates (Stuart *et al.* 2004; Wake and Vredenburg 2008, see also Figure 1).



Percentage of all declining species in family

Figure 1 - Amphibian families and respective species with rapid declining status classified as being caused by overexploitation, habitat loss or enigmatic decline. Percentages of species under decline are referenced in each family, depending both on the high number of species under rapid decline (see right section of the figure) but also on the on the number of species that constitute each family. Adapted from Stuart *et al.* (2004).

Numerous possible causes for this rapid decline have been highly debated in the scientific community (reviewed in Hopkins 2007). There is now a widely accepted agreement that the forthcoming extinction of many amphibian species was and is being caused mostly by extremely complex interactions driven by anthropogenic effects (Collins and Storfer 2003; Pounds *et al.* 2006; Hopkins 2007; Blaustein *et al.* 2011) – see also related Figure 2.

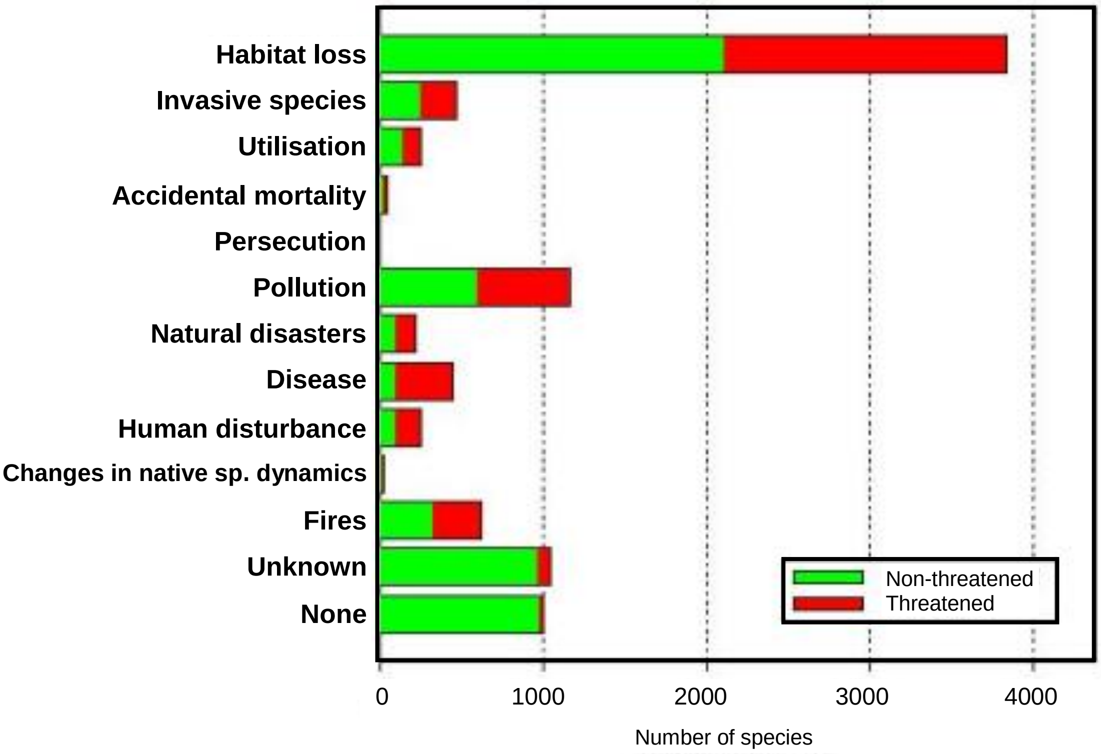


Figure 2 - Recognized major threats associated with the worldwide amphibian decline. Habitat loss is considered the uppermost concern to amphibian conservation, followed by pollution. Almost every threat in this plot is based on anthropogenic disturbances. Adapted from IUCN (2009).

Among the factors more strongly correlated with this decline are habitat destruction and alteration, human exploitation, introduction of invasive species, climate change and disease outbreak (Blaustein and Kiesecker 2002; Beebee and Griffiths 2005; Blaustein *et al.* 2011). Amphibians are known to play an important role in ecosystems for their different life stages (tadpoles and adults) that range from herbivorous to carnivorous regimes in trophic webs (Hopkins 2007), and for their high contribution to the transfer of energy and nutrients through food webs (Wyman 1998; Colón-Gaud *et al.* 2009). Due to their trophic role, high sensitivity to environmental changes (as direct consequence of a great variety of factors including the permeable skin to pollutants or for many species the use of aquatic and terrestrial habitats during their life stages) and their alarming decline, amphibian ecotoxicology has become an essential field of study to investigate and characterize the level of ecological disturbances in ecosystems (Hopkins 2007).

Amphibian larvae often occupy stream ecosystems, facing major pollution risks since agricultural and industrial contaminants from various sources are accumulated in most freshwater habitats. Numerous studies have already quantified and demonstrated a great number of morphological and behavioral changes on larvae under contaminant exposure (e.g. Bridges 1997; García-Muñoz *et al.* 2011a, b, Yu *et al.* 2013). However, the described negative effects have not been fully evaluated or used for quantitative assessments of risk, remaining unable to clarify their role in amphibian decline (see Hayes *et al* .2006 and references therein). In vertebrates, most ecotoxicological tests are usually related to the status of the populations: survival, growth and measures of reproductive success (e.g. Miracle and Ankley 2005). Such standard ecotoxicological studies have proved to be of valuable help in current risk assessments in several life forms (Fedorenkova *et al.* 2010). However, the use of molecular techniques could provide important information on contamination sub-lethal effects with no visible phenotypic manifestation and improve the risk assessment process and (be used as an early warning sign of occurring contamination) (reviewed in Robbens *et al.* 2007). Efficient risk assessment studies on amphibians already used molecular approaches (Veldhoen and Helbing 2001). Molecular biology methods can therefore provide a helpful tool in identifying unknown cellular/genetic effects due to contaminants in the environment that could eventually help to clarify the role of pollution on amphibian global decline.

Molecular Biology in Ecotoxicology

Knowledge on the inter and intra-specific genetic variation together with phylogenetic relationships among taxa can largely contribute to further analyze toxic implications in biological systems (Coutellec and Barata 2011). In some cases, phylogenetic knowledge on the studied group may allow the detection of strong phylogenetic signals for pollution responses, possibly giving further indication whether species can be more or less tolerant (Carew *et al.* 2011). Key-gene families phylogenies can equally be of great importance to clarify at what extent gene duplication and function differentiation may be associated with distinct functions and efficiencies for a function (e.g. Tío *et al.* 2004; Chang and Duda 2012). For example when looking at a gene with known function in organism detoxification from chemical substance, a phylogenetic approach could provide insights into the effects of the contaminant on different taxa.

Another very important and promising scientific area within Ecotoxicology is Genomics. Genomics is a field which encompasses a great number of techniques from genome sequencing, annotation of function to genes, patterns of gene expression (transcriptomics), protein expression (proteomics) and metabolite flux (metabolomics) (as in Kille *et al.* 2003). Gene expression profiling is one of the most relevant genomic approaches to environmental analysis and stress tests. This technology, among other genomic based techniques, has the potential to increase risk assessment power analyses and to facilitate a rapid evaluation of: 1) the toxic compound potential effects and 2) the response of the biological systems to environmental change (Kille *et al.* 2003). In ecotoxigenomics, gene expression analyses is generally carried out by exposing organisms under certain conditions (e.g. concentration of a known pesticide) and profiling their transcriptome by microarrays or quantitative RNA sequencing (van Straalen and Federer 2011).

Amphibians, Genetics and Ecotoxicogenomics

Amphibians have been largely covered in toxicological experiments that focused at genetic or biochemical responses to contaminants (e.g. LeBlanc and Bain 1997; Veldhoen and Helbing 2001; Zocche *et al.* 2013). The most common species for ecotoxicological studies is *Xenopus laevis* (US EPA, 2012 and reviewed in Helbing 2012). Currently, this is also the only amphibian species with a fully sequenced genome available in public databases. This facilitates the use of this species, instead of others, in toxigenomics, even if its representativeness as a model amphibian or even as a natural frog species (most studies rely on animals bred in captivity since many generations) is far from ideal (Helbing 2012).

Ecotoxicological tests: Natural, semi-natural and artificial conditions

To provide meaningful results, ecotoxicological tests should be performed on: 1) individuals collected under carefully designed and controlled experimental conditions, and 2) individuals collected in semi-natural conditions (e.g. microcosms, mesocosms).

Mesocosms, or replicated outdoor artificial systems containing simplified self-sustaining communities, provide a compromise between reductionist laboratory experiments and uncontrolled, difficult-to-interpret field observations, permitting also rigorous experimental design and statistical analyses due to the replication not achieved in natural conditions (Odum 1984). Mesocosm experiments, in an early phase, can also serve as a pilot study to look for differences between laboratory and semi-natural conditions, offering a new perspective on how mesocosm setting changes th treatment results

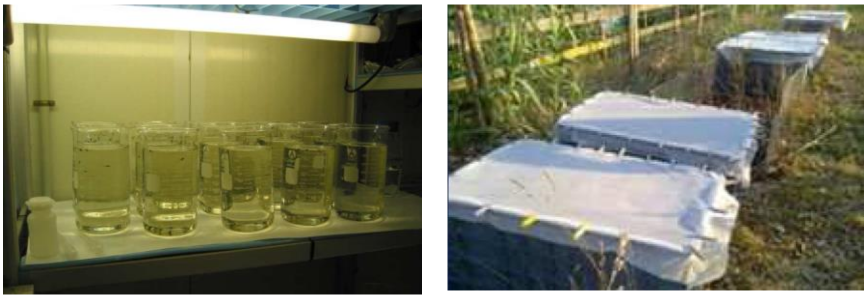


Figure 3 – Laboratory and mesocosm settings used in our study. Photos: Enrique García-Muñoz

Experimental Design - Study species, contaminant and temperature effect, and gene of study

Our study species is an anuran, the Natterjack toad (*Epidalea calamita*). This species is generally found in a wide range of territories in Europe and makes use of shallow temporary ponds and lagoons in early stages of life (short larval development period) and for spawning (IUCN, Beja *et al.* 2009). It is known to survive in medium-high contaminated ecosystems across the Iberian Peninsula (García-Muñoz *et al.* 2009) and it has been demonstrated that it is affected by copper sulfate exposure (García-Muñoz *et al.* 2009; 2010; 2011b) on laboratory conditions. Copper sulfate ($CuSO_4 \cdot 5H_20$) is a highly toxic component often used as fungicide and algaecide in agricultural practices, and it is encountered also in textile, leather and oil industries discharges (U.S. Environmental Protection Agency 1986).

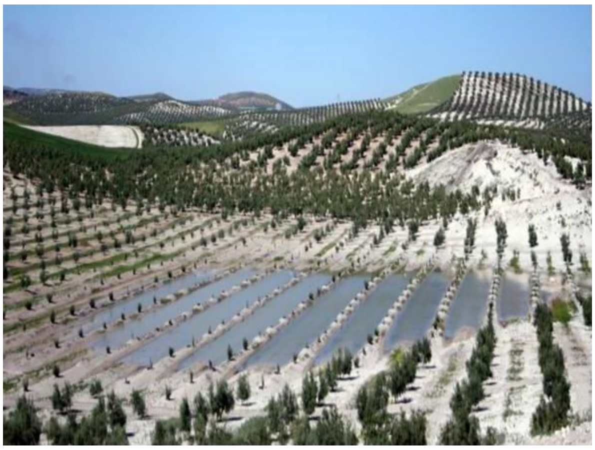


Figure 4 - Natural pond containing amphibian species (including *E. calamita*) showing high levels of copper sulfate contamination. Fungicide containing this toxic compound is used across vast areas of olive grove monocultures, affecting dramatically aquatic ecosystems and biodiversity in every stream or nearby ponds. Photo: Enrique García-Muñoz.

Due to these unwary origins of compounds containing this highly toxic component, high levels of copper contamination have already reached natural aquatic habitats, affecting a great number of species (Herkovits and Helguero 1998; U.S Environmental Protection Agency 2008).

Several studies on amphibians demonstrated the negative effects of copper by testing the toxic effects of copper sulfate or copper exposure in lab conditions to anurans (Porter and Hakanson 1976, Khangarot and Ray 1987, Herkovits and Helguero 1998; García-Muñoz *et al.* 2009, 2010, 2011b; Gürkan and Hayretdağ 2012; Santos *et al.* 2013). Contaminant effects reported for *E. calamita* include morphological, histological and behavioral traits (García-Muñoz *et al.* 2009, 2010, 2011b). However, to date, nothing is known concerning molecular and/or genetic mechanisms triggered in *E. calamita* by the pollutant.

Figure 5 - Natural pond where eggs from *E. calamita* were collected for the study.
Photo: Enrique García-Muñoz

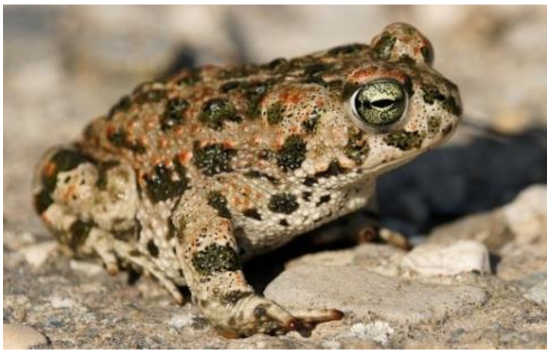


Figure 6 - Adult specimen of *E. calamita.*
Photo: Matthieu Berroneau

Previous studies on several organisms have shown that one immediate effect of copper was to enhance metallothionein (MT) protein production and metallothionein gene expression (Lam *et al.* 1998; Riggio *et al.* 2003; Mosleh *et al.* 2006; Serafim and Bebianno 2009). This is most likely correlated to the detoxification function of these proteins in presence of heavy metals. A demonstration of MT effect on amphibians is still to be studied. Until date, only MT protein production in amphibians has been demonstrated and not by copper effect cadmium exposure (Suzuki *et al.* 1986; Pérez-Coll *et al.* 1997). Despite the evident pertinence that the knowledge of amphibian MT gene expression (under different experimental conditions) could bring to understand amphibian sensibility to pollution or environmental change, MT gene expression was never studied in any amphibian species.

MTs are metal-containing proteins that bind zinc, copper and other metallic ligands thanks to the cysteine residues of their polypeptide chain (Palmiter 1998). MTs fulfill a wide range of functions including detoxification of toxic metals, scavenging of harmful reactive oxygen species, and others (reviewed in Carpenè *et al.* 2007; Blindauer and Leszczyszyn 2010). MTs have therefore been largely used in biomonitoring programs to characterize metal contamination in the environments (Linde *et al.* 2001). In addition, MT gene expression under different metal exposure scenarios has been proposed as a sensitive and efficient biomarker for evaluating the cumulative biological effects of metal exposure (Ceratto, *et al.* 2002; Tom *et al.* 2004; George *et al.* 2004).

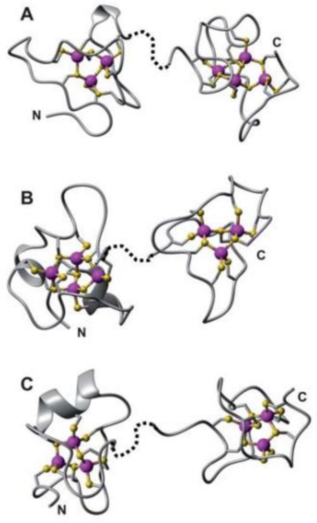


Figure 7 – Three-dimensional models of MT proteins from three distant species (A – rat; B – sea urchin; C – blue crab). Adapted from Blindauer and Leszczyszyn, 2010.

An interesting, yet to be fully understood, relationship concerning the copper effect on the MT gene expression, consists on adding the effect of temperature (T) variation to the equation. Temperature patterns vary significantly year to year and it is expected to increase in its variation due to global climate change, which represents a great concern in terms of how global biodiversity will respond to it. Amphibians are among the species for which changing temperatures are considered to represent a strong threat to animal survival (Blaustein and Kiesecker 2002, Blaustein *et al.* 2010; Wake and Vredenbourg 2008). Previous studies have already demonstrated a synergetic effect of temperature with the contaminant, negatively influencing the impact of the contaminant in many aquatic organisms, like fish (Macek *et al.* 1969; Mayer and Ellersieck 1986; Capkin *et al.* 2006; Osterauer and Köhler 2008) and amphibians (reviewed in Blaustein *et al.* 2010; e.g. Boone and Bridges 1999). When the opposite effect is observed (temperature reduces toxic effect, see Rohr *et al.* 2011) some authors have proposed to be due to the increased metabolic rates associated with the rising temperature that can lead to a more quick response against the contaminant as in some insecticides (Mayer and Ellersieck 1986). Another possibility is that increasing temperature determines an accelerating embryonic and larval development, which might decrease the amount of exposure of the organisms at these sensitive stages (Rohr *et al.* 2011). Additional studies on more species and populations under the same contaminant are needed to improve predictions and underlying causes of the synergetic mechanisms on temperature versus copper effect mutual interference. Ideally this should be done to understand patterns of response to temperature and pollutants among species and populations.

Objectives

A gene family (metallothionein), known to be involved in metal detoxification, will be studied to provide us an approximation of the number of gene copies present in our study-species. It will also to give us an indication of how tolerance or sensitivity may have evolved through many vertebrates groups including amphibians.

To isolate for the first time the MT gene in the species studied for my thesis and to study the evolution of this gene family in vertebrates to further investigate its correlation to distinct functions (including metal detoxification), we carried out a phylogenetic analysis (Chapter 1 and paper submitted to Journal of Molecular Evolution). The main relevant topics investigated in Chapter 1 regarding the phylogeny of this gene family explore the MT CDS sequence conservation among: selected species, introns or sequence characteristics, number of MT copies in each group (mammals, birds, fish, etc.), estimation of gene gain and loss events, and detection of gene sites correlated with functional divergence between MT groups. Some of these gene parameters could be useful to better understand the effects of pollution due to heavy metal (copper, as copper sulfate) in future studies. To complement this, in

Chapter 2 we assessed the differential copper sulfate LC50 under distinct temperature regimes in amphibians to find a phylogenetic signal.

On the other hand, we carried out another analysis which can broaden the study to a prediction of the copper sulfate versus temperature effect on other species. The study of the patterns of sensitivity on amphibian already available data (in our case, anurans) on copper sulfate exposure at different temperatures, can be extremely useful to predict how other species may respond to the same contaminant through a phylogenetic signal approach. Ultimately, this study permitted to test the influence of temperature on amphibian sensitivity to copper sulfate (LC50) and if could affect amphibian populations survival. This is of paramount importance, especially taking in consideration that most amphibian species are declining and using these species in experiments could result in serious conservation threats.

A case-study evoking these questions is addressed in Chapter 3. In this chapter, I described the work carried out to obtain the MT gene from the study species *E. calamita* by using degenerated primers and PCR amplification on a cDNA sample. The isolated gene can therefore be used for future studies in ecotoxicogenomics on *E. calamita* into studying gene expression following treatment with copper sulfate and different temperature in *E. calamita.* For this reason, in Chapter 4, I developed a study on optimization of endogenous control genes (reference genes) with the required characteristics for control in future qRT-PCR gene expression experiments. In this chapter it is described the gene selection, primer design and methods applied in laboratory to isolate the reference genes from cDNA samples

The aims of my work are therefore:

1) Study the evolution of MT family gene in vertebrates, using species with full genomic data available.
2) Carry out laboratory and mesocosm experiments with copper and temperature testing conditions in *E. calamita*.
3) Infer if there is a phylogenetic signal for copper/temperature effects in anurans.
4) Isolate the MT gene(s) in *E. calamita.*
5) Select appropriate reference genes for endogenous control to test their gene expression stability in our experimental conditions using qRT-PCR.

1. Molecular evolution and functional divergence of the metallothionein gene family in vertebrates

Abstract

The metallothionein superfamily consists of metal binding proteins involved in various metal-detoxification and storage mechanisms. The evolution of this gene family in vertebrates has been previously studied mostly in mammals using a sparse taxon or gene sampling. Genomic databases and available data on metallothionein protein functions and expression allow a better understanding on the evolution and functional divergence of the different metallothionein types. We downloaded 77 metallothionein coding sequences from 20 representative vertebrates with annotated complete genomes. We found multiple metallothionein genes, also in reptiles, which were thought to have only one metallothionein type. Phylogenetic analyses both on nucleotide and amino acid data recovered metallothionein clades corresponding to the eutherian MT1/MT2, potential tetrapod MT3 and amniote MT4, and fish MT. The optimal gene-tree/species-tree reconciliation analysis identified the best root in the fish clade. Functional analyses reveal variation in hydropathic index among protein regions, likely correlated with distinct flexibility and function (or metal affinity) of the protein domains. Analyses of functional divergence identified amino acid sites correlated with functional divergence among metallothionein types. Uncovering the number of genes and sites possibly correlated with functional divergence will help to design cost-effective metallothionein protein functional studies in other organisms. This will permit further understanding of the distinct roles and specificity of these proteins as well as to properly target specific metallothionein types for different types of functional studies. In the genomic-era comparative functional genomic studies of gene families are possible, improving our understanding on the underlying mechanisms of functional divergence after gene duplication.

Keywords: Functional analysis, Gene duplication, Gene tree, Genomic database, Pseudogene, Reconciliation

Introduction

Gene families are a set of genes sharing sequence, and often functional, homology. The evolution of gene families is considered an important source driving species evolution (Ohno 1970; e.g. Demuth *et al.* 2006 and references therein). Gene families mainly evolve as a result of duplication and loss events, often associated with gain of adaptive function (Chang and Duda 2012; Kondrashov 2012; Zhang 2003). This mechanism may be due to neofunctionalization (Ohno 1970) according to which recent duplicated genes can suffer an accelerated rate of mutations in one of the recently duplicated copies once free from selective constraints, with these changes potentially leading to new function (e.g. Zhang *et al.* 1998). Another hypothesis proposes that the paralogs gradually substitute the multiple functions once maintained by the original single copy gene, leading to specialized genes with no overlapping functions (subfunctionalization model, Force *et al.* 1999; e.g. Prince and Pickett 2002; see also Kondrashov *et al.* 2002). Independently of the underlying mechanisms driving functional divergence after gene duplication, this process may promote increased gene diversity and new gene functions (Kondrashov *et al.* 2002; Prince and Pickett 2002; Zhang 2003), possibly leading or facilitating organismal adaptation to various environmental conditions (e.g. Kondrashov 2012). In yeast, it has been suggested that selection has favored retention of gene copies of yeast hexose transport genes under low nutrient environments (Brown *et al.* 1998). Furthermore, mosquitoes' populations show high frequency of duplications on genes involved in pesticide-resistance, apparently due to selection induced by insecticide treatment (Lenormand *et al.* 1998).

The metallothionein (MT) gene superfamily represents an interesting case study, known for the high turnover of gene duplication and loss determining its evolution (Capdevila and Atrian 2011). In some mammals, such as mouse and human, the presence of multiple MTs has been connected to different gene expression and gain and loss of function (e.g. Garrett *et al.* 1998; Moleirinho *et al.* 2011; Tío *et al.* 2004; reviewed in Blindauer and Leszczyszyn 2010; see also below). Metallothioneins are ubiquitous low molecular weight proteins and polypeptides of extremely high metal and sulfur content (Nordberg and Nordberg 2009). These inducible proteins encompass essential metal-binding properties and have several roles in metabolism, homeostasis, and kinetics of metals such as transport, storage and detoxification of metal ions in cells (e.g. Carpenè *et al.* 2007; Nordberg & Nordberg 2009; Palmiter 1998). Metal affinity varies among the different MT types (Nordberg 1989 and see below). MTs have recently also gained more attention in biomedical studies, due to their proposed involvement in cancer or neurological diseases (reviewed in Hidalgo *et al.* 2009). Metal resistance is known to be induced by gene duplication events in several species (Kondrashov *et al.* 2002; Kondrashov and Kondrashov 2006). For example, it has been shown that bacteria highly adapted to extreme metal concentrations retain several duplicated genomic regions containing other metal transporter and resistance genes. This suggests that rapid adaptation may be driven by these duplication events (von Rozycki and Nies 2009).

The wide representation of MTs genes across all three domains of life does not mean that they have been equally studied in different taxonomic groups. In vertebrates, a large number of biochemical, molecular, and chemical studies have been carried on these multi-functional genes in mammals (mostly in human and mouse), contrasting with a scarce knowledge and the very limited data available for other organisms (reviewed in Blindauer and Leszczyszyn 2010; Hidalgo *et al.* 2009). Different MTs classification systems have been developed for all organisms based on protein structure (e.g. Fowler *et al.* 1987; Nordberg and Kojima 1979; Palacios *et al.* 2011; Valls *et al.* 2001; Vašák and Armitage 1986; reviewed in Nordberg and Nordberg 2009) or using both protein structure and phylogenetic relationships (Binz and Kägi 1999; Moleirinho *et al.* 2011). The earlier classification, based on protein structure, divides MTs in three classes, including proteinaceous MTs closely related to those in mammals (called class I), proteinaceous MTs that lack this close resemblance (class II), and non-proteinaceous MTs (MT-like polypeptides form, class III) (Fowler *et al.* 1987; reviewed in Blindauer and Leszczyszyn 2010; Miles *et al.* 2000). However, the currently adopted classification in available genomic and protein database of MT genes is mostly based on the phylogenetic relationships among mammalian MT sequences, which are known for their great functional diversity (e.g. Capdevila and Atrian 2011; Vašák and Meloni 2011). According to this classification, MTs fall at least into four subgroups, MT1, MT2, MT3, and MT4, which are generally differentially expressed, induced, and show diverse metal binding affinities (e.g. Tío *et al.* 2004; reviewed in Davis and Cousins 2000; Miles *et al.* 2000; Vašák and Meloni 2011). Only a partial correspondence is shown between the early classification and the more recent mammalian MT subgroups (e.g. MT1/2 to class I) (reviewed in Palacios *et al.* 2011). Recent studies highlight the lack of suitability of this latest classification based on mammalian MTs for molecular evolution studies at a taxonomically large scale, claiming the dissemblance of physiological selective pressures between mammals and other organisms (reviewed in Capdevila and Atrian 2011). For example, studies on other taxonomic groups (including plants and bacteria) underline a departure from the classical mammalian (human and mouse) amino acid composition, biochemical metal-binding characteristics, and protein folding (reviewed in Vašák and Meloni 2011; see also Villarreal *et al.* 2006).

The increasing availability of genomic annotated databases provides an incredible resource to study functional diversification and evolution of many genes and gene families (e.g. Koonin 2009; Yanai *et al.* 2000). In vertebrates, available gene and protein databases have been recently used to infer the MT gene family origin and evolution through comparative analyses (e.g. in mammals, mostly on human, Moleirinho *et al.* 2011 and in other vertebrates, Guirola *et al.* 2012; Trinchella *et al.* 2008, 2012). However, these studies have been based either on sparse taxon or gene sampling when species with fully sequenced genomes have been considered (e.g. Moleirinho *et al.* 2011) or they have been based on cDNA or protein data (Guirola *et al.* 2012; Trinchella *et al.* 2008, 2012). In this latter case, proper distinction between different genes versus different isoforms cannot be assessed without genomic sequencing of the target gene and for the cDNA data, all existing genes cannot easily be detected if not expressed, for example as a result of no response to a metal treatment used or differential expression in time and space. This could produce misleading estimation of duplication and loss events and of the evolutionary history and functional divergence of the studied gene family. The goal of our work is therefore to complement the current and sparse knowledge on the molecular evolution and functional divergence of this gene family in vertebrates, aiming at: 1) identifying actual MT genes in the studied species, 2) estimating the number of duplication/loss events, 3) inferring the root of the MT gene tree in vertebrates, 4) inferring possible different levels of selective pressure following the duplication events and among the main MT types, and 5) studying functional divergence among MT types and identifying amino acid sites potentially correlated with this difference.

Materials and Methods

Dataset assembly and characteristics

The dataset was initially constructed based on metallothionein genes retrieved from the Ensembl 68 database (Flicek *et al.* 2011) (data collected on September 25, 2012). Data were further double-checked using the BLAT tool on UCSC Genome Bioinformatics (Kent 2002), the BioMart, BLAST/BLAT tools of Ensembl, the NCBI genomic database (data collected on January 29, 2013), and the Ensembl 70 release (data checked on January 28, 2013). Sequences were further double-checked to account for annotation discrepancies between databases. When necessary due to the comparison between the Ensembl and NCBI databases, for sequences showing partial bad annotation on each of the databases, we combined the information retrieved from the two databases (Supplementary Materials 1 and 2). We selected only representative vertebrate species spanning across distinct vertebrate groups (Supplementary Material 1). MT genes for all selected species were first retrieved using Ensembl Comparative Genomics search tool for orthologs and paralogs of all human identified functional MT genes (MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1M, MT1X, MT2A, MT3, MT4) except for MT5, which is testis-specific and was not included in our work. Obtained genes were then further checked by using the other databases and tools listed above.

To properly identify the CDS (coding sequences) products of distinct genes instead than derived from different gene splicing events (therefore different transcripts of the same gene), we compared exon and intron sequences and length. Only CDS corresponding to different genes (distinct exons and intron sequences) were retained for our analyses. Furthermore, for genes with multiple CDS only the ones codifying for a product above 50 and below 70 amino acids were retained, accordingly to the amino acid length of characteristic MT proteins in vertebrates. When this parameter was matched by more than one transcript, we maintained only the transcript tagged as CCDS, the Consensus CDS project (Pruitt *et al.* 2009) (e.g. Hom_MT1G and Hom_MT1F for human). A complete list of species, CDS (with relative Ensembl and NCBI accession numbers), chromosome location when available, and intron/exon gene characteristics of the CDS used for this work are indicated in Supplementary Material 1. The final dataset contained ten mammals, *Bos taurus* (Linnaeus, 1758)*, Canis lupus* *familiaris* (Linnaeus, 1758)*, Equus caballus* Linnaeus, 1758*, Homo sapiens* Linnaeus, 1758*, Monodelphis domestica* (Wagner, 1842)*, Mus musculus* Linnaeus, 1758*, Ornithorhynchus anatinus* (Shaw, 1799)*, Pan troglodytes* (Blumenbach, 1799)*, Rattus norvegicus* (Berkenhout, 1769)*,* and *Sus scrofa* (Linnaeus, 1758), three birds, (*Gallus gallus* (Linnaeus, 1758), *Meleagris gallopavo* Linnaeus, 1758, and *Taeniopygia guttata* (Vieillot, 1817), two reptiles, *Anolis carolinensis* (Voigt, 1832) and *Pelodiscus sinensis* (Wiegmann, 1835), one amphibian, *Xenopus tropicalis* (Gray, 1864) and four fish, *Danio rerio* (Hamilton, 1822)*, Oryzias latipes* Temminck & Schlegel, 1846*, Takifugu rubripes* (Temminck & Schlegel, 1850), and *Tetraodon nigroviridis* Marion de Procé, 1822.

Sequence alignment was carried out on CDS nucleotide sequences in MEGA 5 (Tamura *et al.* 2011) using the Clustal W option. The alignment was further checked by eye. Terminal stop codons were removed from all CDS prior analyses. Nucleotide alignment of the dataset used in this work can be accessed on Dryad. The number of variable and conserved nucleotide and amino acid sites was calculated in MEGA.

Phylogenetic analyses

Prior to phylogenetic analyses, the degree of saturation was estimated for all the codon positions together and for the 3rd codon position alone in DAMBE 5.3 (Xia 2013) as the presence of substitution saturation in the data, if not taken into account, may produce misleading phylogenetic results (e.g. Chiari *et al.* 2012). The estimate of the degree of saturation present in the dataset was based on the comparison between the index of substitution saturation (ISS), calculated from the data and a critical value (ISS.c) at which the sequences signal fails to recover the true tree. The calculation was performed under different topologies (symmetrical and asymmetrical); if ISS was not recovered to be smaller than ISS.c, this was interpreted as indication of substitution saturation in the dataset (see Xia *et al.* 2003 for further details). Phylogenetic analyses were run on the nucleotide and amino acid datasets. Maximum Likelihood (ML) analysis was performed in PhyML 3.0 (Guindon *et al.* 2010). ML analysis on CDS was carried out with a K80+G substitution model (tr/tv = 1.7917; gamma shape = 0.7250; proportion of invariable sites = 0) as estimated by the AICc (Akaike information criterion corrected for finite sample size), to account for the small size of the dataset used, in jModeltest2 (Darriba *et al.* 2012; Guindon and Gascuel 2003). ML analysis on amino acid was carried out with FLU substitution model as estimated by the AICc in Prottest3 (Darriba *et al.* 2011) allowing PhyML to estimate the gamma factor and the proportion of invariable sites. ML analysis was run with 1000 bootstrap replicates for both nucleotide and amino acid data. Bayesian analyses were carried out on the nucleotide and amino acid datasets in MrBayes 3.2. (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Bayesian analyses on the nucleotide dataset were run applying 1) the same model of evolution to all codon positions or 2) a two-partitioned mixed model (1st + 2nd codon and 3rd codon positions). The partitioned mixed model was applied to our dataset as an alternative model of substitution to take into account the higher substitution rate of the 3rd codon position comparatively to the 1st and 2nd. For these analyses we used number of substitutions (nst), proportion of invariable sites (pinvar), and rates according to jModeltest2. In the analysis with the partitioned mixed model we used nst = 6, gamma = equal, and pinvar = 0 for the 1st + 2nd codon position, and nst = 6, rates = gamma, pinvar = 0 for the 3rd codon position. The Bayesian amino acid analysis was run with rates = gamma and pinvar = 0. The other parameters were left to be estimated by MrBayes. Phylogenetic analyses were run on nucleotide and amino acid datasets as well as on the nucleotide dataset using a codon-based partition strategy (Bayesian analysis) to further take into account differences in the recovered tree topologies that may be due to potential saturation occurring in the data (e.g. see Chiari *et al.* 2012) and not detected by the saturation test.

All Bayesian analyses (on CDS and amino acid) were performed with two runs each of four Markov MonteCarlo chains (MCMC), of which one cold and three hot. We ran the analyses for 50 million generations to allow the standard deviation of split frequencies to reach a value below 0.01. Trees and associated model parameters were sampled every 1000 generations. The first 25% of the obtained trees were discarded by the burnin and the 50% majority-rule Bayesian consensus retained. Admixture and convergence of chains and runs were checked with Tracer v1.5 (Rambaut and Drummond 2009). To compare the best model of evolution strategy used for the Bayesian analysis ran on the nucleotide dataset, we calculated the Bayes factor for the two distinct models (one single or a partitioned mixed model). Bayesian factor was also used to compare among distinct tree topologies concerning the main MT clades as recovered by the nucleotide and amino acid Bayesian analyses. Bayesian analyses with a constrained monophyletic tetrapod MT3 clade and tetrapod MT3 – amniote MT4 (as recovered in the Bayesian analysis on the amino acid dataset, see Results) were ran separately on the nucleotide dataset. The Bayes factor value (K) was calculated by the ratio between harmonic means (average for all runs) of likelihoods for the two models/tree topologies comparison (e.g. A and B, see (Nylander *et al.* 2004 for further information). An A/B ratio with K>1 indicates that the A model is more strongly supported than B, while a value of K<1 states the opposite. A value of K=1 suggests that the difference between the two models is not important.

Reconciliation analysis

To understand the gene evolution of the MT gene family, including duplication and loss events, and to infer gene orthology relationships (see Doyon *et al.* 2011 for a review), we carried out a reconciliation analysis of the gene and species trees. The reconciliation was performed by a parsimony-based approach as implemented in Notung 2.6. (Chen *et al.* 2000; Durand *et al.* 2005; Vernot 2008) using an unrooted gene tree with multifurcations (uncertainties) and a built binary species tree. A species tree including all the species in our dataset was built based on Chiari *et al.* (2012), Li *et al.* (2007), and the Tree of Life web project (accessed on 2 October, 2012). The used gene tree corresponded to the one obtained from the Bayesian analysis ran on the nucleotide dataset with one model of evolution (best strategy model, see 3.2 Results and discussion). For the reconciliation analysis, we chose the default parameters and the posterior probability values as obtained from the Bayesian analysis for the used tree (edge weight threshold / posterior probability values (pp) = 0.9, duplication = 1.5, loss = 1.0). The edge weight threshold identifies nodes that are not supported with a posterior probability equal or above 0.9 (chosen threshold for this analysis in our study) and that can be rearranged during the reconciliation. This allows obtaining the optimal reconciliation (see below) considering also different tree topologies from the one used as input, at least for nodes with support lower than the used threshold. The cost/weight of gene loss was considered lower than the one for duplication so that losses may occur more frequently than duplications in the inferred reconciliation. This allows accounting for possible non-sequenced or non-retrieved data in our dataset. Because there may be many possible reconciliations of a gene tree within a species tree, the optimal reconciliation corresponds to the one with the lower cost of duplication and loss (see Doyon *et al.* 2011 for further information). The obtained optimal reconciliation was used to root the gene tree, in order to obtain the lower cost of gene duplication and loss, and to solve the polytomies in the gene tree. This process allows inferring a tree with optimal reconciliation cost among all binary gene trees that are consistent with the input multifurcated gene tree. A reconciliation analysis to confirm the results obtained with the nucleotide-based gene tree was also performed using the Bayesian amino acid gene tree.

Analysis of variation in selective pressure among lineages

To estimate the possible variation in selective pressure associated with duplication events in the MT gene family, we applied a model of coding sequence evolution allowing variation of the selective pressure among branches. Selective pressure is calculated by comparing synonymous (dS) versus non-synonymous (dN) substitution rates. Synonymous are silent substitutions as they do not involve an amino acid change, differently from non-synonymous substitutions. The analysis of variation in selective pressure among main MT clades (as in Figure 8 and Supplementary Material 4) was performed with the codeml program of the PAML 4.7 package (Yang 2007). The analyses were performed both on the unrooted Bayesian nucleotide tree (one model of evolution) and on the binary gene tree resulting from the optimal reconciliation (basal tricotomy in the gene tree was not solved according to the codeml application requirements). Analyses were run on the two tree topologies to infer the possible influence of polytomies and different gene topologies at not-well supported nodes on parameters estimates. This strategy allows us to also take into account uncertainties concerning the phylogenetic relationship among the main MT clades. This Maximum Likelihood-based analysis can, based on the data, estimate different ω (dN/dS) within the tree by letting the user apply different weights of selective pressure among evolutionary lineages. The parameter ω is therefore first estimated by running the model with one single ω across all lineages (model = 0 option; hypothesis H = 0), and then by allowing the program to estimate from the data distinct ω parameters for chosen clades (model = 2 option; other hypotheses). This permits to test the different selection rates among branches following duplication by assigning different ω estimation to these branches. Branch lengths and transition/transversion are also estimated separately for each analysis. Both ambiguity characters and alignment gaps were treated as undetermined nucleotides (option Cleandata = 0) and the analyses were run with one single ω across sites (option Nsites = 0). To test for convergence of the runs, several simulations were run with multiple initial starting values of ω (0.2 and > 1) and kappa (transition/transversion rate; k = 2.0241 obtained with ω = 0.2 and k = 3.5834 as previously calculated in jModeltest2), separately, in H0 (hypothesis with one single ω across the tree). Alternative hypotheses (H1-H4, Table 1a and Supplementary Material 3) were formulated to test ω between branches. We aimed to test for: 1) a difference in selective pressure in the branches immediately following the duplication events (Hps H1 and H2), to assess functional divergence following duplication, and 2) a difference in the mutation rates of one of the main MT clades (Hps H3 and H4), to assess if the distinct main MT types evolve under similar selective pressure (Table 1a and Supplementary Material 3). To statistically compare the different evolutionary hypotheses we applied the LRT (Likelihood Ratio Test), which is a statistic test based on the likelihood ratio between the null and alternative hypotheses (LR) following the χ2 distribution of this statistic with degrees of freedom (fd) equal to the difference between the number of parameters (*np*) of the alternative hypothesis and the *np* of H0. The LRT rejects the H0 when the LR is considered too small by the given significance level (p value < 0.05).

Bayesian and ML phylogenetic analyses ran on the amino acid dataset recovered a tetrapod MT3 clade (see Results). To test if the selection pressure results obtained using the Bayesian tree would be confirmed in case of an alternative tree topology, we repeated the analyses described above using as input tree the Bayesian tree obtained on the nucleotide dataset (one model of evolution) with a tetrapod, instead than a eutherian MT3 clade (input tree represented in Supplementary Material 6).

Functional analyses

To further analyze the existence of functional protein divergence among main MT clades (as in Figure 8 and Supplementary Material 4; see also Results and Discussion), we calculated the GRAVY (grand average of hydropathicity) index using the referenced hydropathic index for amino acids as in Kyte and Doolittle (1982). The hydropathic index consists of the attribution of a fix value to an amino acid according to the hydrophobic or hydrophilic properties of its side chain (Kyte and Doolittle, 1982). The GRAVY index of a sequence corresponds to the sum of the hydropathic value of each amino acid in the sequence divided by the number of residues in the sequence. This calculation was performed using the GRAVY Calculator web application. Increasing positive score indicates greater hydrophobicity meaning higher water repellency to non-polar molecules. Since the structure of the protein and its folding define its function, differences in overall GRAVY index and in the hydropathic plot (see below) can provide information about functional divergence among protein types and have been used as an indication of the flexibility of the protein (e.g. Capasso *et al.* 2003, 2005). We calculated the average, maximum, minimum, and SD (standard deviation) GRAVY index for the main MT clades (see below) corresponding to the arithmetic mean of the obtained GRAVY index of all the sequences contained within each main MT clade. Main MT clades (Supplementary Material 4) correspond to the clade defined by node 3 (amphibian, bird, and reptile MT), node 31 (eutherian MT1/2), node 40 (eutherian MT3), node 46 (mammalian MT4), node 49 (potential amniote MT4), and node 53 (fish MT) (node numbers as in Supplementary Material 4). Furthermore, for each of the above mentioned clades, a "clade" amino acid consensus sequence was manually built by eye. For these consensus amino acid clade sequences, the hydrophobicity plots were obtained following the Kyte-Doolittle method using the Protein Hydrophobicity Plots Generator.

Functional divergence among main MT clades after duplication events was further investigated using DIVERGE v.2 (Gu and Vander Velden 2002) by assessing the Type I and Type II functional divergences among clusters (main MT clades, see below). Gu (2001) recognizes two main types of functional divergence for duplicated genes: Type I (Gu 1999) is characterized by amino acids that are highly conserved in one cluster, but variable in the other, taking into account the phylogeny and sequences variation across the tree. Type I divergence is correlated to different functional constraints between duplicate genes with consequent site-specific rate differences (Gu 1999). Type II (Gu 2006) is characterized by a "cluster-specific functional divergence" (Lichtarge *et al.* 1996) due to site-specific changes of amino acid physiochemical property (e.g. charge, hydrophobicity). For this analysis, the sequences Equ_b_MT, Mel_MT1_, Orn_a_MT, Orn_b_MT, OrnMT3, and all sequences of *P. sinensis* were removed from the dataset due to either missing data at the beginning of the sequences or very divergent amino acid sequences and unresolved phylogenetic placement (see dataset file deposited in Dryad, Figure 8, and Supplementary Material 4). Sites with missing data would be excluded from the divergence analysis, thus reducing the amino acid sites for which the estimate of sequence divergence among the distinct MT clades would be calculated, for example by eliminating half of the functional β-domain of the protein (see Results and Discussion for MT protein domains). Very distinct sequences for which phylogenetic placement was not well recovered (Orn_a_MT, Orn_b_MT, and Orn_MT3) were removed as they could interfere with divergence estimates. The DIVERGE analysis was run using the rooted binary gene tree obtained after reconciliation (Figure 9), since the software only operates with phylogenetic trees without polytomies. The three-dimensional (3D) structure of the MT2 protein of *R. norvegicus* (Uniprot protein database, accession number P04355 and corresponding to Rat_MT2A in our dataset) was used as MT protein reference. Divergence was tested between 1) eutherian clades MT1/2 and MT3 (corresponding to node 35 in Supplementary Material 4), and 2) eutherian clades MT1/2 and MT3 (MT1/2/3) versus potential amniote MT4 (the amniote MT4 clade is identified by node 49 in Supplementary Material 4), corresponding to major gene duplication events (see Results and Discussion and Figure 9). The coefficient of functional divergence, theta (θ), corresponding to the proportion of sites to be expected functionally divergent, was determined for all gap-free amino acid positions. θ is directly linked to the coefficient of rate correlation between the evolutionary rates of a site within each gene cluster (Gu 1999; Wang and Gu 1999). It varies between 0 and 1, with $\theta = 0$ indicating no observed functional divergence. DIVERGE provides a ML statistical estimate of θ (ThetaML) (Gu 2001). The statistically significant functional divergence among clusters ($\theta > 0$) is evaluated by a LRT with $\theta = 0$ representing the null hypothesis. The LRT was used for each of the pairwise comparisons eutherian MT1/2 versus eutherian MT3 and eutherian MT1/2/3 versus potential amniote MT4, with the null hypothesis being rejected for $p < 0.05$. Once the statistical evidence for functional divergence after gene duplication is provided, sites that are likely to influence this divergence may be identified by applying a cut-off value. The cut-off value corresponds to the posterior probability of functional divergence at a site (see Gu 1999 for further details). In our analyses, we applied a conservative cut-off value of 0.9 for all comparisons. Analysis of functional divergence and search for sites involved in functional divergence was repeated using the constructed tree topology including a tetrapod MT3 (see tetrapod MT3 clade in Supplementary Material 6) to assess if the influence of this clade versus a eutherian MT3 clade one the results obtained with the analyses indicated above.

Putative pseudogene inference

Species for which MT sequences were recovered as a within-species duplication event (Figure 9 and Results and Discussion) or showed very divergent amino acid sequences and were recovered within the same MT clade (e.g. Can, Can_MT2A, Pan_a, Pan_c and Pan_h, Figs 1 and 2, see also alignment available on Dryad) were comparatively investigated in order to infer potential pseudogenization. All known mammalian functional MTs are characterized by 20 conserved metal-binding cysteine residues and no or little aromatic amino acids (Kägi *et al.* 1984; reviewed in Vašák and Meloni 2011, see also Figure 10 in Moleirinho *et al.* 2011). MT proteins folding depend on these metal-binding cysteines and a mutation involving one of these amino-acids may incapacitate the protein main function (Han and Lee 2006). Hence, to infer the presence of pseudogenes in our dataset, we looked for sequence variation in cysteines for 24 candidate pseudogene MT sequences (as defined at the beginning of this section). Human MT sequences were chosen as reference to look for sequence variation due to the large knowledge in protein structure and function in human MTs. Furthermore, the 20 conserved metal-binding cysteine residues are the same as in the chicken sequences, permitting to use the human MTs also for comparison of non-mammalian MT sequences. Among human MTs, the choice of reference sequence to use was based on phylogenetic relatedness according to the Bayesian nucleotide analysis (one model of evolution, see Results and Discussion) rather than on nomenclature resemblance (when nomenclature was available), to avoid incorrect results due to misleading nomenclature. For the potential pseudogene sequences, the GRAVY index was calculated as a further indication of possible functional divergence from classical MT proteins.

Results and Discussion

Dataset assembly and characteristics

The initial retrieved dataset contained 86 sequences. After removal and replacement of sequences due to incorrect annotation or annotation problems (Supplementary File 2) the final dataset consisted of 77 sequences (Supplementary Material 1). Approximately half of the removed sequences were intronless (data not shown). On duplication events, intronless genes may be generated by retroposition when the mRNA is retrotranscribed into the genome and may still represent functional MT proteins. Despite this possibility, intronless sequences were removed from the dataset after the lack of introns was confirmed according to both used databases (Ensembl and NCBI) (Supplementary Material 2). In our dataset assembly, we recovered cases of incorrect annotation (Supplementary Material 2) in the Ensembl database, as it has been previously observed (e.g. McEwen *et al.* 2006; see also Wang *et al.* 2003).

A great diversity of MT genes was recovered among mammalian species, supporting what previously found in human (e.g. Moleirinho *et al.* 2011; Tío *et al.* 2004), ranging from three in *M. domestica* to twelve in *P. troglodytes* (see below for additional comments on one of these twelve MTs). In other vertebrates, we found two MT genes for each bird species (see below), two or three genes in reptiles (lizard and turtle; see also below), one gene in amphibian, and one or two genes in fish. Previous studies carried out to characterize MT genes in squamates and amphibians from cDNA, recovered only one MT gene per species (Riggio *et al.* 2003; Trinchella *et al.* 2008, 2012), confirming for amphibians what already observed in *Xenopus* (Saint-Jacques *et al.* 1995). In mammals, multiple MT genes/proteins are associated with expression in distinct tissues and different metal affinity, with MT genes considered to be more ubiquitous and other more specific (see below and Guirola *et al.* 2012). Data on MT gene characterization in reptiles were, to our knowledge, until our study only limited to squamated. In the squamate species *Podarcis sicula* (Rafinesque, 1810), Riggio *et al.* (2003) and Trinchella *et al.* (2006, 2008) observed only one MT type in the different tissues studied (brain, liver, ovary). The same MT type was also expressed in another squamate, a snake, in the venemon glands (Junqueira-de-Azevedo and Ho 2002). In chicken, similarly to what found in our work for reptiles, a second gene copy was recovered only recently after the full genome of this species was released (Villarreal *et al.* 2006). Biochemical analyses support for this gene/protein functional spectrum in between the ones characterized for mammalian MT1 and MT4. Villarreal *et al.* (2006) proposed that the second chicken MT gene may have remained undiscovered until the full genome of this species was released due a restricted or limited expression in time or space or specific metal-induction mechanisms (which may also differ among species, see Nam *et al.* 2007 for expression of the two MT genes in two avian species). A similar hypothesis could explain why only one MT gene has been characterized so far in squamates. Our results, together with future newly sequenced complete reptile genomes and biochemical studies will permit to test for differential expression and induction among distinct species, tissues, developmental stages and metal response. Furthermore, future fully sequenced genomes, including data from crocodilians and tuatara, will provide further data to understand if a second MT duplication event equally interested all amniotes, in comparison to the single MT gene recovered in fish and amphibians. In fish, the two MT gene copies recovered in our dataset only in *T. nigroviridis* are most likely due to the whole-genome duplication observed in this species (Jaillon *et al.* 2004). Teleost fish (all the fish included in our dataset) genomes are characterized by a whole genome duplication and large gene loss (Brunet *et al.* 2006; Taylor *et al.* 2003). Although we cannot completely exclude the possibility of not having recovered a second MT gene for the other fish species in our dataset, whole genome duplication and secondary gene loss can explain the different number of MT genes recovered in our dataset for fish and reported in other studies (Bargelloni *et al.* 1999; see also datasets in Nam *et al.* 2007; Trinchella *et al.* 2008, 2012; one of the MT genes used for *D. rerio* in these last two studies have been removed from Genbank due to the chimeric origin of the sequence). Additional fully sequenced fish genomes (e.g. Howe *et al.* 2013) will provide further insights on this subject.

Most MT CDS contained 162 nucleotides (61 amino acids), excluding the terminal stop codon (Materials and Methods). MT3 type has an additional seven amino acids in comparison to the other MTs, as already previously observed (reviewed in Vašák and Meloni 2011). The majority of MT sequences included in our dataset consisted of three exons, following the classical structure of mammalian MT (reviewed in Hidalgo *et al.* 2009) (see sequence alignment). Two exons encode for the β-domain, while the third exon for the α-domain of the protein (Vašák and Meloni 2011). These thiol-rich domains bind with high affinity a different number and types of metal ions (e.g. Zn^{2+}, Cd^{2+}, Cu^{2+} and others) consequently folding into two dumbbell-like shaped connected by a flexible region constituted by lysine amino acids (reviewed in Hidalgo *et al.* 2009; see also amino acid positions 33 and 34 in our alignment and below). The β-domain is generally characterized by the amino acids 1-30, while α- domain by amino acids 31-61 (Braun *et al.* 1992; Romero-Isart *et al.* 1999).

Sequences in our dataset had 13% and 17% of conserved nucleotides and amino acids (183 and 58 variable sites), respectively (missing data and gaps considered as different states). 1/3 of the conserved amino acids are within beta domain, and 2/3 in the alpha domain. The double number of conserved amino acids observed in the α-domain of the protein, a pattern previously reported for mammals for MT1 versus MT4 (Tío *et al.* 2004), is probably correlated with the higher structural constrain of this domain (reviewed in Hidalgo *et al.* 2009).

Within main MT clades (see below) sequence identity was 72 and 21 sites (120 and 43 variable) for eutherian MT1/2, 146 and 68 (58 and 16 variable) for eutherian MT3, 77 and 26 sites (127 and 42 variable) for tetrapod MT3, 78 and 29 (111 and 34 variable) for potential amniote MT4, 67 and 26 (122 and 37 variable) for amphibian, bird, and reptile MT and 113 and 41 (67 and 19 variable) for fish MT for the nucleotide and amino acid sequences, respectively. Finally, there was no evidence of saturation when assuming symmetrical and asymmetrical topology on the complete dataset as well as for the 3rd codon position alone (data not shown).

Phylogenetic analyses

Tree topologies obtained using distinct phylogenetic reconstruction methods and the nucleotide or amino acid dataset were largely similar. The Bayes factor calculation ratio between the partitioned and non partitioned Bayesian analyses was 0.98, suggesting a barely worth to mention evidence that the non partitioned strategy was better than the partitioned one. Almost all analyses identified distinct major MT clades in fish MT, eutherian MT1/2, eutherian MT3, mammalian MT4, a reptile/bird MT clade, and a potential amniote MT4 (Figure 8 and Supplementary Material 4).

According to our phylogenetic results, currently used nomenclature to distinguish among different MT types is not necessarily meaningful. In fact, we recovered a reptile/bird MT clade including MT2, MT3 and MT4 called sequences, as well as a well-supported eutherian MT1/2 clade consisting of many sequences with unknown MT characterization (Figure 8, Supplementary Materials 1 and 4). In this paper, we will refer to main MT clades following the predominant MT types included in the clade as delineated above. The distinct fish versus tetrapod MT clades were already recovered in previous analyses based on reduced MT type sequences (e.g. Nam *et al.* 2007; Trinchella *et al.* 2008, 2012). Clade A (Figure 8, and node 31 in Supplementary Material 4), the clade with most sequences (39), encloses all eutherian MTs annotated as MT1, MT2 and some unknown from *C. lupus*, *E. caballus*, *P. troglodytes*, and *S. scrofa*. This clade is highly supported by all trees, with the exception of the ML and Bayesian amino acid trees (bootstrap = 18 and pp = 0.51, data not shown).

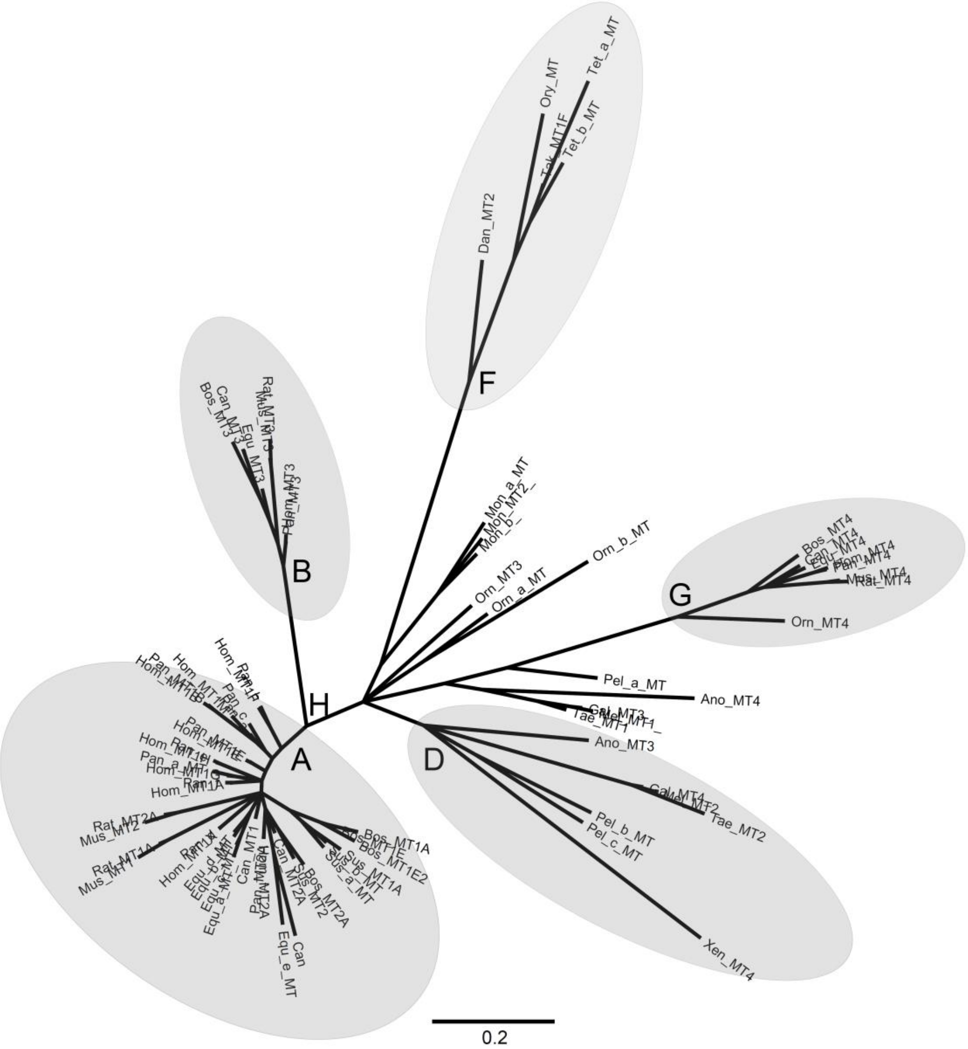


Figure 8 - Unrooted Bayesian consensus tree (50% majority-rule) based on one single model of evolution. Bootstrap for ML (in %) and Posterior Probability values are given for specific clades in the following order: ML nucleotide/ML amino acid/Bayesian nucleotide no partition/Bayesian nucleotide partition/Bayesian amino acid. Values replaced by "-" when below 60% bootstrap or 0.70 pp. When a clade is not recovered by the analysis it is indicated with "#". Clade A (eutherian MT1/2) 78/-/0.79/1/#; Clade B (eutherian MT3) 99/94/1/1/1; Clade D (bird+reptile+amphibian MT) -/#/-/0.75/#; Clade F (fish MT) 93/99/1/1/1; Clade G (mammalian MT4) 99/100/1/1/1; Clade H (eutherian MT1/2/3) #/#/0.85/0.96/#.

Ornithorynchus and *Monodelphis* MT sequences were not recovered to belong to this clade (Figure 8 and Supplementary Material 4). Clade B (Figure 8 and node 40 in Supplementary Material 4) includes MT3 from eutherian mammals (since *O. anatinus* MT3 is outside this clade) and it is recovered with high support values from all analyses (Figure 8 and Supplementary Material 4). Mammalian MT1/2 and eutherian MT3 clades were also recovered in previous studies using smaller datasets (in terms of species or MT types) (Moleirinho *et al.* 2011; Nam *et al.* 2007; Trinchella *et al.* 2008, 2012). Differently from our results, Moleirinho *et al.* (2011) also resolved a mammalian MT3 clade, with the inclusion of *Ornithorynchus* and *Monodelphis*, although this clade was not strongly supported (pp< 0.6 and < 0.8 for MT3 clades including *Monodelphis* and *Ornithorynchus*, respectively). The ML and Bayesian analyses ran on the amino acid dataset recovered a tetrapod MT3 (eutherian MT3 + bird/reptiles MT + amphibian) (bootstrap = 12 and pp = 0.87, data not shown). Bayes factor calculation to compare for alternative tree topologies (Bayesian trees obtained with constrained clades) suggests that a tree topology including a tetrapod MT3 clade (as in the trees obtained on the amino acid dataset) or a tree with a eutherian MT3 and a reptile/bird MT clades (as obtained in the trees obtained with the nucleotide dataset) are equally probable (BF = 1; data not shown). Clade D (Figure 8 and node 3 in Supplementary Material 4) includes seven MTs only from amphibian, reptiles, and birds. This clade containing unknown MTs was recovered by all phylogenetic analyses using nucleotide sequences, despite showing relatively weak support (ML nucleotide bootstrap value = 27, Bayesian no partition pp = 0.58, and Bayesian with partition pp = 0.75). The ML and Bayesian amino acid analyses recovered a reptile/bird clade, with the exclusion of the amphibian sequence, which instead belonged to the tetrapod MT3 clade (bootstrap = 49, pp = 0.94, data not shown). Trinchella *et al.* (2012) using more species of squamate reptiles (but no other reptiles) and amphibians recovered a reptile/bird clade corresponding to clade D in our study, but to the exclusion of amphibian sequences. Clade F (Figure 8 and node 53 in Supplementary Material 4) corresponds to fish MT and it is always recovered with high support values. Clade G (Figure 8 and node 46 in Supplementary Material 4), which includes the mammal MTs annotated as MT4 is always recovered with maximum support by all analyses (Supplementary Material 4). The rooted tree indicates a potential amniote MT4 clade (node 49 in Supplementary Material 4), which is recovered by all phylogenetic analyses, but the ML amino acid one. A potential mammals/birds MT4 clade was also previously recovered, although with no significant statistical support, using ML analyses on amino acid sequences of a reduced dataset by Trinchella *et al.* (2012). Based on phylogenetic analyses, the MT4 clade has been proposed to be of a more ancient origin than the rest of the MT types (e.g. mammalian MT4 in Moleirinho *et al.* 2011, and mammals/birds MT4 in Trinchella *et al.* 2012). Our results do not clearly solve MT4 as the ancestral MT type (Supplementary Material 4, see also gene tree obtained after reconciliation, Figure 9) and independently on the dataset used (nucleotide or amino acid) phylogenetic relationships among main MT clades are generally poorly resolved. The MT4 mammalian relationship was, however, recovered as basal to all the other MT types by the ML analyses on the amino acid dataset (bootstrap value = 99, data not shown). Furthermore, the binary gene tree obtained after reconciliation supports the amniote MT4 clade as ancestral to eutherian MT1/2 and MT3 (Figure 9 and reconciliation results below). Clade H (node 35 in Supplementary Material 4) representing the duplication event between MT1/2 and MT3 genes (see also reconciliation results below) is recovered with high statistical support only by the Bayesian analyses on the nucleotide dataset. This clade has not been clearly recovered in previous studies in which all MT types were included (Moleirinho *et al.* 2011; Trinchella *et al.* 2012). In Moleirinho *et al.* (2011), the sister relationship between MT1/2 and MT3 was disrupted only by the inclusion of a sequence from *Anolis*; however the node corresponding to the relationship MT1/2 and MT3/*Anolis* was recovered with high posterior probability (pp. 0.95). In Trinchella *et al.* (2012), phylogenetic relationships among main MT types are generally poorly resolved.

Reconciliation analysis

The optimal unrooted reconciliation had a D/L (duplication/loss) cost of 89, with 32 duplications and 41 losses, confirming the high turnover of gene duplication and loss predicted for this family. The "*a priori*" best outgroup chosen to root the phylogenetic vertebrate MT tree in previous studies (e.g. Moleirinho *et al.* 2011; Trinchella *et al.* 2012), the fish MT clade, was confirmed by our analysis (D/L score = 85, number of duplications = 32, number of losses = 37). This result was also confirmed by the optimal reconciliation obtained using the Bayesian amino acid gene tree (data not shown). Figure 9 shows the rooted binary (with solved polytomies from the input gene tree) gene tree resulting from the optimal reconciliation, with putative duplication and loss events. According to the optimal reconciliation, several duplication events occurred before the MT gene expansion within the eutherian mammals (clade MT1/2 and MT3). Furthermore, our results indicate that MT duplications mostly predate speciation events. A duplication event characterized the divergence of the clades containing MT from birds, reptiles, and amphibian from the rest. Another duplication was responsible for the diversification of the putative amniote MT4 from mammalian MT1/2 and MT3 (with the exclusion of *M. domestica*). Finally, a duplication event interested eutherian mammals only and separated the eutherian MT3 and MT1/2. The obtained reconciliation was recovered within species duplication events (duplication located at the tip of that species) for *B. taurus*, *E. caballus*, *M. domestica*, *O. anatinus*, *P. sinensis*, and *S. scrofa* (Figure 9). These within species duplication were further investigated to infer the possible existence of pseudogenization events (see Materials and Methods).

Analysis of variation in selective pressure among lineages

The estimated *ω*, likelihood values and obtained LRT according to the different tested hypotheses of variation in selective pressure after duplication events and among main MT types (Table 1a and Supplementary Material 3) are shown in Table 1b.

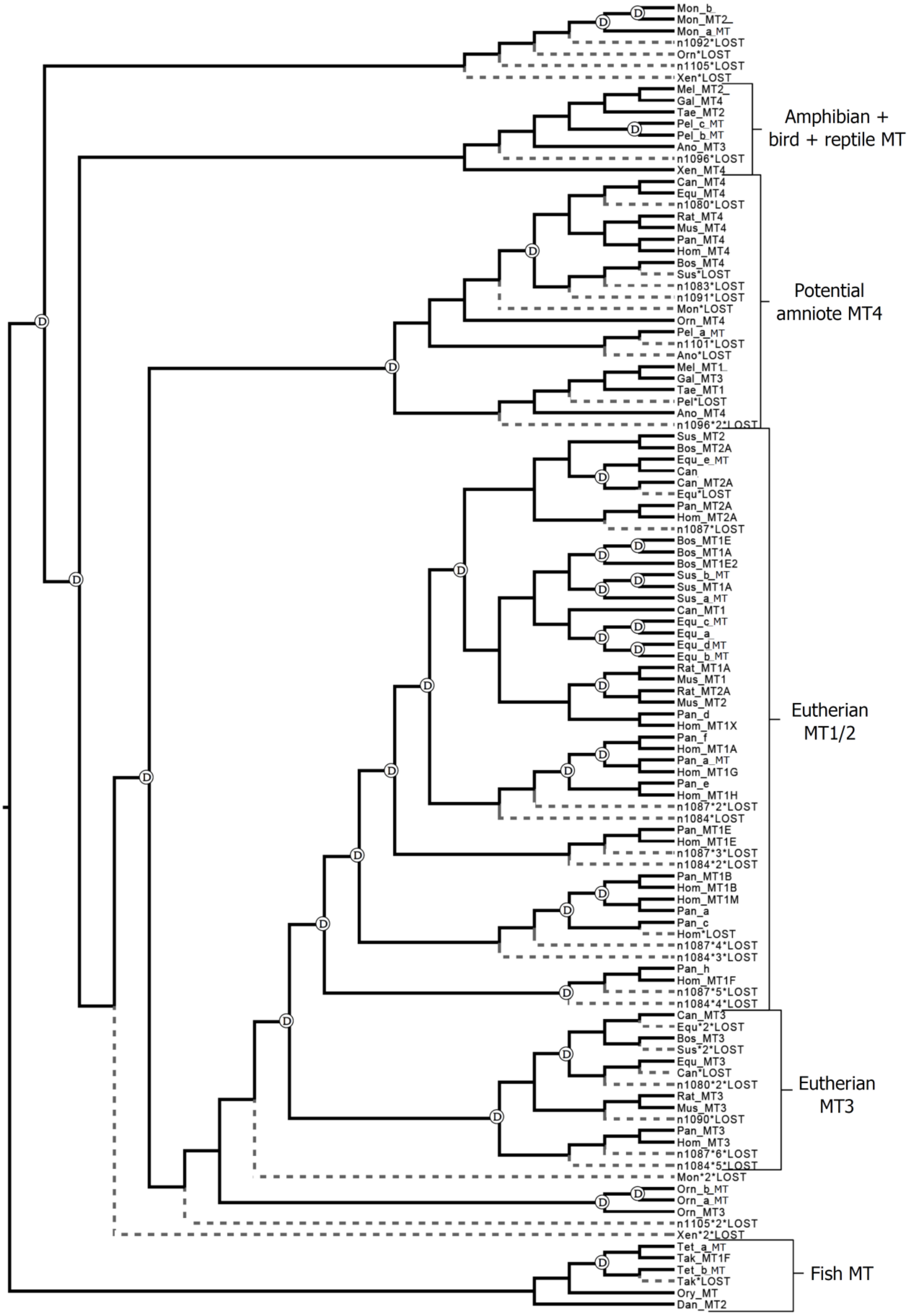


Figure 9 - Rooted gene tree resulting from the optimal reconciliation. Gene duplications are indicated with (**D**), while gene loss is indicated by a dashed line

Table 1- Test of selective pressure. **a)** Models chosen to test variability of selective pressure (H0 – H4) for the following tree topologies: unrooted Bayesian consensus tree/unrooted gene tree obtained after reconciliation/unrooted Bayesian consensus tree with inclusion of a tetrapod MT3 clade (see Materials and Methods for additional information). "ω" indicates dN/dS. "A0, B0, C0 and E0" indicate branches corresponding to a duplication event, while "A1, B1, C1 and E1" indicate main MT branches (Supplementary Material 3). "H0" represents the null hypothesis with equal ω across the tree; "H1" indicates that ω is different between the branches A0, B0 and different from all the remaining branches (indicated as "others"), which have instead equal ω ; "H2" indicates that ω is different between D0, E0 and different from all the remaining branches (indicated as "others"), which have instead equal ω ; "H3" indicates that ω is different between A (A0+A1) and B (B0+B1) branches and different from all the remaining branches (indicated as "others"), which have instead equal ω ; "H4" indicates that ω is different between C (C0+C1) and E (E0+E1) branches and different from all the remaining branches (indicated as "others"), which have instead equal ω.

H0	$\omega_{A0} = \omega_{A1} = \omega_{B0} = \omega_{B1} = \omega_{others}$
H1	$\omega_{A0} \neq \omega_{B0} \neq \omega_{others}$
H2	$\omega_{C0} \neq \omega_{E0} \neq \omega_{others}$
H3	$\omega_{A0} = \omega_{A1} \neq \omega_{B0} = \omega_{B1} \neq \omega_{others}$
H4	$\omega_{C0} = \omega_{C1} \neq \omega_{E0} = \omega_{E1} \neq \omega_{others}$

b) Results on variable selective pressures among main MT clades according to the different hypotheses listed above. Values are given as results using Bayesian tree topology/results using gene tree topology after reconciliation. "|l|" indicates the Likelihood absolute value; "np" indicates the number of parameters; "LRT" indicates the Likelihood Ratio Test value standing for significant when LRT < 0.05 (significant values indicated in bold).

	ω_{A0}	ω_{B0}	ω_{C0}	ω_{E0}	ω_{others}	**|l|**	***np***	**LRT**
H0	0.1113/ 0.1099/ 0.1000	$= \omega_{A0}$	$= \omega_{A0}$	$= \omega_{A0}$	$= \omega_{A0}$	5044.01/ 5030.14/ 5046.91	134/ 153/ 134	-
H1	0.0104/ 0.0101/ 0.0013	0.0045/ 0.0041/ 14.308	$= \omega_{others}$	$= \omega_{others}$	0.1134/ 0.1115/ 0.1013	5040.16/ 5026.20/ 5043.76	136/ 155/ 136	**2.1×10^{-2}/** **1.9×10^{-2}/** **4.3×10^{-2}**
H2	$= \omega_{others}$	$= \omega_{others}$	+ ∞/ + ∞/ 0.8864	+ ∞/ + ∞/ + ∞	0.1077/ 0.1065/ 0.0989	5037.44/ 5023.47 5043.220	136/ 155/ 136	**1.4×10^{-3}/** **1.3×10^{-3}/** **2.5×10^{-2}**
H3	0.1453/ 0.1464/ 0.1390	0.0915/ 0.0922/ 0.0535	$= \omega_{others}$	$= \omega_{others}$	0.0875/ 0.0846/ 0.0827	5040.51/ 5026.37/ 5040.06	136/ 155/ 136	**3.0×10^{-2}/** **2.3×10^{-2}/** **1.1×10^{-3}**
H4	$= \omega_{others}$	$= \omega_{others}$	0.1289/ 0.1328/ 0.1186	0.1218/ 0.1197/ 0.1080	0.0162/ 0.0163/ 0.0156	5033.54/ 5019.82/ 5038.53	136/ 155/ 136	**2.8×10^{-5}/** **3.3×10^{-5}/** **3.3×10^{-4}**

The obtained values are similar, independently of the tree used (Bayesian or binary gene tree obtained after reconciliation). For coding sequences, the variation of selective pressure may reflect an acceleration of non synonymous substitutions and indicate functional divergence following the duplication event, eventually decreasing secondarily as an effect of purifying selection, which permits that the duplicated genes maintain related but distinct functions (e.g. Gu 1999; Kondrashov *et al.* 2002; Li *et al.* 1985). Depending on when functional divergence among the paralog genes occur, different patterns of evolutionary rates may be detected immediately after the duplication event or among paralogs (in our case among the main MT clades) (see also Gu 1999 for further theoretical details). The null hypothesis, H0 that the selective pressure and the mutation rate remain constant along the tree is rejected in all alternative hypotheses tested (H1-H4, $p << 0.05$, Table 1b). Our analyses indicated that the evolution of the MT genes under the null hypothesis is generally characterized by purifying selection (H0, $\omega < 1$). Because the estimation of ω is based on the average across all sites, our results do not discard the possibility that positive selection and adaptation may occur at specific amino acid sites, as suggested by our functional analysis results (see below). Substitution rates and selective pressure change immediately following the duplication events (H1 and H2). The divergence of MT3 from MT1/2 resulted in a two-fold increase in ω in MT1/2 compared to MT3 (e.g. H1, ω = 0.0104 versus ω = 0.0045, Table 1b). While there may not be an overall indication of positive selection ($\omega < 1$) and functional adaptation associated with this duplication event, it has been reported that human MT3 shows different biological proprieties in comparison to MT1/2 (see also Functional analyses below). For example, in humans MT3, but not MT1/2, has been correlated to Alzheimer disease (reviewed in Vašák and Meloni 2011). Our results support, however, a burst of positive selection for the duplication event associated with the divergence of MT4 from all the rest ($\omega >> 1$) (H2, Table 1b). This could suggest the evolution of functional divergence between MT4 and the other MTs. This result would support structural and biochemical data concerning the distinct metal binding affinity of MT4 versus MT1 in mammals and the more specific versus ubiquitous tissue expression of the two forms (Tío *et al.* 2004; reviewed in Vašák and Meloni 2011, see also below). Finally, our analyses suggested that following gene duplication, MT1/2 experienced an increase of ω of about one time and half in comparison to all the rest of the MT genes (H3, ω = 0.1453, Table 1b). This result seems to be in agreement with the large number of duplication events occurring within this clade and the differential tissue and temporal expression of distinct MT1 genes observed in human and mouse (e.g. Moleirinho *et al.* 2011; Schmidt and Hamer 1986). On the other hand, after the duplication event, the evolution of MT4 from the other MTs was not observed to be associated with any change in *ω* (H4, Table 1b). When the analyses were ran using the alternative topology including a tetrapod MT3 clade (see Materials and Methods for further specifications), we recovered a different result for the duplication event associated with MT1/2 and MT3 and for the divergence of MT4 from the rest. In fact, when considering a tetrapod MT3 clade, this shows a burst of selective pressure (H2, *ω* = 14.308) corresponding to the duplication event from MT1/2, differently from what previously observed. Furthermore, when running the analyses with this alternative tree topology, the divergence of MT4 from the rest of the MTs is not associated with an equal increase in selective pressure as observed with the other analyses. This suggests caution in interpreting the results concerning variation in selective pressure, as this parameter was found to change depending on the tree topology used.

Functional analyses

Minimum, maximum, average and standard deviation of hydropathic GRAVY index for the main MT clades are provided in Table 2a. MT4 clades (potential amniote MT4 and mammal MT4) have similar slightly negative average GRAVY scores, comparable with what obtained for the Fish MT clade and different from what calculated for the other main MT clades (Table 2a).

However, while the two MT4 clades have also similar hydrophobic profiles across the sequences, these profiles differ for the one observed for fish MT clade (Figure. 9).

Our results confirm a negative and large variation at hydropathic value (-0.117, in Capasso *et al.* 2003; Trinchella *et al.* 2008, 2012) in fish MT. We found large variation in hydropathic index to generally occur across all MT types (Table 2a). When considering average hydropathic values (GRAVY indices), MT1/2 and MT3 are at the opposite extremes of the range, with MT1/2 being the only MT clade showing a positive average hydropathic value and MT3 representing the most negative averaged value obtained (Table 2). Scudiero *et al.* (2005) by comparing only mammalian versus fish MTs suggested that the average hydropathic value may be phylogenetically correlated. The hydrophobicity plots for the main MT clades (Figure 10) also show higher variability among clades in the beginning of the amino acid sequence, which correlated with the β-domain being more variable (see also below) and the one mostly involved in the functional divergence among MT types (e.g. Hidalgo *et al.* 2009; Tío *et al.* 2004, see also below). The two domains also strongly differ in their metal binding affinity (e.g.

Jiang *et al.* 2000; reviewed in Tío *et al.* 2004). The hydropathic/hydrophobic value gives indications about the flexibility of the protein, which is correlated to higher capacity of undertaking conformational changes and therefore may be an indication of functional divergence. In fact, the protein folds around the metal(s) it binds, and the ligand accessibility and release are dependent on the flexibility of the whole protein. Our results would therefore suggest a higher functional flexibility in the β-domain of the MT protein and possibly a well defined distinction among main MT types.

Table 2 - Hydropathic value results. **a)** GRAVY index calculated for main MT clades (see Materials and Methods for additional information). "Max", "Min", "Average", "SD" indicate respectively the maximum, minimum, average, standard deviation GRAVY indices obtained for sequences within a given clade; "GRAVY +/- 2SD" represents the higher and lower boundaries respectively, limiting the 95% area of the normal distribution of the GRAVY index for all sequences within the main MT clades. b) GRAVY index for potential pseudogenes, sequences used for comparison (see Materials and Methods), and clade to which they belong to. For M. domestica and O. anatinus sequences, the GRAVY index was not calculated because these sequences do not belong to any clade for which other sequences could be used for comparison.

MT Clades	Max	Min	Average	SD	GRAVY +/- 2SD
MT1/2	0.4344	-0.0820	0.1353	0.0957	0.3267 +/- 0.0561
MT3	-0.3029	-0.4691	-0.3849	0.0674	-0.2501 +/- 0.5197
Mammal MT4	0.0758	-0.1571	-0.0225	0.0787	0.1452 +/- 0.1798
Potential amniote MT4	0.0758	-0.1667	-0.0650	0.0840	0.1348 +/- 0.2329
Amphibian, bird, and reptile MT	0.1436	-0.5065	-0.2105	0.1779	0.1058 +/- 0.5662
Fish MT	0.0767	-0.1917	-0.0687	0.0872	0.1029 +/- 0.2431

b) GRAVY index for potential pseudogenes, sequences used for comparison (see Materials and Methods), and clade to which they belong to. For M. domestica and O. anatinus sequences, the GRAVY index was not calculated because these sequences do not belong to any clade for which other sequences could be used for comparison.

Clade	Potential Pseudogenes	Sequence GRAVY	Clade GRAVY +/- 2SD
Clade MT1/2	Bos_MT1A	0.1934	0.3267 / -0.0561
	Bos_MT1E	0.1557	
	Bos_MT1E2	0.0377	
	Can_MT2A	0.1721	
	Can	**0.4344***	
	Equ_a_	0.0607	
	Equ_b_MT	0.1462	
	Equ_c_MT	**-0.0820***	
	Equ_d_MT	0.0525	
	Pan_a	-0.0291	
	Pan_c	0.2574	
	Pan_h	0.0758	
	Pan_MT1B	0.0328	
	Sus_MT1A	0.1295	
	Sus_a_MT	0.2475	
	Sus_b_MT	0.2148	
Clade amphibian, bird and reptile MT	Pel_b_MT	-0.1453	0.1058 / -0.5662
	Pel_c_MT	**0.1436***	
Sequences not recovered in major clades	Mon_MT2	0.0871	(GRAVY index not calculated)
	Mon_a_MT	-0.0516	
	Mon_b	-0.0323	
	Orn_MT3	-0.3190	
	Orn_a_MT	-0.0873	
	Orn_b_MT	-0.0823	

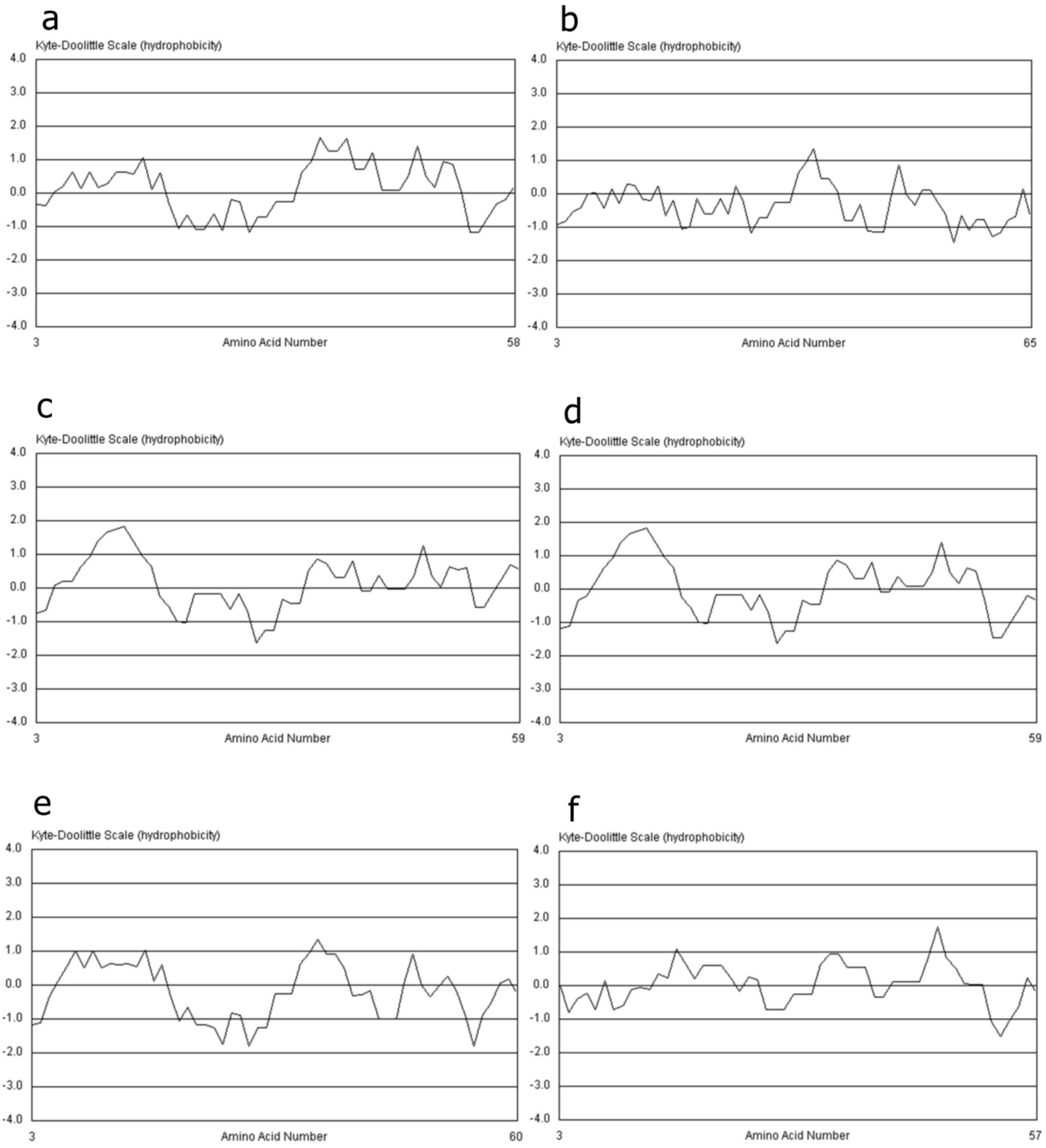


Figure 10 - Hydrophobicity plots using consensus amino acid sequences obtained for the following clades: a) Eutherian MT1/2; b) Eutherian MT3; c) Mammalian MT4; d) Potential amniote MT4; e) Amphibian, bird, and reptile MT; and f) Fish MT. Y-axes indicates hydrophobicity values, whereas x-axes indicates amino acid positions

The DIVERGE analysis, ran to further study the protein functional divergence among main MT types, statistically confirmed Type I, but not Type II, functional divergence (Table 3). Type I involved one and four amino acid sites, for MT1/2/3 versus

MT4 and MT1/2 versus MT3 functional divergence (Table 3, Figure 8, and Supplementary Material 5).

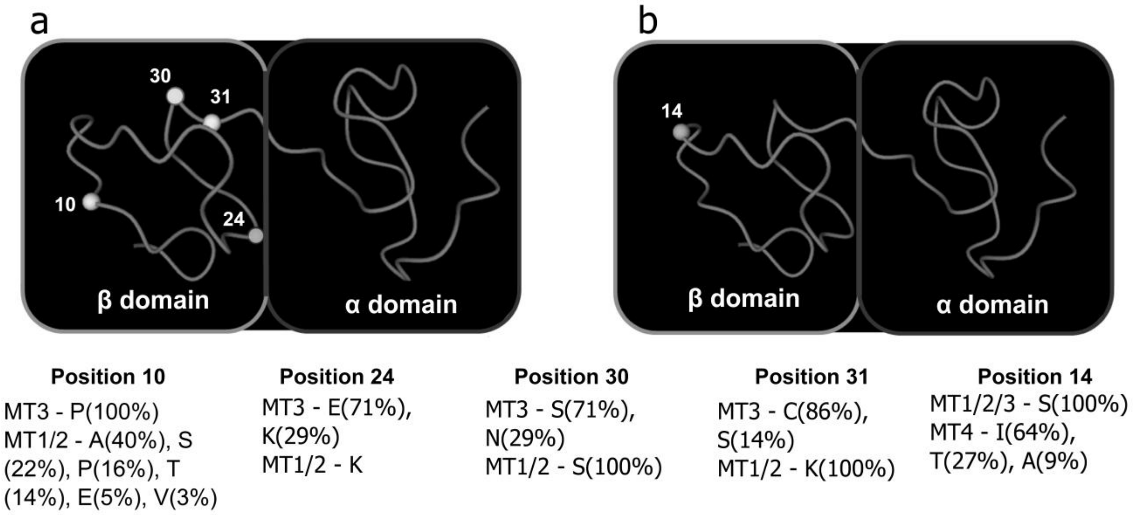


Figure 11 - Metallothionein 3D structure as obtained from DIVERGE using the RatMT2 3D protein as a model. Colored circles on the protein structure indicate Type I divergent amino acids (cut-off value = 0.9) between MT1/2 versus MT3 clades (A) and MT1/2/3 versus MT4 clades (B). Colored rectangles under the figure correspond to the distinct colored amino acids and indicate the type of amino acid change at that position for the compared clades. Amino acid code: "A" – alanine, "R" – arginine, "N" – asparagine, "C" – cysteine, "E" – glutamic acid, "I" – isoleucine, "K" – lysine, "P" – proline, "S" – serine, "T" - threonine, "V"- valine

Table 3 - Type I and II divergence test results. "***θ*** $_{I}$" indicates the coeffient of functional divergence; "**SE**" indicates the standard error; "**LRT**" corresponds to the 2 log-likelihood-ratio against the null hypothesis of $\theta_{I} = 0$; "***p***" indicates the p value; "**Pp cut-off**" represents the posterior probability cut-off for specific amino acid sites. Significant p-values ($p < 0.05$) are indicated in bold.

	θ_{I} ± SE	LRT	*p*	Pp cut-off = 0.9	θ_{II} ± SE	*p*	Pp cut-off = 0.9
MT1/2 vs MT3	7.992×10^{-1} ± 0.310	7.158	**0.007**	4	8.244×10^{-2} ± 0.160	0.20	8
MT1/2/3 vs MT4	2.552×10^{-1} ± 0.107	5.659	**0.017**	1	-6.236×10^{-2} ± 0.201	0.26	3

These sites all occur within the β-domain, to further confirm the less constrained functional activity of this part of the protein compared to the α-domain. The amino acid site recovered at position 10 in our alignment (Figure 11 and

Supplementary Material 5) within the β domain has been previously identified to be involved in different functions in MT3 and MT1/2 in mammals (Faller *et al.* 1999). The change of two conserved proline residues in positions 7 and 10 (position numbers according to Supplementary Material 5) to alanine or serine, which occurs in some sequences in our MT1/2 clade, dissolves the neuroinhibitory activity and cluster dynamics of the MT3 unique function in the brain (Hasler *et al.* 2000; reviewed in Hidalgo *et al.* 2009). Two of the other sites indicated by our study to be involved in functional divergence show an interesting pattern in the MT3 dataset. These divergent amino acids (positions 24 and 30, alignment as in Supplementary Material 5) suggest a species-specific functional divergence of the MT3 protein in rodents compared to MT3 and MT1/2 of other species. The amino acid site at position 24 is divergent for all MT1/2 versus MT3, except for *M. musculus* and *R. norvegicus* (MT3 amino acid in these species is the same as the one conserved in MT1/2), while the one in position 30 is conserved between MT1/2 and MT3 with the exception of these two species. The amino acid site at position 24 is the only one consistent with functional divergence between MT1/2 and MT3 when including in the input tree a tetrapod MT3 clade (see Materials and Methods for further explanations). This would suggest that the other recovered sites would be most likely involved in functional divergence of only eutherian MT3 versus the other MT types. The last site, amino acid position 31 (position number as in Supplementary Material 5) most likely was recovered due to the substitution of a conserved cysteine in Bos_MT3. Expression data for this gene/protein will be necessary to assess if this is a fully functional copy. Finally, the amino acid potentially involved in functional divergence between MT1/2/3 and MT4 at position 14 involves a serine, conserved in MT1/2/3 (and also in most of other sequences not belonging to MT4 clade) (Figure 11 and Supplementary Material 5). This amino acid occurs next to the metal binding cysteine, conserved in all main clades. Intercalating residues among the conserved cysteines in the β-domain are highly dissimilar and associated with functional divergence between MT4 and MT1 types (Tío *et al.* 2004). This result is further confirmed when performing the analysis using an input tree including a tetrapod MT3 clade.

Putative pseudogene inference

To identify potential pseudogenes among the studied sequences we followed two criteria: the occurrence of a replacement of invariant cysteines and, when possible a strong deviation of hydropathic index in comparison to the other sequences recovered in the same phylogenetic clade. In Bos_MT1E2 we observed a substitution of a terminal cysteine with histidine. However, this substitution does not necessarily indicate a non-functional protein (e.g. Romero-Isart *et al.* 1999). In our dataset, the Can sequence shows a substitution of two cysteines by arginine and lysine in the beginning of the amino acid sequence (position seven and nine in our alignment, see alignment in Dryad, along with eight other different neighboring amino acids from position one to 11. The substitution of these metal-binding residues for hydrophilic amino acids (as further confirmed by the GRAVY index = 0.4344 and identified as a potential outlier, Table 2b) may compromise the function of the protein and could therefore indicate that this sequence is a pseudogene. Orn_MT3 showed a cysteine replacement by serine (position 69 in our alignment). However, this change has been proven to maintain the protein function in recombinant MTs (Chernaik and Huang 1991). Pan_c presented a substitution of a moderately conserved serine (position 67 of our alignment) by a cysteine, which could potentially interfere with the optimal protein function. All *P. sinensis* (for the part of the sequences that could be compared, due to the missing part in the beginning), *M. domestica*, *E. caballus,* and *S. scrofa* sequences did not show any cysteine change, although two of these sequences (Equ_c_MT and Pel_c_MT) were scored as outliers according to the GRAVY index (- 0.0820 and - 0.1453, respectively, Table 2b). Further studies on MT gene expression and evolution will help clarifying the possible pseudogenization of the Can, Equ_c, Orn_b_MT, Pan_c, and Pel_c_MT sequences.

Conclusions

The dataset used in this work, which was built based on representative vertebrate species with complete genome annotation and checked by more than one genomic database to improve dataset quality, resulted in some advances in the current sparse and sometimes puzzling knowledge of MT gene family molecular evolution and functional divergence. We were able to recover multiple MT types in all amniotes, suggesting that duplication and functional divergence in MTs is not limited to mammals and birds. Furthermore, our results indicate the existence of a reptile/bird MT clade, a potential amniote MT4 clade, and a mammal MT3 and MT1/2 clades. Future phylogenetic studies including a larger taxa sampling may help to further confirm the existence of a tetrapod MT3 clade, as recovered in this study by the analyses ran on the amino acid dataset. Our results, together with the analyses of functional divergence between main MT clades and sites possibly associated with the functional divergence, permitted us to conclude a likely association of MT functional genes roles in vertebrate groups to duplication/loss events.

In humans, MT1/2 are inducible and expressed in almost every tissues. MT3 and MT4 are, on the other hand, relatively unresponsive to the inducers that stimulate MT1/2 expression and are mostly located in the central nervous system and in the stratified squamous epithelium, respectively (reviewed in Vašák and Meloni 2011). The limited amount of data available for on non-mammalian vertebrate and on a non-vertebrate chordate in which the expression and induction of distinct MT types have been analyzed (e.g. Guirola *et al.* 2012; Nam *et al.* 2007) suggest the existence in non-mammalian vertebrates of two MT types, of which one is more ubiquitous and the other is more specialized. The poor resolution of phylogenetic relationship among the main MT types does not allow us to fully interpret the evolutionary process of functional divergence in this gene family. We may speculatively propose that a less functionally specialized ancestral MT may have occurred in all tetrapods (similar to the current mammalian MT1/2 type), which evolved into more specialized MT types (e.g. the current MT3 and MT4). Although our analyses support the existence of functional divergence among the main MT types, we do not have sufficient indication to support that MT3 and MT4 represent functionally specialized MTs in amniotes other than mammals. More biochemical and expression data are certainly needed to understand the underlying mechanisms of functional divergence after gene duplication in MTs, especially between mammals and other vertebrates. Together with highlighting that MT gene duplication interested all amniotes and that functional divergence occurred among main MT types, our results can also help to design future cost-effective MT functional studies in other vertebrates, beside human and mouse. In fact, while a large body of biochemical and molecular work is currently available for mammalian model species, similar data are currently lacking for the distinct MT types recovered in non-mammalian vertebrates. Furthermore, in vertebrates, metallothionein expression and concentration are often used in ecotoxicological and metal homeostasis studies (e.g. Andreani *et al.* 2007; Kim *et al.* 2013; Riggio *et al.* 2003). As studies on mammalian model species reveal, not all MT types are equally involved in the same function, expressed in the same tissue and at the same time, neither show the same metal affinity. Therefore, the lack of knowledge on similar potential differences among MT types in other vertebrates possessing multiple MT genes may be misleading or provide incomplete conclusions. Comparative genomic and biochemical studies will help filling this gap of knowledge and contribute to our understanding on both metallothionein evolution and functional divergence after gene duplication in vertebrates.

2. Phylogenetic signal in amphibian sensitivity (LC50) to copper sulfate

Abstract

One of the most common objective measures used in toxicological studies to quantify the association between exposure and toxicity is the LC50. This parameter is exclusive for each species and contaminant and may therefore vary considerably between species, providing some indications of species sensitiveness to a contaminant when exposed conditions are the same. In ecological risk assessment (ERA) for ecotoxicological studies, little variation in chemical sensitivity within a taxonomic group (and sometimes among taxonomic groups, e.g. fish and amphibians) is assumed. This relies on a further assumption that taxonomic relationship may be used to infer species sensitivity to a certain contaminant. However, the influence of taxonomic relationships on sensitivity to a certain chemical compound has largely not been tested. We therefore used a phylogenetic comparative approach to assess the phylogenetic signal to sensitivity, using the LC50 parameter, to copper sulfate in amphibians and to also estimate the effect of temperature variation on sensitivity variation.

Our results demonstrate a strong influence of temperature on amphibian LC50 to copper sulfate. The data gathered from published and available comparative data on eleven amphibian species show a phylogenetic signal in LC50 only when taking temperature under consideration, demonstrating the important role of temperature in species sensitiveness to copper sulfate. This result, if confirmed with a larger sample size, can be used as a predictive tool in amphibian conservation and risk assessment studies.

Introduction

To evaluate the effects of chemicals, pesticides, and contaminants on organisms, a repeatable quantitative measure of relationship between exposure and toxicity to the organisms is generally used. This relationship is typically characterized by two variables: 1) the dose or exposure (amount of chemical/pesticide taken up or ingested by the organism, such as the chemical concentration in the exposure medium in a total exposure time), and 2) the response, which may be mortality, growth, reproductive performance or other phenotypic responses (Wright and Welbourn, 2002). One parameter often used as a response is the LC50 (Lethal Concentration 50), which corresponds to the toxicant concentration at which in a certain time, half of the test population/sample is killed (Wright and Welbourn, 2002). LC50 is therefore dependent on the concentration of the chemical used and on the time of exposure, however it may also be influenced by: specific experimental conditions (e.g. temperature, salinity), the studied species, and by the developmental stage of the tested specimens.

The importance of integrating concepts and knowledge from evolutionary biology into ecological risk assessment (ERA) has been recently recognized (e.g. Coutellec and Barata 2011 and references therein). In fact, traditional ecotoxicology and ecotoxicological risk assessment focus mostly on descriptive phenotypic changes (e.g. mortality, LC50, growth, etc.) following treatment with a contaminant, generally considering species and populations as independent units. However, species, and population sensitivity resemblance to a chemical (contaminant/pesticide) may or may not reflect the evolutionary history of the species (or populations). In ERA, the common procedure is to assume little intraspecific and interspecific variation (generally among species belonging to the same taxonomic group, e.g. birds, mammals) in chemical sensitivity. Therefore, the LC50 of a determinate chemical for certain species provides an indication of how distinct species may be more or less sensitive to a contaminant under the same experimental conditions.

The assessment of chemical sensitivity can be carried out on a handful of model species in ecotoxicology that can be easily studied in laboratory conditions to identify the most sensitive taxonomic group (e.g. birds, mammals, fish, and invertebrates). The results are then extrapolated to estimate the impact of a chemical on all the species within the group and sometimes even on other distantly related groups (e.g. ESFA Journal 2013, pag 169; Species Sensitivity Distribution, SSD, see for example Awkerman *et al.* 2008; Interspecific correlation Estimation, ICE, Raimondo *et al.* 2010). However, this approach may ignore evolutionary processes, such as natural selection and neutral evolution (genetic drift), and their outcome. Those include adaptation and plasticity, which may produce variation in sensitivity to a chemical among populations and species (e.g. Hua *et al.* 2013). Hammond *et al.* (2012) reviewed how intra- and inter- specific variation in sensitivity to a pesticide may largely vary depending on the chemical and on the species. In the same work, these authors found that significant phylogenetic signal can be observed in the sensitivity to endosulfan, a common insecticide.

Knowledge on patterns of sensitivity to a contaminant among and within well studied species may help to formulate prediction on how other species may respond to the same contaminant. This approach would be especially useful to predict how toxic a compound may be to endangered species and populations, in order to prevent or limit their decline. When assessing patterns of sensitivity within and among species, identifying confounding factors that may influence the sensitivity parameter is especially important, since these factors could invalidate predictions that ignore them. In this study we aim to use a phylogenetic comparative approach to evaluate the phylogenetic signal in sensitivity (in this case, LC50) to copper sulfate in amphibians and to estimate the influence of temperature variation on this measure of sensitivity. We used published and available comparative data, together with a phylogenetic generalized least-squares model on eleven amphibian species representing different genera and families of Anura.

Materials and Methods

The list of amphibian species to use in this study was selected using three different reference sources: Fryday and Thompson (2012), the Ecotox database (last accessed on Sept. 7, 2013), and a literature search using “LC50”, copper sulfate, and amphibians as keywords (last search Sept. 7, 2013). Data from Ecotox and Fryday and Thomson (2012) were double checked on the original papers (see Table 4). To retain the maximum number of amphibian species for which comparative data were available for copper sulfate, we selected only studies that were carried under static conditions without renewal, with technical test material, at the stage of tadpole (or larva) and for studies with duration of 96 hours (four days). The only parameters we allowed to vary in this analysis were species, temperature at which the study was carried out, and LC50 (see Table 4).

Table 4 – List of eleven species for which the effect of temperature and phylogenetic signal on LC50 was calculated.

Species	Family	T (ºC)	LC50	Original source data
Epidalea calamita (Laurenti, 1768)	Bufonidae	20±0.5	0.08	García-Muñoz *et al.* 2010
		20±0.5	0.10	García-Muñoz *et al.* 2010
Bufo boreas Baird & Girard, 1852	Bufonidae	22	0.12	Dwyer *et al.* 1999
		22	0.12	Dwyer *et al.* 2005
Bufo bufo (Linnaeus, 1758)	Bufonidae	20±0.5	0.08	García-Muñoz *et al.* 2010
		20±0.5	0.09	García-Muñoz *et al.* 2010
Pseudepidalea viridis (Laurenti, 1768)	Bufonidae	20.6	0.10	Gürkan M and Hayretdağ, 2012
Duttaphrynus melanostictus (Schneider, 1799)	Bufonidae	31.5	0.32	Khangarot and Ray 1987
Pelophylax perezi (López-Seoane, 1885)	Ranidae	20±0.5	0.36	García-Muñoz *et al.* 2010
		20±0.5	0.57	García-Muñoz *et al.* 2010
Rana sphenocephala (Cope, 1889)	Ranidae	22	0.23	Bridges *et al.* 2002
Euphlyctis hexadactylus (Lesson, 1834)	Ranidae	15	0.04	Khangarot *et al.* 1985
Hoplobatrachus tigerinus (Daudin, 1802)	Ranidae	26.5	0.39	Khangarot *et al.* 1981
Pelobates cultripes (Cuvier, 1829)	Pelobatidae	20±0.5	0.22	García-Muñoz *et al.* 2010
		20±0.5	0.22	García-Muñoz *et al.* 2010
Discoglossus jeanneae Busack, 1986	Alytidae	20±0.5	0.08	García-Muñoz *et al.* 2010
		20±0.5	0.10	García-Muñoz *et al.* 2010

The species for which more than one data point was available, the mean study temperature and mean LC50 were retained. To infer the strength of the phylogenetic signal in association with variation in temperature of the study, we used the phylogenetic least-squares models (PGLS) (Pagel 1997, 1999; Freckleton *et al.* 2002). The PGLS methods rely on a known phylogeny for which branch lengths are known.

The phylogeny is transformed into a variance-covariance matrix, in which the diagonal of the matrix, representing the variance, is given by the path length from the root to the tips of the tree, while the off-diagonal values, representing the covariance, are given by the path length from the root to the most common ancestor between two taxa (for further explanations see Figure 1 in Rohlf 2001). The ʎ value, representing the strength of the phylogenetic signal, is estimated by ML in PGLS. It varies between 0 and 1, with 0 indicating no phylogenetic signal in the data and 1 indicating that the pattern observed in the data can be predicted by phylogeny (for further detailed explanations see Capellini *et al.* 2010 and references therein). PGLS was run in R environment using the "Caper" package (Orme 2012). We used the amphibian phylogenetic tree with branch lengths obtained by Pyron and Wiens (2011). Caper allows estimating ʎ based on ML and compares the likelihood value of this model with the one obtained on regression models ran with fixed ʎ = 0 and ʎ = 1.

Results and Discussion

Figure 12 shows LC50 values plotted in function of the temperature. An increase in temperature is generally correlated with a decrease in sensitivity to copper sulfate (higher LC50).

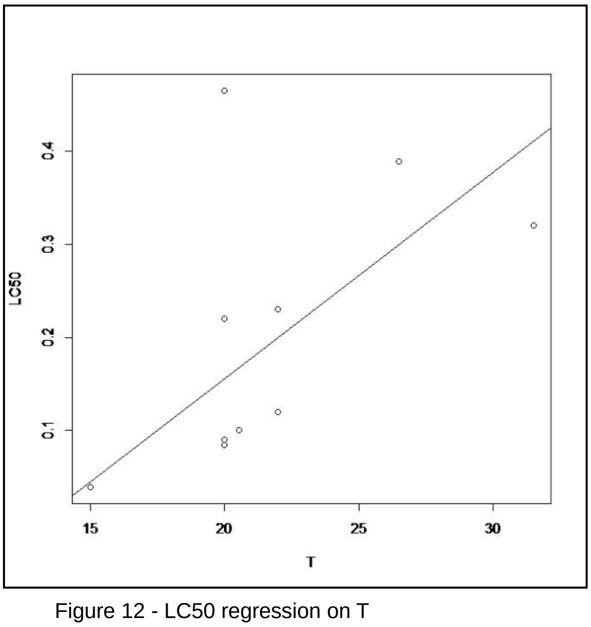


Figure 12 - LC50 regression on T

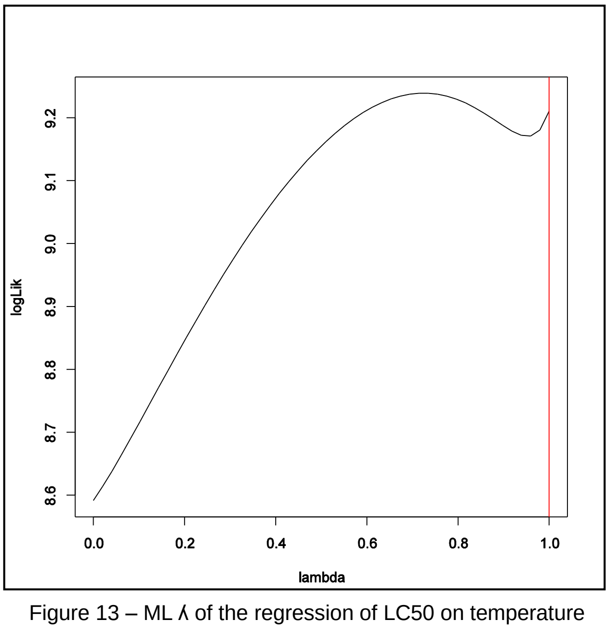


Figure 13 – ML ʎ of the regression of LC50 on temperature

ML λ of the regression of LC50 on temperature (Figure 13) was found to exhibit a strong phylogenetic signal (ML λ = 1), with temperature accounting for 63% of the variance observed in LC50 (Multiple R-squared: 0.6279, Adjusted R-squared: 0.5865, F-statistic: 15.18 on 2 DF, p = 0.001306).

Figure 14 shows the model assumptions graphs. Figure 13, which reflects the precision of our estimates, indicates a large curvature near the maximum value, suggesting a small variance and a good estimate. A similar indication that the assumptions of the models are well applied is offered in Figure 14. The residuals are generally normally distributed, while in the Q- Q plot the residuals lie along a line.

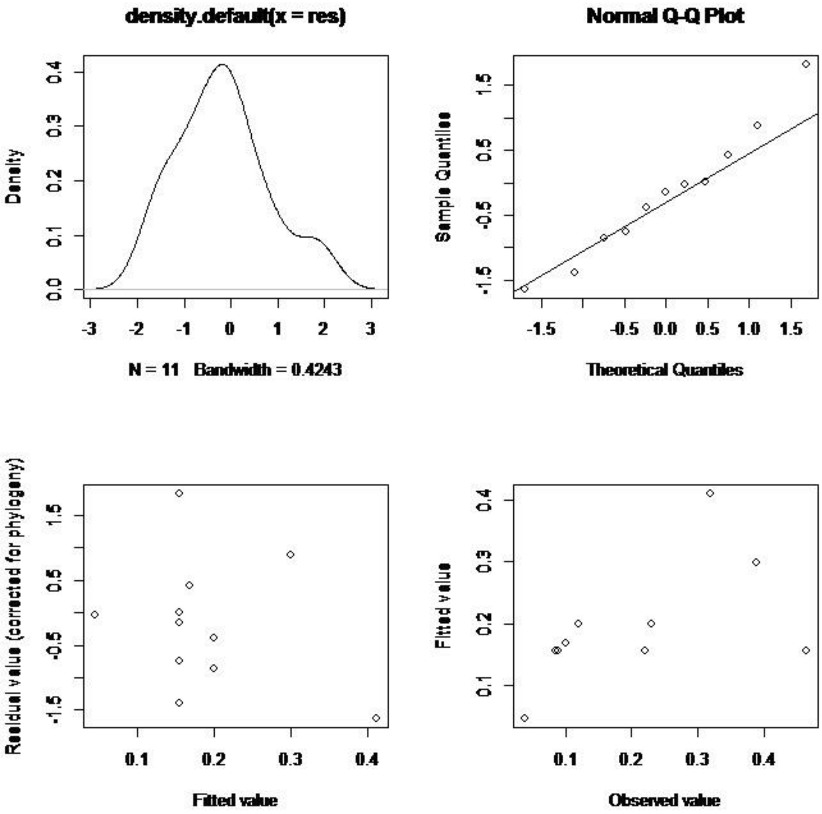


Figure 14 – Evaluation of the model settings: top left, density plot, top right Q-Q plots, bottom left analysis of residuals corrected for phylogeny, and bottom right, fitted values.

Furthermore, no obvious pattern can be observed in the residuals corrected for phylogeny and in the fitted values. Regression models with λ fixed to either 0 or 1 did not show any statistical difference from the model with ML λ (p = 0.26591 and 1, respectively). The fact that the estimated ML λ did not result different from a model indicating no phylogenetic signal in species sensitivity to copper sulfate is most likely due to the low number of species used in this study. Freckleton *et al.* (2002) reported that in datasets where the sample size is below 20 taxa, it is often difficult to obtain a statically significant difference between ʎ equal to 0 or 1. However, this does not invalidate the result of the regression, which suggests that once the variance in LC50 explained by temperature is removed, there is a strong phylogenetic signal in the lethal concentration at which 50% of the animals are killed following copper sulfate treatment.

If these results would be confirmed using a larger taxon sampling (e.g. including other amphibians, such as Urodela), a larger sample size, and studies carried out at different temperatures also for the same species (for a known temperature) phylogenetic relationships could be used to predict LC50 in other species of amphibians. This would be especially important for species that due to either their endangered status or their difficulty in being sampled or raised in laboratory conditions could not be experimentally manipulated to obtain data on their sensitivity to copper sulfate. Finally, future works could focus on the molecular basis correlated with the phylogenetic signal observed in LC50 following copper sulfate treatment in amphibians. One future direction would be to study if amino acid variation in the sequence of target genes involved in metal detoxification (e.g. metallothioneins) could be associated with functional divergence among species and therefore explain the phylogenetic signal observed in the LC50.

3. Isolation of the metallothionein gene in *E. calamita*

Abstract

Ecotoxicological studies often use morphological and behavioral changes on amphibians to estimate the effect of contaminant exposure. However, the use of molecular techniques in this field could provide a first line of contamination detection since it identifies sub-lethal effects with no visible phenotypic manifestation.

The metallothionein genes, which are involved in organismal detoxification processes, are known to be expressed in high doses under copper exposure in some organisms. A demonstration of MT high gene expression on amphibians in response to copper sulfate treatment remains to be studied. In my study, I will develop tools to study metallothionein gene expression in order to fill this knowledge gap since it is of great importance to further understand the rapid implications at molecular level caused by a common contaminant exposure in this sensitive animal group. The first step in any gene expression study is to identify and isolate the gene of interest in the target species. In this study, we isolated the metallothionein gene in *E. calamita* from cDNA samples obtained from an animal treated with copper sulfate in laboratory condition.

Introduction

This chapter focuses on the isolation of the metallothionein gene (most likely a single gene copy, see further information in Chapter 1 and below) in *E. calamita.*

Despite the great abundance and diversity of MT gene copies in some vertebrates (namely mammals; Capdevila and Atrian 2011; Vašák and Meloni 2011; Guirola *et al.* 2012, see Chapter 1), earlier characterization of MT genes in squamates and amphibians from cDNA, succeed to recover only one MT gene per species (Riggio *et al.* 2003; Trinchella *et al.* 2008, 2012, see also Chapter 1 for what concerns the number of copies in reptiles). This confirms for amphibians what already observed in *Xenopus* (Saint-Jacques *et al.* 1995). Generally all MT ortholog and paralog genes present a relatively short and similar CDS sequence across vertebrate taxa (see Chapter 1, this Thesis). Data on MT in amphibians are still limited. However MT amphibian gene sequences have been previously isolated in five species, including Anura and Urodela (Saint-Jacques *et al.* 1998; Trinchella *et al.* 2012). Sequence similarity among these five species has been reported as 70% taking in consideration the number of similar nucleotide sites between sequences (Trinchella *et al.* 2012). In our work, we used the conserved status of the MT gene among amphibians to isolate the MT gene directly from the cDNA of one of our samples (see below), by using degenerated primers designed on the basis of CDS amphibian sequences obtained from Genbank.

Hence, this step of our work in is aimed for:

1) Design specific primers to use in qRT-PCR experiment to study gene expression following treatment with copper sulfate (see Future Work);
2) Isolate the gene from cDNA to make sure that the designed primers amplify one single band (the target gene) before running the qRT-PCR, and that the MT gene in *E. calamita* is a single copy.

Materials and Methods

Lab settings

One complete egg mass of *E. calamita*, (a single clutch of genetically similar individuals) was collected in May, 2012 from a pond site with no record of historical pollution (Ardal´s pond; UTM 30S 448100, 4220986; located on Jaén province, south-eastern Spain, on siliceous bedrock and surrounded by Mediterranean forest, see Figure 5). Eggs, embryos and tadpoles were always kept in compartments or aquariums in unpolluted highland spring from the same source and with same chemical characteristics (pH: 7.2–7.8; alkalinity: 170–250 mg L^{-1}; hardness: 129 mg L^{-1}; NO^{-3} < 0.1 mg L^{-1}). Eggs were kept at 20ºC (±0.5ºC), on a 12 h light vs 12 h dark cycle in a temperature-controlled chamber. Individuals were allowed to develop to Gosner Stage 25 (equal to a tadpole stage).

The test was performed on distinct experimental sets consisting of five tadpoles each placed in separate glass vessels of 15 cm diameter) under different temperatures (20 and 25ºC) and each with 1000 mL of solution containing different concentration of copper sulfate, $CuSO_4{\cdot}5H_2O$ (0; 0.02 and 0.04 mg L^{-1}) or without the contaminant. Samples were collected according to the following criteria: 1) individuals raised with no contaminant in water were used as a control, while 2) and 3) samples were collected one hour after adding copper sulfate. Samples were immediately flash frozen in liquid nitrogen to preserve RNA quality and conserve effective gene expression of individuals. These samples were then shipped to CIBIO in dry ice (-80ºC) to maintain RNA stability (prevent degradation). All collected samples (tadpoles in Gosner Stage 20 and Gosner Stage 25 or toadlets) were stored at -80ºC after flash frozen untill the extraction procedure. Obtained samples were used to isolate the metallothionein gene in *E. calamita* (see Table 5) as well as to carry out the experiments described in Chapter 4.

RNA extraction

RNA was extracted from a total of 45 samples (entire animal) using TRIzol Reagent (Life Technologies®) following manufacturer instructions. Animals were ground with a pestle in a mortar frozen by pouring liquid nitrogen on it (see also Gayral *et al.* 2011 for additional information). Extractions were commissioned to AllGenetics® (La Coruña, Spain). The required RNA extraction technique was selected due to the high efficiency (in terms of yield and success of extraction) of this method (Y. Chiari, pers. comm.) and first tested and optimized by AllGenetics on specimens of *Rana temporaria* (tadpoles) and on a newt (species not identified, J Vierna, AllGenetics, pers. comm.) available at the facility. Available test samples shared the same storing conditions as ours (flash frozen in liquid nitrogen immediately after collection and stored at -80ºC before the extractions). After RNA extraction, samples were treated with RQ1 RNase-Free DNase (Promega®) according to manufacturer instructions and run on the Agilent 2100 Bioanalyzer (Agilent Technologies®) to check for RNA integrity and yield. RNA samples were shipped to us in dry ice and stored at -80ºC until retro-trascription (see below).

Reverse transcription of RNA

RNA samples for which the extraction had been carried out successfully (see Table 5) and with a minimal total RNA quantity of 47 ng/µL were reverse transcribed to single strand cDNA using the iScript cDNA Synthesis Kit (Bio-Rad®). 0.7 µg of total RNA for each sample (see Chapter 4 for additional information regarding the use of the same RNA quantity for this step) were retro-transcribed following manufacturer instruction. cDNA samples were stored at -80ºC.

Primer design and gene isolation

Available amphibian MT sequences were downloaded from Genbank and aligned in MEGA. Sequences were available for *Pelophylax esculentus (*Linnaeus, 1758)*, Xenopus laevis* (Daudin, 1802)*, Xenopus tropicalis* (Gray, 1864)*, Triturus carnifex* (Laurenti, 1768) and *Ambystoma mexicanum* (Shaw & Nodder, 1798) (see Figure 15 for Genbank accession numbers); and we also added two MT sequences of a single bird species, *Gallus gallus* (Linnaeus, 1758) (see Chapter 1) to take into account sequence divergence across taxa. Search of available MT CDS was primarily organized by hierarchical taxonomic level: first we looked for available sequences for the same species, than the same genera, family, and class. Primers were designed in Primer3 Plus (Untergasser at al., 2007) and/or by eye on the beginning and end of the CDS sequence. Primers were further checked by MFEprimer-2.0 (Qu *et al.* 2009) for possible unspecific binding (given by the number of predicted amplicons), annealing temperature, primer-dimer formation, and hairpins using the Chicken RNA and genomic background database selection. The primers used for PCR amplification and gene isolation for the *E. calamita* metallothionein gene were: forward, 5' ATGGACCCTAA[A/G]GACTGCG 3' (Ta 57-59.7ºC), and reverse, 5' GTTGCA[A/G]CAGCTGCACTTCT 3' (Ta 59.4-64.4ºC). The expected amplified fragment has 189 bp.

Final primer alignment of the CDS used for MT primer design is shown below

```
Pel_esc_MT ATGGACCCTAAAGACTGCGGCTGTGCTGCCGGTGGCTCATGCTCCTGCGGTGATTCCTGCAAGTGC
Xen_lae_MT ATGGACCCTCAGGACTGCAAATGCGAAACAGGTGCTTCTTGCTCCTGTGGTACTACCTGCAGTTGC
Xen_tro_MT ATGGACCCCCAGGACTGCAATTGTGAAACAGGTGCTTCCTGCTCCTGTGCAAATAAATGTGTATGC
Tri_car_MT ATGGACCCTAAAGACTGCGGCTGTGCCTCCGGTGGCTCTTGTTCATGTGCTGGGTCGTGCAAGTGC
Amb_mex_MT ATGGAC---------TGCGCATGCGCCACTGGCGGCTCCTGCTCTTGTGCTGGGTCATGCAAGTGT
Gal_ga_MT4 ATGGACCCTCAGGACTGCACTTGTGCTGCTGGTGACTCCTGCTCCTGTGCTGGGTCGTGCAAGTGC
Gal_ga_MT3 ATGGACTCCCAGGACTGCCCTTGTGCCACCGGCGGCACCTGCACGTGTGGAGACAACTGCAAATGT
           ******         ***   ** *   * ** *   * **  * ** *        **    **

Pel_esc_MT AAAGACTGCAAATGCAAGGGCTGCAAGAAGAGTTGCTGCTCCTGCTGTCCAACAGACTGCACCAAA
Xen_lae_MT AGCAATTGCAAGTGCACATCATGCAAGAAAAGCTGCTGTTCCTGCTGTCCAGCTGAATGCAGCAAA
Xen_tro_MT AGCAATTGCAAGTGCACATCTTGCAAGAAAAGCTGCTGTTCCTGCTGTCCAGCTGAGTGCAGTAAA
Tri_car_MT GAGAACTGCAAATGTACCTCCTGCAAAAAAAGCTGCTGTTCCTGTTGCCCTGCCGGATGCGATAAA
Amb_mex_MT GAGAACTGCAAGTGCACATCCTGCAAAAAAAGTTGCTGTTCCTGCTGCCCATCGGAATGTGAGAAG
Gal_ga_MT4 AAGAACTGCCGCTGCCGGAGCTGCCGCAAGAGCTGCTGCTCCTGCTGCCCCGCCGGCTGCAACAAC
Gal_ga_MT3 AAAAACTGCAAATGCACATCGTGCAAAAAAGGCTGCTGCTCCTGCTGCCCTGCAGGATGTGCCAAG
               * ***   **       ***   **  * ***** ***** ** **  * *  **    **

Pel_esc_MT TGCAGCCAGGGCTGTGAATGTGCAAAGGGATGT---GATACCTGCAGTTGTTGCAAGTGA
Xen_lae_MT TGCAGCCAGGGCTGCCACTGTGAAAAGGGAAGC---AAGAAGTGCAGCTGCTGTAACTGA
Xen_tro_MT TGCAGCAAGGGCTGCCACTGTGAAAAGGAAAGC---AAGAAGTGCAGCTGCTGTAACTGA
Tri_car_MT TGTGGCCAGGGTTGTGTGTGCAAAGGAGGGTCGACTGAGAAATGCAGCTGTTGCACCTAA
Amb_mex_MT TGTGGCCAGGGATGTGTTTGCAAAGGAGGGTCATCCGAGAAATGCAGCTGTTGCAACTAA
Gal_ga_MT4 TGTGCCAAGGGCTGTGTCTGCAAGGAACCGGCCAGCAGCAAGTGCAGCTGCTGCCACTGA
Gal_ga_MT3 TGTGCACAGGGCTGCGTCTGCAAAGGGCCCCCCTCCGCCAAGTGCAGCTGCTGCAAGTGA
           **     **** **    **                   *  ****** **  **   * *
```

Figure 15 - Alignment performed on the CDS of amphibian and bird species used to design primers to isolate the MT gene in *E. calamita*. The location of the designed primers is highlighted in grey. The corresponding species name and Genbank accession number of the selected MT sequences are the following: "Pel_esc_MT" - *Pelophylax esculentus,* HE681912 ; "Xen_lae_MT" - *Xenopus laevis,* X69380; "Xen_tro_MT" - *Xenopus tropicalis*, NM_001171679; "Tri_car_MT" - *Triturus carnifex*, HE681911; Amb_mex_MT - *Ambystoma mexicanum*, AF008583; Gal_ga_MT4 and Gal_ga_MT3 – *Gallus gallus*, NM_205275 and NM_001097538 respectively. Sequences accessed in Genbank at 19th July, 2013.

For the gene isolation we used the sample 23G30H and 12G30H already converted to cDNA. We selected the first sample among the ones exposed to 0.04 mg L^{-1} to ensure that the metallothionein gene was expressed and therefore could be isolated by PCR. 12G30H was selected for its high RNA amount so that can be easily saved for posterior gene expression experiments which require a greater dilution of the samples with high RNA concentration.

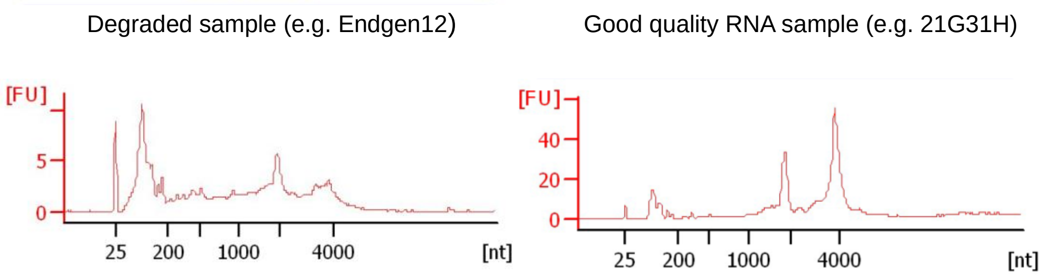
PCR amplification was carried out using 12.5 µl of Multiplex PCR Master Mix (Qiagen®) and 0.5 µM of each designed degenerated primer for 35 ng of template cDNA. PCR reaction was performed with an initial activation step of 95ºC followed by 40 cycles of 94ºC for 30 seconds (denaturation), a gradient between 50-60ºC (temperature of annealing (Ta) tested were 53.2ºC and 56.8ºC) for 90 seconds (annealing), and 72ºC for 90 seconds (extension). It was also implemented a final step of 72ºC for 10min (final extension). Due to the unknown level of gene expression for this gene, PCR reactions were also ran on a 1:10 dilution of the original cDNA following the conditions written above. Finally, to obtain a cleaner PCR product (see Results and

Discussion section in this chapter), PCR reactions were optimized after reducing 1x to 0.7x of Multiplex PCR Master Mix, decreasing each primer concentration from 0.5 µM to 0.4 µM, and by running a PCR on the previously obtained PCRs. Hence, PCRs described above were sequentially diluted to 1:10, 1:50, 1:100, and 1:1000 and 1 µL of each was used as a template for further reactions, using three test annealing temperatures, 51ºC, 54.6ºC, and 57ºC, 60 seconds instead of 90 seconds extension at 72 ºC and 35 PCR cycles. A negative control, consisting of the PCR mix without the template, was always added to each PCR run.

Gel electrophoresis was performed with TBE buffer solution (5%) on an agarose gel (2%). The ladder used to determine the base pair length of our amplified fragments was NZYDNA Ladder V (Nzytech®).

Results and Discussion

Results for all RNA extractions are available with detailed description in Table 3. Some of the samples showed RNA degradation most likely due to bad sample collection or storage above -80ºC (Figure 16). Total RNA quantity varies among samples, largely due to an improvement of the RNA extraction technique among extracted batch of samples (J. Vierna, AllGenetics, pers. comm.). Good quality DNA was obtained from 35 out of 50 samples (Table 5, see also Figure 16 for good quality RNA example profile).


Degraded sample (e.g. Endgen12)

Good quality RNA sample (e.g. 21G31H)

Figure 16 – Agilent profile obtained from two samples tests, one with degraded RNA (in the left, Endgen12) and the other containing good RNA quality. A good RNA quality is indicated by two clear 18S and 28S peaks. Indication of no degradation of large mRNA is represented with a 28S peak higher than 18S.

Table 5 - Total sample list after RNA extraction procedure. Light grey rows represent samples showing RNA degradation, small RNA concentration recovery or sample lost. First column on the left indicates the sample code we attributed to each sample. “Treatment” indicates the copper sulfate concentration, time and temperature variables in the experiments. “Regime” indicates the experimental conditions from which the samples were obtained (laboratory or mesocosm). Concentration and quality of RNA were determined by Agilent 2100 Bioanalyzer.

Sample code	Treatment			Regime	Development Stage	RNA extraction integrity	
	CuSO4·5H2O (mg L^{-1})	Time (h)	Temperature (ºC)			Concentration (ng/µL)	Quality
GS201	0	-	20	Laboratory	Gosner 20	25	Slightly degraded
GS202	0	-	20	Laboratory	Gosner 20	-	Degraded
GS203	0	-	20	Laboratory	Gosner 20	26	Good
GS204	0	-	20	Laboratory	Gosner 20	20	Degraded
GS205	0	-	20	Laboratory	Gosner 20	114.5	Good
GS251	0	-	20	Laboratory	Gosner 25	27.5	Slightly degraded
GS252	0	-	20	Laboratory	Gosner 25	63.5	Good
GS253	0	-	20	Laboratory	Gosner 25	110.5	Good
GS254	0	-	20	Laboratory	Gosner 25	272	Good
Endgen1	0	-	20	Mesocosm	Toadlet	100	Good
Endgen12	0	-	20	Mesocosm	Toadlet	-	Degraded
Endgen13	0	-	20	Mesocosm	Toadlet	-	Degraded
Endgen14	0	-	20	Mesocosm	Toadlet	-	Degraded
11G10H	0	-	20	Laboratory	Gosner 25	96.5	Good
11G20H	0	-	20	Laboratory	Gosner 25	354	Good
11G30H	0	-	20	Laboratory	Gosner 25	288	Good
12G10H	0.02	0	20	Laboratory	Gosner 25	66	Good
12G20H	0.02	0	20	Laboratory	Gosner 25	500	Good
12G30H	0.02	0	20	Laboratory	Gosner 25	555	Good
13G10H	0.04	0	20	Laboratory	Gosner25	27	Good
13G20H	0.04	0	20	Laboratory	Gosner 25	363	Good
13G30H	0.04	0	20	Laboratory	Gosner 25	92	Good
21G10H	0	-	25	Laboratory	Gosner 25	92	Good
21G20H	0	-	25	Laboratory	Gosner 25	398	Good
21G30H	0	-	25	Laboratory	Gosner 25	166	Good
22G1OH	0.02	0	25	Laboratory	Gosner 25	-	Degrated
22G2OH	0.02	0	25	Laboratory	Gosner 25	353	Good
22G3OH	0.02	0	25	Laboratory	Gosner 25	142	Good
23G10H	0.04	0	25	Laboratory	Gosner 25	-	Degrated
23G20H	0.04	0	25	Laboratory	Gosner 25	-	Degrated
23G30H	0.04	0	25	Laboratory	Gosner 25	84	Good
11G11H	0	1	20	Laboratory	Gosner 25	86	Good
11G21H	0	1	20	Laboratory	Gosner 25	434.5	Good
11G31H	0	1	20	Laboratory	Gosner 25	213	Good
12G11H	0.02	1	20	Laboratory	Gosner 25	47	Good
12G21H	0.02	1	20	Laboratory	Gosner 25	453	Good
12G31H	0.02	1	20	Laboratory	Gosner 25	84	Good
13G11H	0.04	1	20	Laboratory	Gosner 25	57	Good
13G21H	0.04	1	20	Laboratory	Gosner 25	~300	Slightly degrated
13G31H	0.04	1	20	Laboratory	Gosner 25	246	Good
13G41H	0.04	1	20	Laboratory	Gosner 25	-	Degrated
21G11H	0	1	25	Laboratory	Gosner 25	558	Good
21G21H	0	1	25	Laboratory	Gosner 25	90	Good
21G31H	0	1	25	Laboratory	Gosner 25	443	Good
22G11H	0.02	1	25	Laboratory	Gosner 25	392	Good
22G21H	0.02	1	25	Laboratory	Gosner 25	374	Good
22G31H	0.02	1	25	Laboratory	Gosner 25	-	Degrated
23G11H	0.04	1	25	Laboratory	Gosner 25	262	Good
23G21H	0.04	1	25	Laboratory	Gosner 25	450	Good
23G31H	0.04	1	25	Laboratory	Gosner 25	299	Good

PCRs resulted in a clear MT band only for reactions ran either with 1 µl of cDNA or from previous PCRs (Figures 17 and 18). No clear band was obtained using a 1:10 cDNA dilution (Figure 19), however, PCR ran on these products produced an amplification (Figure 18) indicating that, although not visible, all the PCRs we ran amplified the required fragment. We selected one of the cleaner PCR products (see Figure 18) to send it for sequencing to Macrogen®.

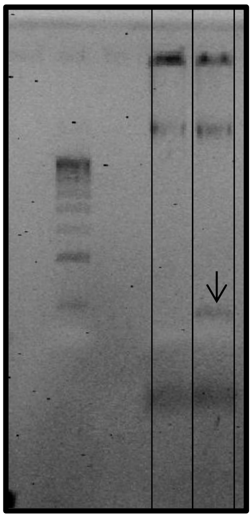


Figure 17 – Electrophoresis gel run with 1 µl of cDNA from sample 23G30H for MT amplification. (Ta of 53.2 and 56.8ºC). A clear MT band is shown for one of the temperatures tested (56.8ºC).

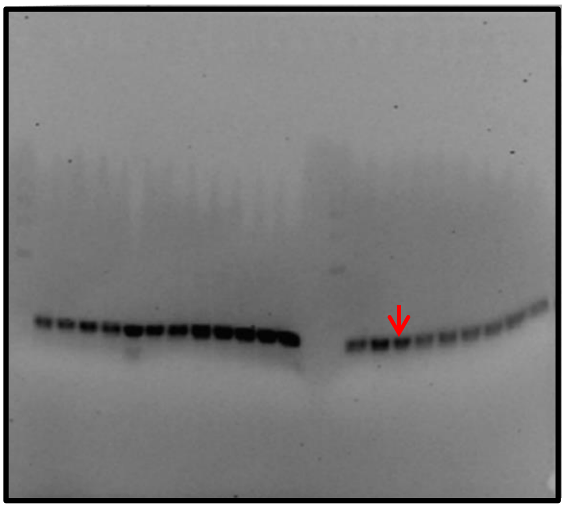


Figure 18 – Electrophoresis gel run with PCR template obtained from 1:10 cDNA dilution (Figure 19) and wiith PCR template obtained with no dilution (Figure 18) with clear MT band in all dilutions of PCR templates (1:10; 1:50; 1:100 and 1:1000). Ta used are 53.2 and 56.8 ºC. A clear MT band is shown for all loaded wells with amplified cDNA. Arrow indicates the band for which corresponding sample was selected for sequencing.

An extra RNA sample obtained under treatment of 0.02 mg L^{-1} of copper sulfate (12G30H) was tested for MT amplification to look for MT expression from 2 µl of cDNA to check if a MT band is still visible in the gel when the sample treatment applied presented a lower contaminant concentration (a visible but faiding band was visible on sample with 0.04 mg L^{-1} copper sulfate treatment, see Figure 17). The gel did not show any bands (see Figure 24 in Chapter 4) possibly indicating lower MT mRNA amount on samples retrieved from 0.02 mg L^{-1} but only further gene expression qRT-PCR tests can confirm if MT gene is less expressed in this sample.

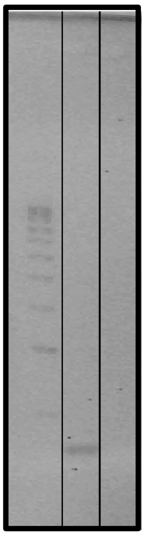


Figure 19 – Electrophoresis gel run with 1:10 dilution of cDNA from sample 23G30H for MT amplification, highlighted (Ta of 53.2 and 56.8 ºC). No MT bands appeared in the gel.

4. Development and test of endogenous control genes for qRT-PCR experiments in *E. calamita* following treatment with copper sulfate and temperature variation

Abstract

qRT-PCR is a revolutionary technique used for precise and rapid quantification of gene expression. To benefit from qRT-PCR, and especially due to its highly sensitive nature to cDNA detection, several control procedures must be carried out in parallel with the main analysis to calibrate several sources of variation. The endogenous control method relies on a selection of genes that do not change expression among tissues and developmental stages and as a consequence of different treatments. The use of these reference genes in qRT-PCR experiments permits to equalize uncontrolled sources of variation. However, stability of the reference genes for the specific experimental conditions needs to be tested. We started from a list of 28 genes to select the reference genes for stability testing in our experimental setting among the ones generally used as endogenous controls. In the end, six candidate reference genes were selected for our experimental setting of copper sulfate exposure and temperature variation in the Natterjack frog (*Epidalea calamita*). Newly designed primers were used to isolate the six referenced genes in our target species. We successfully isolated from cDNA five of the six genes: the Beta actin, 18S, Annexin A2, GAPDH, and Elongation factor 1 alpha 1. This study enables future studies on gene expression using qRT-PCR in the experimental conditions tested in *E. calamita*

Introduction

This chapter focuses on identifying the most suitable endogenous control genes for qRT-PCR in *E. calamita* following treatment with copper sulfate and temperature variation. The initial settings would include several tests of gene expression for the reference genes in varying contaminant concentration (0, 0.02, and 0.04 mg L^{-1}), temperature (20 and 25ºC), developmental stage (Gosner Stages 20 and 25 and toadlet), and natural/semi-natural environment (laboratory and mesocosm). Due to some complications during the treatment of some samples (e.g. RNA degradation or minimal quantity obtained, see Table 4, Chapter 3), we were able to test the most important characteristics proposed (concentration and temperature), but failed to obtain enough specimens to obtain the samples for testing the differences in expression among developmental stages and natural/semi-natural conditions.

In order to understand the relevance of the endogenous control study in gene expression experiments in qRT-PCR, it is important to elucidate briefly how this technology operates.

qRT-PCR is often used for quantification of gene expression, as it is a highly sensitive, accurate and relatively fast tool in quantifying (even very small concentrations) of RNA or cDNA (reviewed in Li *et al.* 2012). It allows a quantification of the products during each cycle so it can control the quantification between the initial and final product or abundance, since it varies significantly with reaction efficiency (VanGuilder *et al.* 2008). This methodology can ease some associated error with former types of quantitative PCR (e.g. it is more sensitive and more precise in quantification). However, in order to take full advantage of qRT-PCR technique, and especially due to its highly sensitive nature to detect mRNA/cDNA, several control procedures must be carried out in parallel with the main analysis. This allows to calibrate the inner technical variability associated with the collection of samples, inherent differences across samples, RNA degradation or extraction efficiency, extracted RNA quantity or quality, and reverse transcription reaction efficiency (Li *et al.* 2012). Also, the variation found between biological and technical replicas can interfere with the interpretation of the analysis and therefore requires a normalization step – endogenous control with reference gene stable expression (VanGuilder *et al.* 2008).

Ideal endogenous control testing, by suitable(s) reference gene(s), allows a subtle calibration of the variations detected in the amount of cDNA or RNA generated depending on the quality of the starting material, since small concentration differences in the starting material and in RNA or cDNA preparation will be amplified many levels, and so the error associated with it (Radonić *et al.* 2004). The endogenous control method must rely, therefore, on a selection of genes that have certain number of stable characteristics and at the same time, go through the exact same conditions as the target gene undergone experimentation. These genes (reference genes) should have a high RNA transcription level in all cells and tissues and more importantly, its transcription levels should remain stable under the different tested experimental conditions (Li *et al.* 2012). Of course, in qRT-PCR, specific RNA quantification for a target gene cannot be carried out before the experiment, so the same quantity and quality of cDNA or RNA should be used for all the samples. This helps to already reduce variability due to different cDNA/RNA quantity and quality among samples.

A great number of genes are used on endogenous control, and despite many are demonstrated as suitable in some cases, they have also been shown to express differently following different treatments, developmental stages, or experimental condition (Chiari *et al.* 2010; Li *et al.* 2012). This highlights the need of previous testing of the stability of the reference gene for specific experimental conditions before carrying out qRT-PCR studies. For this reason, in this step our work is focused on testing the stability of gene expression for some of the commonly used reference genes available.

Materials and Methods

For the reference genes, sequences of amphibians (and when necessary from other vertebrates) were obtained from Genbank and aligned in MEGA by the same methodology adopted and explained in Chapter 3. Primers were designed when unavailable for the studied species or closely related and genes were amplified from cDNA on the same samples described in Chapter 3.

Primer design

A total of 28 genes have been sorted from commonly used reference genes for qRT-PCR in vertebrates, and in amphibian in particular (e.g. *Xenopus laevis,* Sindelka *et al.* 2006, *Xenopus tropicalis*, Dhorne-Poullet *et al.* 2013, *Rattus* sp., Rocha-Martins *et al.* 2012, and *Danio rerio,* Casadei *et al.* 2011. We added also one house-keeping gene to our 28 reference genes that has already known primer sequences for our target species, defined in Harris *et al.* (2001).

The vast majority (18 genes) was excluded immediately after browsing Genbank database due to the little number of sequences available for these genes in closely related species (Amphibia) and in the hierarchical taxonomic level above, using the same search strategy as described in Chapter 3. These searches revealed that these genes (partial and/or full sequence) had less than four amphibian representatives, two of which belonging to the genus *Xenopus,* failing therefore any chance of alignment (when the partial gene fragments were not overlapping) and primer design for our species. The remaining genes were also further selected in the alignment due to: 1) partial gene sequences that prevent a good base alignment across all sequence (e.g. ornithine decarboxylase 1 gene); 2) high dissimilarity between available sequences (e.g. ubiquitin and phosphoglycerate kinase); and 3) small sequence length. Sequence length is an important factor to take into account when working with qRT-PCR. In fact, different fragment length can differently amplify during the qRT-PCR run (as in any PCR, shorter fragments amplify quicker than longer fragments), therefore to avoid noise in the results due to differently longer gene fragments, it is advisable to select primers that amplify similar fragment lengths. Fragment amplification above 200bp was selected as a criterion to obtain fragments on which to further design specific primers for qRT-PCR reactions amplifying similar gene fragment across the reference genes and the metallothionein (see Future work section). The remaining six reference genes showed good alignments and therefore primers could be successfully designed. The selected genes were: 18S, Beta actin (βActin), GAPDH, ribosomal protein L8 (rpL8), Annexin A2 (ANXA2) and eukaryotic Elongation factor 1 alpha 1 (eef1a1). Sequences from closely related species used to design these primers and their accession numbers are indicated in Supplementary File 7.

The primers were designed following the same methods explained in Chapter 3, and the final sequences for gene PCR amplification are shown in Table 6.

Table 6 - Endogenous control selected primers sequences and characteristics. GC content represents the percentage of guanines and cytosines in the sequence (40-60% GC content ensures stable binding of primer/template). "Bp" stands for base pair and "Tm" represents the melting temperature of the primers.

Gene acronym	Forward Primer 5'-3' (F)	Reverse Primer 5'-3' (R)	GC content (F/R) in %	Bp lenght (F/R)	Tm (F/R) in ºC
18S	AGCTCGTAGTTGGATCTTGG	GTCGGAACTACGACGGTATC	45% / 55%	20 / 20	57.6 / 58.2
βActin	AGCTATGA[A/G]CTGCCTGA[C/T]GGACA	TTGCTGATCCACATCTGCTGGAA	52% / 48%	23 / 23	64.4* / 63.1
GAPDH	CCAACATCAAATGGGGAGAT	TTCACTGCAGCCTTGATGTC	45% / 50%	20 / 20	55.9 / 59.1
rpL8	GGCTCTGTTTT[C/T]A[A/G]AGCCCACGT	CAGGATGGG[C/T]TT[A/G]TCAATACG	52% / 47%	23 / 21	64.3* / 57.2*
ANXA2	CCATTAA[A/G]AC[A/T]AAAGGTGTGGA	TA[G/T]GGRCTGTAGCTCTTGTA	40% / 44%	22 / 20	56.0* / 56.4*
Eef1a1	ATGT[C/G]TACAA[A/G]ATTGG[A/G]GGTATTG	AACTTGCAAGCAATGTGAGC	38% / 45%	24 / 20	58.3* / 58.4

* Mean Tm of degenerated primers (between the minor and maximum Tm of all primer combinations)

We found one house-keeping gene, 16S, with already available primers tested on our species (Harris *et al.* 2001). We choose to not test this gene for its stable expression since there is no reference in the literature on 16S suitability as reference gene for endogenous control in vertebrates (only in bacteria, see for example Chang *et al.* 2009).

Isolation of reference genes

All genes were isolated from the same samples used for MT isolation in Chapter 3. PCR amplification was carried out in the same conditions as in MT relatively to the volume of reagents and concentrations for the PCR from cDNA and for the PCRs using as template the previous PCR. The PCR cycle steps for PCR template amplification varied only in gradient of the annealing step for each gene. Ta tested for each pair of primers was: 18S (52 and 55.6ºC); β-Actin (55.6 and 60º); GAPDH (51, 52, 54.4, 55.1, 55.6; 58.2ºC); rpL8 (50, 53.2, 54.4, 55.6, 58ºC); ANXA2 (50, 50.8, 51, 52.9, 53.2, 54.4, 55.1, 55.6ºC); and eef1a1 (52, 54.2, 55.6, 57, 59ºC). If the band of the target gene was not visible from PCR of cDNA, we diluted the PCR product in a proportion of 1:10, 1:50, 1:100 and 1:1000 and tested for other temperatures and other improved PCR components concentrations, as in Chapter 3 (1x to 0.7x of PCR Master Mix and 0.5 µM to 0.4 µM). When we performed a PCR on a previous PCR product with no visible band of the target gene, we select the sample ran in middle temperature, or in case of only two temperatures, the higher temperature to prevent unspecific amplification product. In some PCRs we used 1 µl, 1.5 µl or 2 µl of the sample cDNA with 0.035 µg/µl concentration.

Gene electrophoresis specifications and ladder were equal to the ones described in Chapter 3, and a negative control was also added to each electrophoretic run. The cleaner PCR products of the isolated reference genes were sent for sequencing to Macrogen®.

Table 7 - Samples selected for endogenous control tests

Sample code	Technical replicates	Treatment			RNA extraction
		CuSO4·5H2O (mg L^{-1})	Time (h)	Temp. (ºC)	Concentration (ng/µL)
GS252	GS252A	0	-	20	63,5*
	GS252B				
	GS252C				
GS253	GS253A	0	-	20	110,5*
	GS253B				
	GS253C				
GS254	GS254A	0	-	20	272
	GS254B				
	GS254C				
21G10H	21G10HA	0	-	25	92
	21G10HB				
	21G10HC				
21G20H	21G20HA	0	-	25	398
	21G20HB				
	21G20HC				
21G30H	21G30HA	0	-	25	166
	21G30HB				
	21G30HC				
13G21H	13G21HA	0.04	1	20	~300
	13G21HB				
	13G21HC				
13G31H	13G31HA	0.04	1	20	246
	13G31HB				
	13G31HC				

Results and Discussion

The two developmental stages (Gosner Stage 20 and 25) of *E. calamita* proposed to be compared for the stability of gene expression for the reference genes will not be further tested because three of the five available biological samples for Gosner stage 20 were degraded, and one of the last two (GS203) had such a low concentration of RNA that was discarded for the test (see Table 3). Because all the samples are required to have the same total RNA quantity for the gene expression analysis, and low total RNA amount could result in very little quantity of low expression genes, their amplification would be very difficult. Since, we could not know *a priori* how expressed the genes we work on are, we decided to select samples with a minimum total RNA quantity of 47 ng/µl. Furthermore, to properly interpret qRT-PCR results at least one biological replicate is needed. Therefore, samples that were available only as a single data point will not be considered for the experiments. Therefore, in this specific case, the remaining biological sample has no statistical meaning in the study and will not be used in qRT-PCR experiments.

GS252, GS253 and GS254 samples will be tested side-to-side with 21G10H, 21G20H and 21G30H to check for stability of reference gene expression under different temperature regime (20 and 25ºC). 13G21H and 13G31H will be tested for gene expression stability under treatment with copper sulfate. These are only two biological replicates because one of the extracted samples was lost in the procedure and the other exhibited RNA degradation.

From the six selected genes for endogenous control, five were successfully isolated from cDNA: 18S and β-Actin (Figure 20), ANXA2 (Figure 21), GAPDH (Figure 22) and eef1a1 (Figure 23). PCR of PCR template containing all genes (Figure 20) with dilutions from first gel and three different Ta, failed to amplify any of the remaining target genes (results not shown). For that reason we changed the settings of the PCR (number of cycles, extension time and PCR Master Mix components concentration as described in the Methods of Chapter 3) and started a PCR for each gene from the cDNA. ANXA2 was isolated without requiring a PCR of another PCR. The other GAPDH and eef1a1 needed an extra PCR cycle to show visible band fluorescence (see Figure 21 and 22 respectively). Rpl8 was not successfully isolated by any of these methods.

A last gel was run directly on 2 µl of cDNA from the 21G30H sample to better check GAPDH and MT gene identity in the gel (see Figure 24). MT band didn’t appear in the gel (possibly related with the lower contaminant esposure in this sample, and therefore weaker gene expression) and GAPDH showed a high band (see Figure 24) which can represent another isoform of GAPDH gene, since this gene is known to show alternative splicing and generating different mRNA transcripts, for example GAPDH gene has 10 different transcripts in *Homo sapiens* and two for *Xenopus tropicalis* (Ensembl database).

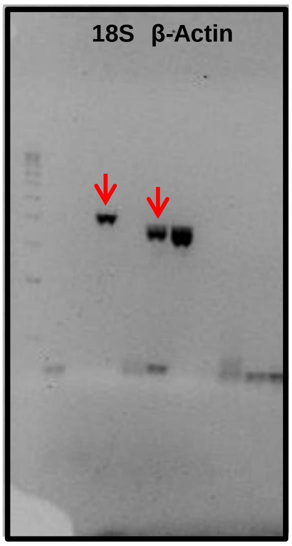


Figure 20 – Electrophoresis gel run with 1:10 dilution of cDNA from sample 23G30H for all endogenous genes (two Ta tested). Clear bands only visible for 18S and β-Actin (Ta of 52ºC for 18S and 55.6 and 60ºC for β-Actin). Arrows indicate the band for which corresponding sample was selected for sequencing.

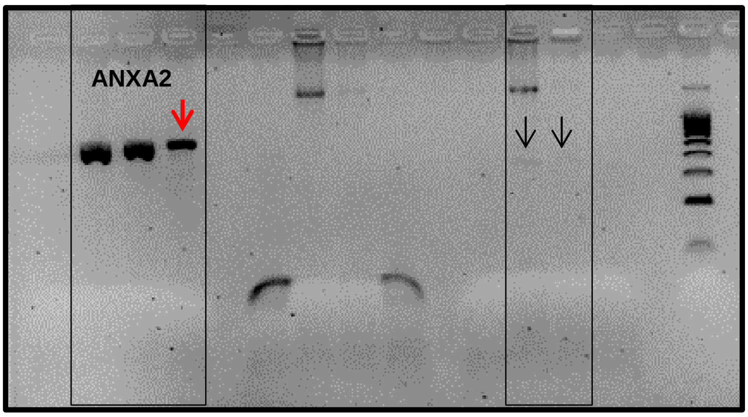


Figure 21 – Electrophoresis gel run with no dilution of 1.5 µl of cDNA from sample 12G30H for ANXA2, and 23G30H for rpL8 and eef1a1 (three Ta tested). Clear bands only visible for ANXA2 in the three temperatures (51, 53.2, 55.6 ºC). Two fading bands appear in the lower tested Ta of eef1a1 (see thin arrows). The only sample to sequence (ANXA2 gene) is indicated by the red arrow.

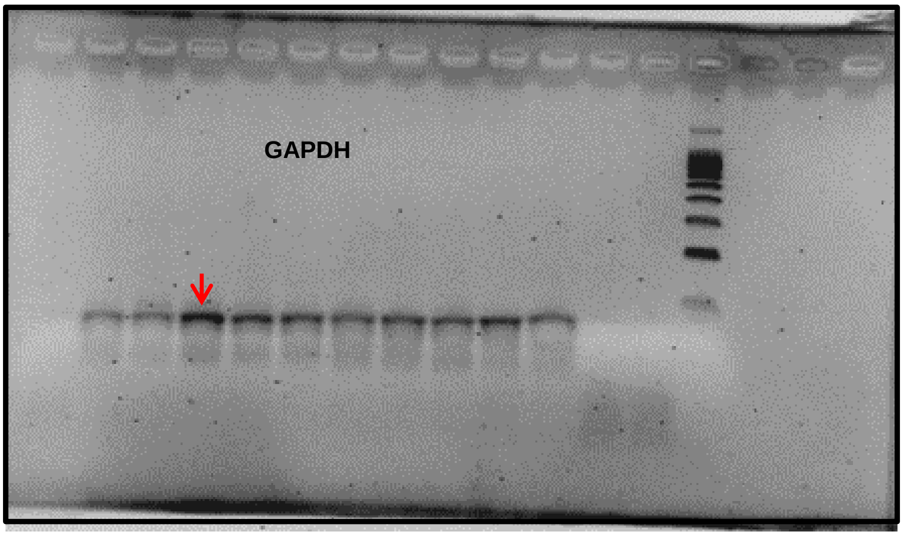


Figure 22 – Electrophoresis gel run of PCR from PCR result on GAPDH (no dilutions) with 1 µl cDNA from sample 23G30H (Ta from the initial PCR is 55.1ºC, no bands appeared). A band of small bp appeared for all tested temperatures in the new PCR (55.1 and 58,2ºC). Arrow indicates the band for which corresponding sample was selected for sequencing.

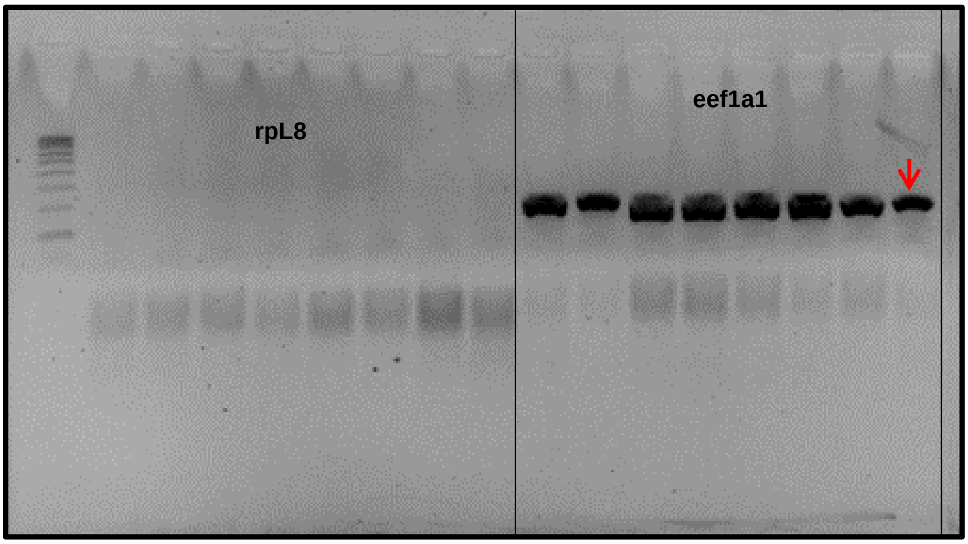


Figure 23 – Electrophoresis gel run with PCR template obtained from 1.5 µl CDNA of sample 23G30H (see respective gel in Figure 21) for eef1a1 amplification under 1:10, 1:100 and 1:1000 dilutions. Ta for this gene were (55.6, 59ºC). Sample with highest temperature of the gene was selected to this PCR. rpL8 gene, also loaded in this gel under several dilutions. Arrow indicates the band for which corresponding sample was selected for sequencing.

Figure 24 - Electrophoresis gel run with no dilution of 2 µl of cDNA from sample 12G30H with PCR, for MT and GAPDH amplification. MT bands are absent, and GAPDH presents one band above 500 base pair.

Futurely we will design specific primers for the five gene sequences to ensure that variation in qRT-PCR experiments is not caused by different annealing of the primers due to different specificity. Also, primers will be designed to amplify a fragment of approximately 200bp in each gene. All samples for which the gene was successfully isolated will run in qRT-PCR and results analyzed for gene stability using geNorm (Vandesompele *et al.* 2002).

Future work developed from this study

Our work until date provided sufficient information and working material on a non-model amphibian species (*E. calamita*), on which future works could be developed to study the genetic response to heavy metal contamination (copper sulfate) and how genetic variation can be correlated to different sensitivity to the contaminant and to temperature variation.

Consequently, in the future we will continue our study on *E. calamita* to look at metallothionein gene expression variation with the already available material (copper sulfate treated tadpoles in laboratory and mesocosms, specific primers designed on our species) by qRT-PCR. The lists of the available samples that have already been extracted or are currently in the process of being extracted are indicated in Table 8 and Table 9 (below). We will study also the samples of mesocosms (currently being extracted) which we sampled in Aveiro, Portugal, from four mesocosms containers installed near a natural lake of the University of Aveiro (divided by two controls, without contaminant, and two with sublethal concentration of 0.04 mg L^{-1} of copper sulfate.

The type of quantification of genetic responses in the cells required for this qRT-PCR consists in the primary mechanism through which organisms respond to environmental changes (within or between populations). Being the first evidence of effect on the individuals, this quantification and characterization of the genetic response at individual/population level could provide monitoring and conservation tools for key-populations possibly by identifying an occurring threat due to contamination before any traceable morphological or behavioral effect of the contaminant could be observed.

Table 8 - Sample selection for MT gene expression future studies

Sample code	Technical replicates	Treatment			RNA extraction		Future qt-PCR testing conditions
		CuSO4·5H2O (mg L-1)	Time (h)	Temp. (ºC)	Concentration (ng/uL)	Condition	
11G10H	11G10HA 11G10HB 11G10HC	0	-	20	96,5	Clean	Test stability of MT baseline gene expression (non contaminated medium) under different temperatures.
11G20H	11G20HA 11G20HB 11G20HC	0	-	20	354	Clean	
11G30H	11G30HA 11G30HB 11G30HC	0	-	20	288	Clean	
12G10H	12G10HA 12G10HB 12G10HC	0.02	0	20	66	Clean	Test for MT gene expression differences
12G20H	12G20HA 12G20HB 12G20HC	0.02	0	20	500	Clean	
12G30H	12G30HA 12G30HB 12G30HC	0.02	0	20	555	Clean	
13G20H	13G20HA 13G20HB 13G20HC	0.04	0	20	363	Clean	Test for MT gene expression differences
13G30H	13G30HA 13G30HB 13G30HC	0.04	0	20	92	Clean	
21G10H	21G10HA 21G10HB 21G10HC	0	-	25	92	Clean	Test stability of MT baseline gene expression (non contaminated medium), under different temperatures.
21G20H	21G20HA 21G20HB 21G20HC	0	-	25	398	Clean	
21G30H	21G30HA 21G30HB 21G30HC	0	-	25	166	Clean	
22G2OH	22G2OHA 22G2OHB 22G2OHC	0.02	0	25	353	Clean	Unnecessary, backup for brown
22G3OH	22G3OHA 22G3OHB 22G3OHC	0.02	0	25	142	Clean	
11G11H	11G11HA 11G11HB 11G11HC	0	1	20	86	Clean	Unnecessary, backup for blue
11G21H	11G21HA 11G21HB 11G21HC	0	1	20	434,5	Clean	
11G31H	11G31HA 11G31HB 11G31HC	0	1	20	213	Clean	
12G11H	12G11HA 12G11HB 12G11HC	0.02	1	20	47	Clean	Test for MT gene expression differences after 1 hour contaminant exposure, under different concentrations, at 20ºC.
12G21H	12G21HA 12G21HB 12G21HC	0.02	1	20	453	Clean	
12G31H	12G31HA 12G31HB 12G31HC	0.02	1	20	84	Clean	
13G21H	13G21HA 13G21HB 13G21HC	0.04	1	20	~300	Slightly degrated	Test for MT gene expression differences after 1 hour contaminant exposure under differen concentrations, at 20ºC.
13G31H	13G31HA 13G31HB 13G31HC	0.04	1	20	246	Clean	
21G11H	21G11HA 21G11HB 21G11HC	0	1	25	558	Clean	Unnecessary, backup for blue
21G21H	21G21HA 21G21HB 21G21HC	0	1	25	90	Clean	
21G31H	21G31HA 21G31HB 21G31HC	0	1	25	443	Clean	
22G11H	22G11HA 22G11HB 22G11HC	0.02	1	25	392	Clean	Test for MT gene expression differences after 1 hour contaminant exposure, under different concentrations, at 25ºC.
22G21H	22G21HA 22G21HB 22G21HC	0.02	1	25	374	Clean	
23G11H	23G11HA 23G11HB 23G11HC	0.04	1	25	262	Clean	Test for MT gene expression differences after 1 hour contaminant exposure, under different concentrations, at 25ºC
23G21H	23G21HA 23G21HB 23G21HC	0.04	1	25	450	Clean	
23G31H	23G31HA 23G31HB 23G31HC	0.04	1	25	299	Clean	

Table 9 – Samples currently being extracted for mesocosm and laboratory settings

Sample code	Treatment			Regime
	CuSO4·5H2O (mg L^{-1})	Time (h)	Temperature (ºC)	
11110H	0	-	-	Mesocosm
11210H	0	-	-	Mesocosm
11310H	0	-	-	Mesocosm
11111H	0.04	1	-	Mesocosm
11211H	0.04	1	-	Mesocosm
11311H	0.04	1	20	Mesocosm
11411H	0.04	1	20	Mesocosm
41110H	0	-	20	Mesocosm
41210H	0	-	20	Mesocosm
41310H	0	-	20	Mesocosm
41111H	0.04	1	20	Mesocosm
41211H	0.04	1	20	Mesocosm
41311H	0.04	1	20	Mesocosm
41411H	0.04	1	20	Laboratory
21G4OH	0	0	25	Laboratory
13G51H	0.04	1	20	Laboratory
12G40H	0.02	0	20	Laboratory
13G40h	0.04	0	20	Laboratory
13G50H	0.04	0	20	Laboratory
22G40H	0.02	0	25	Laboratory
22G50H	0.02	0	25	Laboratory
23G40H	0.04	0	25	Laboratory
23G50H	0.04	0	25	Laboratory
12G41H	0.02	1	25	Laboratory
22G41H	0.02	1	25	Laboratory
22G51H	0.02	1	25	Laboratory
23G41H	0.04	1	25	Laboratory

General conclusions

I believe the work I developed in this study helped increase the understanding on how two different fields, phylogenetics and ecotoxicology, can be used together to help identify major problems in amphibian conservation. There is already a vast body of knowledge in the field of amphibian ecotoxicology due to extensive research on the causes of rapid amphibian decline, even if some of the conclusions can still be subject to debate due to the innumerous and complex problems inherent to each case/species studied, as well as the conditions tested. On the other hand, we are continuously discovering new tools that could address or predict important genetic features of a wide array of taxa, by looking and comparing available genomes (which are continuously increasing in number) to study key-gene family evolution and analyze functional divergence important to further understand the role of distinct protein functions, which had different evolutionary paths across taxa.

I additionally reinforced this connection by demonstrating how important is the influence of phylogenetic relationships in risk assessment studies, by testing for the weight of this influence in the parameters experienced, which were sensitivity to a toxic (measured by LC50) and temperature.

By addressing this important relationship between these two fields of expertise within the context of metal contamination and sensitivity of amphibians, I believe I was able to develop important tools which are still not used or developed to their full capacity in most studies to assess and research very serious conservation issues in the world.

For the final chapters the initial steps of a major gene expression study were developed, aimed at showing the importance of gathering information on gene expression in ecotoxicology and risk assessment, using the non-model amphibian species *Epidalea calamita*. I describe the experimental design, sample collection, RNA extraction, cDNA retro-transcription, target gene isolation and endogenous control gene isolation necessary for further work with qRT-PCR. Future results may show the importance of the organismal genetic response to a contaminant, thus providing traceable contamination responses even before visible sub-lethal effects arise, very important to monitor key-populations.

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Supplementary Materials

Supplementary Material 1 - Vertebrate metallothionein CDS dataset used in this work (see Materials and Methods for further details). “**Annotation**” reflects names according to the database used to retrieve this sequence. “**Sequence Code**” refers to names used for this work. “**Length (bp-aa)**” refers to length of the full exon when known (CDS+UTR or CDS) in number of base pairs (bp) and the number of amino acids; “**Gene Type – Ensembl**” with the classification “known” if there is a sequence match to the CDS or protein for the same species, “novel” if there is no match and "known by projection" classification if the transcript is a match for another species. “**RefSeq Status – NCBI**” as defined according to www.ncbi.nlm.nih.gov/RefSeq/key.html#status; “**Location**” indicates the interval of basepairs where the CDS is located in the chromosome; “**Direction**” means the direction of expression of the CDS in the double stranded DNA chromosome. Acession numbers of the sequences are indicated as full gene in Ensembl and NCBI (Gene Acess No and NCBI annotation) and as CDS in Ensembl (Transcript Acess No.).

Mammals

Annotation	Species	Popular name	Genomic database	Sequence Code	Description (Ensembl)	Description (NCBI)	Gene Type (Ensembl)	RefSeq status (NCBI)	Transcript Access No. (Ensembl)	Gene Acess No. (Ensembl)	NCBI annotation	Lenght (bp-aa)	Chromosome	Exons No.	Lenght Exons only CDS (bp)	Introns No.	Lenght introns	Location	Direction
MT1A	*Bos taurus*	Cow	Ensembl+NCBI	Bos_MT1A	MT1A	MT1A	Known protein coding	Validated	ENSBTAT00000002092	ENSBTAG00000038067	Gene ID: 404071	(394-61)	18	3	28+66+92	2	698+709	24,106,722-24,108,521	⟵
MT1E			Ensembl+NCBI	Bos_MT1E	MT1E	MT1E	Known protein coding	Provisional	ENSBTAT00000002088	ENSBTAG00000001595	Gene ID: 613358	(387-61)	18	3	28+66+92	2	596+364	24,117,441-24,118,787	⟵
MT1E			Ensembl+NCBI	Bos_MT1E2	MT1E	MT1E	Known protein coding	Provisional	ENSBTAT00000046456	ENSBTAG00000038706	Gene ID: 768319	(401-61)	18	3	28+66+92	2	691+231	24,112,351-24,114,381	⟵
MT2			Ensembl+NCBI	Bos_MT2A	MT2	MT2A	Known protein coding	Validated	ENSBTAT00000034373	ENSBTAG00000023659	Gene ID: 404070	(421-61)	18	3	28+66+92	2	292+256	24,125,366-24,126,333	⟵
MT3			Ensembl+NCBI	Bos_MT3	MT3	MT3	Known protein coding	Provisional	ENSBTAT00000022460	ENSBTAG00000016886	Gene ID: 613320	(397-68)	18	3	31+66+110	2	232+244	24,134,095-24,135,544	⟵
MT4			Ensembl+NCBI	Bos_MT4	MT4	MT4	Known protein coding	Provisional	ENSBTAT00000020072	ENSBTAG00000015084	Gene ID: 782851	(394-62)	18	3	31+66+92	2	1394+518	24,163,233-24,165,538	⟵
MT1	*Canis lupus familiaris*	Dog	Ensembl+NCBI	Can_MT1	MT1	MT1E	Known protein coding	Provisional	ENSCAFT00000014488	ENSCAFG00000009113	Gene ID: 100686073	(1032-61)	2	3	28+66+92	2	574+110	56,602,961-59,604,676	⟵
MT2A			Ensembl+NCBI	Can_MT2A	MT2A	MT1H	Known protein coding	Provisional	ENSCAFT00000014487	ENSCAFG00000023759	Gene ID: 403768	(414-61)	2	3	28+66+92	2	288+267	59,607,926-59,608,825	⟵
MT3			Ensembl+NCBI	Can_MT3	MT3	MT3-like	Known by_projection protein_coding	Model	ENSCAFT00000043233	ENSCAFG00000028459	Gene ID: 100855992	(204-68)	2	3	31+66+107	2	245+809	59,624,978-59,626,235	⟵
MT4			Ensembl+NCBI	Can_MT4	MT4	MT4	Known protein coding	Provisional	ENSCAFT00000014504	ENSCAFG00000009118	Gene ID: 403769	(420-62)	2	3	31+66+92	2	1619+548	59,640,784-59,643,370	⟵
unknown			Ensembl+NCBI	Can	?	MT1E	Novel protein coding	Provisional	ENSCAFT00000014491	ENSCAFG00000009110	Gene ID: 403800	(195-64)	?	2	103+92	1	662	470-1,326	⟶
MT3	*Equus caballus*	Horse	Ensembl+NCBI	Equ_MT3	MT3	MT3-like	Known by_projection protein_coding	Model	ENSECAT00000015904	ENSECAG00000015212	GeneID: 100630322	(322-68)	3	3	31+66+110	2	247+833	8,998,215-8,999,616	⟶
MT4			Ensembl+NCBI	Equ_MT4	MT4	MT4-like	Known by_projection protein_coding	Model	ENSECAT00000014386	ENSECAG00000013831	GeneID:100630148	(285-62)	3	3	31+66+92	2	1476+541	8,978,911-8,981,212	⟶
MT			NCBI	Equ_a_	MT	MT1A-like	Known protein coding	Model	-	NC_009146.2	GeneID:100630653	(334-61)	3	3	28+66+92	2	561+737	9,014,955-9,016,582	⟶
MT			Ensembl+NCBI	Equ_b_MT	MT	MT2-like	Known protein coding	Model	ENSECAT00000000282	ENSECAG00000000363	GeneID:100630645	(167-52)	3	2	67+92	1	705	9,031,592-9,032,463	⟶
MT			Ensembl+NCBI	Equ_c_MT	MT	MT1-a-like	Known protein coding	Model	ENSECAT00000000321	ENSECAG00000000368	GeneID:100630794	(230-61)	3	3	28+66+92	2	561+726	9,034,395-9,035,911	⟶
MT			Ensembl+NCBI	Equ_d_MT	MT	MT2-like	Known protein coding	Model	ENSECAT00000001640	ENSECAG00000001555	GeneID:100630858	(382-61)	3	3	28+66+92	2	564+644	9,043,475-9,045,064	⟶
MT			Ensembl+NCBI	Equ_e_MT	MT	MT1-b-like	Known protein coding	Model	ENSECAT00000015995	ENSECAG00000015275	GeneID:100630543	(378-61)	3	3	28+66+92	2	298+200	9,010,353-9,011,228	⟶
MT1A	*Homo sapiens*	Human	Ensembl+NCBI	Hom_MT1A	MT1A	MT1A	Known protein coding	Validated	ENST00000290705	ENSG00000205362	Gene ID: 4489	(396-61)	16	3	28+66+92	2	497+529	56,672,578-56,673,999	⟶
MT1B			Ensembl+NCBI	Hom_MT1B	MT1B	MT1B	Known protein coding	Provisional	ENST00000334346	ENSG00000169688	Gene ID: 4490	(380-61)	16	3	28+66+92	2	589+337	56,685,811-56,687,116	⟶
MT1E			Ensembl+NCBI	Hom_MT1E	MT1E	MT1E	Known protein coding	Validated	ENST00000306061	ENSG00000169715	Gene ID: 4493	(704-61)	16	3	28+66+92	2	586+348	56,659,387-56,661,024	⟶
MT1F			Ensembl+NCBI	Hom_MT1F	MT1F	MT1F	Known protein coding	Validated	ENST00000334350	ENSG00000198417	Gene ID: 4494	(690-61)	16	3	28+66+92	2	588+332	56,691,606-56,694,610	⟶
MT1G			Ensembl+NCBI	Hom_MT1G	MT1G	MT1G	Known protein coding	Provisional	ENST00000444837	ENSG00000125144	Gene ID: 4495	(406-61)	16	3	28+66+92	2	588+341	56,700,643-56,701,977	⟵
MT1H			Ensembl+NCBI	Hom_MT1H	MT1H	MT1H	Known protein coding	Validated	ENST00000332374	ENSG00000205358	Gene ID: 4496	(397-61)	16	3	28+66+92	2	593+326	56,703,726-56,705,041	⟶
MT1M			Ensembl+NCBI	Hom_MT1M	MT1M	MT1M	Known protein coding	Reviewed	ENST00000379818	ENSG00000205364	Gene ID: 4499	(829-61)	16	3	28+66+92	2	580+345	56,666,145-56,667,898	⟶
MT1X			Ensembl+NCBI	Hom_MT1X	MT1X	MT1X	Known protein coding	Validated	ENST00000394485	ENSG00000187193	Gene ID: 4501	(450-61)	16	3	28+66+92	2	596+727	56,716,336-56,718,108	⟶
MT2A			Ensembl+NCBI	Hom_MT2A	MT2A	MT2A	Known protein coding	Validated	ENST00000245185	ENSG00000125148	Gene ID: 4502	(786-61)	16	3	28+66+92	2	308+205	56,642,111-56,643,409	⟶
MT3			Ensembl+NCBI	Hom_MT3	MT3	MT3	Known protein coding	Validated	ENST00000200691	ENSG00000087250	Gene ID: 4504	(569-68)	16	3	28+66+110	2	248+904	56,622,986-56,625,000	⟶
MT4			Ensembl+NCBI	Hom_MT4	MT4	MT4	Known protein coding	Provisional	ENST00000219162	ENSG00000102891	Gene ID: 84560	(294-62)	16	3	31+66+92	2	2591+1024	56,598,961-56,602,869	⟶
MT	*Monodelphis domestica*	Opossum	Ensembl+NCBI	Mon_a_MT	MT	MT-1-like	Known protein coding	Model	ENSMODT00000040200	ENSMODG00000025753	Gene ID: 100015476	(183-61)	1	3	28+66+89	2	450+427	447,543,240-447,544,299	⟶
MT			Ensembl+NCBI	Mon_MT2_	MT	MT-2-like	Novel protein coding	Model	ENSMODT00000040201	NC_008801.1	Gene ID: 100015441	(189-62)	1	3	28+66+95	2	533+242	447,510,292-447,511,251	⟶
unknown			NCBI	Mon_b_	-	MT-1B-like	predicted protein	Model	-	NC_008801.1	Gene ID: 100015559	(189-62)	1	3	28+66+95	2	855+217	447,585,014-447,586,270	⟶
MT1	*Mus musculus*	Mouse	Ensembl+NCBI	Mus_MT1	MT1	MT1	Known protein coding	Validated	ENSMUST00000034215	ENSMUSG00000031765	Gene ID: 17748	(540-61)	8	3	28+66+92	2	484+213	94,179,089-94,180,325	⟶
MT2			Ensembl+NCBI	Mus_MT2	MT2	MT2	Known protein coding	Validated	ENSMUST00000034214	ENSMUSG00000031762	Gene ID: 17750	(556-61)	8	3	28+66+92	2	251+143	94,172,618-94,173,567	⟶
MT3			Ensembl+NCBI	Mus_MT3	MT3	MT3	Known protein coding	Validated	ENSMUST00000034211	ENSMUSG00000031760	Gene ID: 17751	(538-68)	8	3	31+66+110	2	194+810	94,152,607-94,154,148	⟶
MT4			Ensembl+NCBI	Mus_MT4	MT4	MT4	Known protein coding	Validated	ENSMUST00000034207	ENSMUSG00000031757	Gene ID: 17752	(391-62)	8	3	31+66+92	2	958+479	94,137,204-94,139,031	⟶
MT3	*Ornithorhynchus anatinus*	Platypus	Ensembl+NCBI	Orn_MT3	MT3	MT-1-like	Known by_projection protein_coding	Model	ENSOANT00000029863	ENSOANG00000020495	Gene ID: 100073566	(442-63)	?	3	31+66+95	2	1290+1015	7,611,349-7,614,095	⟶
MT4			Ensembl+NCBI	Orn_MT4	MT4/MT4like	MT4-like	Known by_projection protein_coding	Model	ENSOANT00000000897	ENSOANG00000000565	Gene ID: 100073518	(192-63)	?	3	31+66+95	2	2169+2340	7,593,725-7,598,425	⟶
MT1X			Ensembl+NCBI	Orn_a_MT	MT1X	MT-like	Known by_projection protein_coding	Model	ENSOANT00000000895	ENSOANG00000000563	Gene ID: 100079807	(359-63)	?	3	31+66+95	2	830+1156	7,641,737-7,644,081	⟶
MT			Ensembl+NCBI	Orn_b_MT	MT	MT-1-like	Novel protein coding	Model	ENSOANT00000000896	ENSOANG00000000564	Gene ID: 100079781	(189-62)	?	2	94+95	1	793	7,630,202-7,631,183	⟶
MT1B	*Pan troglodytes*	Chimpanzee	Ensembl+NCBI	Pan_MT1B	MT1B	MT1B	Known protein coding	Model	ENSPTRT00000015003	ENSPTRG00000023341	Gene ID: 467981	(384-61)	16	3	28+66+92	2	589+232	55,725,337-55,726,64	⟶
unknown			Ensembl+NCBI	Pan_a	?	MT1B-like	Novel protein coding	Model	ENSPTRT00000075532	ENSPTRG00000031203	Gene ID: 101059555	(105-35)	16	3	28+48+29	2	593+347	55,714,373-55,715,417	⟶
MT2A			Ensembl+NCBI	Pan_MT2A	MT2A	MT2A	Known protein coding	Validated	ENSPTRT00000014994	ENSPTRG00000008136	Gene ID: 471221	(418-61)	16	3	28+66+92	2	308+205	55,687,305-55,688,235	⟶
MT3			Ensembl+NCBI	Pan_MT3	MT3	MT3	Known protein coding	Model	ENSPTRT00000014993	ENSPTRG00000008135	Gene ID: 736124	(396-68)	16	3	31+66+110	2	249+905	55,668,455-55,670,005	⟶
MT4			Ensembl+NCBI	Pan_MT4	MT4	MT4	Known protein coding	Model	ENSPTRT00000014992	ENSPTRG00000008134	Gene ID: 735864	(294-62)	16	3	31+66+92	2	2461+948	55,643,634-55,647,336	⟶
unknown			Ensembl+NCBI	Pan_a_MT	?	MT1G	Known protein coding	Validated	ENSPTRT00000015007	ENSPTRG00000008140	Gene ID: 736169	(395-62)	16	3	28+69+92	2	583+342	55,740,193-55,741,511	⟵
MT1E			NCBI	Pan_MT1E	-	MT1E	-	Validated	-	NC_006483.3	Gene ID: 100609276	(398-61)	16	3	28+66+92	2	587+349	55,704,392-55,705,721	⟶
unknown			Ensembl+NCBI	Pan_c	?	uncharacterized	Known protein coding	Model	ENSPTRT00000015002	ENSPTRG00000031205	Gene ID: 100609360	(372-61)	16	3	28+66+92	2	592+348	55,711,209-55,712,520	⟶
unknown			Ensembl+NCBI	Pan_d	?	MT1X-like	Known protein coding	Model	ENSPTRT00000015008	ENSPTRG00000008141	Gene ID: 100616506	(400-61)	16	3	28+66+92	2	596+727	55,756,363-55,758,085	⟶
unknown			Ensembl+NCBI	Pan_e	?	MT1H	Known protein coding	Inferred	ENSPTRT00000014999	ENSPTRG00000031202	Gene ID: 736361	(396-61)	16	3	28+66+92	2	590+326	55,743,456-55,744,767	⟶
unknown			Ensembl+NCBI	Pan_f	?	uncharacterized	Known protein coding	Model	ENSPTRT00000015005	ENSPTRG00000031204	Gene ID: 100609521	(392-61)	16	3	28+66+92	2	497+530	55,717,203-55,718,621	⟶
unknown			Ensembl+NCBI	Pan_h	?	MT1F	Known protein coding	Model	ENSPTRT00000015004	ENSPTRG00000008139	Gene ID: 100609808	(445-62)	16	3	28+66+92	2	581+337	55,731,372-55,732,734	⟶
MT1A	*Rattus norvegicus*	Rat	Ensembl+NCBI	Rat_MT1A	MT1A	MT1A	Known protein coding	Provisional	ENSRNOT00000038212	ENSRNOG00000025764	Gene ID: 24567	(389-61)	19	3	28+66+92	2	462+166	11,277,133-11,278,149	⟵
MT2A			Ensembl+NCBI	Rat_MT2A	MT2A	MT2A	Known protein coding	Provisional	ENSRNOT00000067391	ENSRNOG00000043098	Gene ID: 689415	(382-61)	19	3	28+66+92	2	263+129	11,283,094-11,283,867	⟵
MT3			Ensembl+NCBI	Rat_MT3	MT3	MT3	Known protein coding	Provisional	ENSRNOT00000025669	ENSRNOG00000018958	Gene ID: 117038	(375-66)	19	3	31+66+104	2	188+1285	11,300,015-11,301,422	⟵
MT4			Ensembl+NCBI	Rat_MT4	MT4	MT4	Known protein coding	Provisional	ENSRNOT00000025694	ENSRNOG00000019004	Gene ID: 498911	(384-62)	19	3	31+66+92	2	1222+491	11,315,073-11,317,169	⟵
MT1A	*Sus scrofa*	Pig	Ensembl+NCBI	Sus_MT1A	MT1A	MT1A	Known protein coding	Provisional	ENSSSCT00000031974	ENSSSCG00000023684	Gene ID: 397417	(389-61)	6	3	28+66+92	2	599+686	26,383,677-26,385,350	⟶
MT2A			Ensembl+NCBI	Sus_MT2A	MT2A	MT-2B isoform	Known protein coding	Model	ENSSSCT00000029059	ENSSSCG00000030300	Gene ID: 396827	(421-61)	6	3	28+66+92	2	279+204	26,420,805-26,421,708	⟶
MT3			Ensembl	Sus_a_MT	?	-	Novel protein coding	-	ENSSSCT00000031542	ENSSSCG00000023305	not annotated	(186-61)	6	3	28+66+92	2	599+158	26,369,804-26,370,746	⟵
MT			Ensembl+NCBI	Sus_b_MT	?	MT-1C-like	Novel protein coding	Model	ENSSSCT00000029094	ENSSSCG00000024911	Gene ID: 100739663	(340-61)	6	3	28+66+92	2	579+702	26,374,348-26,375,968	⟵

Birds

Annotation	Species	Popular name	Genomic database	Sequence Code	Description (Ensembl)	Description (NCBI)	Gene Type (Ensembl)	RefSeq status (NCBI)	Transcript Access No. (Ensembl)	Gene Acess No. (Ensembl)	NCBI annotation	Lenght (bp-aa)	Chromosome	Exons No.	Lenght Exons only CDS (bp)	Introns No.	Lenght introns	Location	Direction
MT1/MT3	*Gallus gallus*	Chicken	NCBI	Gal_MT3	-	MT3	-	Provisional	NM_001097538.1	AB258231/NC_006098	Gene ID: 770592	(848-63)	11	3	31+66+95	2	167+493	2,113,550 - 2,114,397	←
MT2/MT4			NCBI	Gal_MT4	-	MT4	-	Provisional	NM_205275	NC_006098	Gene ID: 396212	(1502-63)	11	3	67+66+95	2	84+1056	2,110,600 - 2,112,101	←
MT1	*Meleagris*	Turkey	NCBI	Mel_MT1_	MT1	-	-	-	-	NC_015023	not annotated	(752-50)	13	2	66+92	2	?+492	636,663-637,415	←
MT2	*gallopavo*		NCBI	Mel_MT2_	MT2	-	-	-	-	X62513	Gene ID: 678662	(642-62)	13	3	31+66+92	2	77+171	638,933 - 640,024	←
MTI	*Taeniopygia*	Zebra finch	Ensembl+NCBI	Tae_MT1	MTI	MT-I-like	Known protein coding	Provisional	ENSTGUT00000006787	ENSTGUG00000006540	Gene ID: 100190094	(192-63)	11	3	31+66+95	2	1075+576	6,083,968-6,085,810	←
MTII	*gutatta*		Ensembl+NCBI	Tae_MT2	MTII variant 2	MT-II-like	Known protein coding	Provisional	ENSTGUT00000006779	ENSTGUG00000006533	Gene ID: 100190731	(549-63)	11	3	31+66+95	2	89+534	6,079,530-6,080,701	←

Non-avian reptiles

Annotation	Species	Popular name	Genomic database	Sequence Code	Description (Ensembl)	Description (NCBI)	Gene Type (Ensembl)	RefSeq status (NCBI)	Transcript Access No. (Ensembl)	Gene Acess No. (Ensembl)	NCBI annotation	Lenght (bp-aa)	Chromosome	Exons No.	Lenght Exons only CDS (bp)	Introns No.	Lenght introns	Location	Direction
MT4	*Anolis*	Anole lizard	Ensembl	Ano_MT4	MT4	-	Known by_projection protein_coding	-	ENSACAT00000023600	ENSACAG00000025066	not annotated	(189-63)	?	3	31+66+92	2	3609+2472	166,582-172,851	→
MT3	*carolinensis*		Ensembl	Ano_MT3	MT3	-	Known by_projection protein_coding	-	ENSACAT00000007496	ENSACAG00000007511	not annotated	(186-62)	?	3	31+66+89	2	2395+1332	182,526-186,438	→
MT4	*Pelodiscus*	Chinese	Ensembl	Pel_a_MT	MT4	-	Known by_projection protein_coding	-	ENSPSIT00000015922	ENSPSIG00000014140	not annotated	(183-61)	?	3	28+66+89	2	665+1589	713,752-716,188	→
MT	*sinensis*	softshell	Ensembl	Pel_b_MT	MT	-	Novel protein coding	-	ENSPSIT00000014308	ENSPSIG00000012779	not annotated	(159-53)	?	2	70+89	1	1641	363,907-365,706	←
MT		turtle	Ensembl	Pel_c_MT	MT	-	Novel protein coding	-	ENSPSIT00000014320	ENSPSIG00000012786	not annotated	(165-55)	?	2	76+89	1	1202	378,186-379,552	←

Amphibians

Annotation	Species	Popular name	Genomic database	Sequence Code	Description (Ensembl)	Description (NCBI)	Gene Type (Ensembl)	RefSeq status (NCBI)	Transcript Access No. (Ensembl)	Gene Acess No. (Ensembl)	NCBI annotation	Lenght (bp-aa)	Chromosome	Exons No.	Lenght Exons only CDS (bp)	Introns No.	Lenght introns	Location	Direction
MT4	*Xenopus tropicalis*	Xenopus frog	Ensembl+NCBI	Xen_MT4	MT4	MT4	Known protein coding	Provisional	ENSXETT00000064664	ENSXETG00000030675	Gene ID: 100135413	(757-62)	?	3	31+66+92	2	1092+984	82,895-85,727	←

Fish

Annotation	Species	Popular name	Genomic database	Sequence Code	Description (Ensembl)	Description (NCBI)	Gene Type (Ensembl)	RefSeq status (NCBI)	Transcript Access No. (Ensembl)	Gene Acess No. (Ensembl)	NCBI annotation	Lenght (bp-aa)	Chromosome	Exons No.	Lenght Exons only CDS (bp)	Introns No.	Lenght introns	Location	Direction
MT2	*Danio rerio*	Zebrafish	Ensembl+NCBI	Dan_MT2	MT2	MT2	Known protein coding	Validated	ENSDART00000061007	ENSDARG00000041623	GeneID:100174951	(594-60)	18	3	25+66+92	2	94+665	17,193,795-17,195,147	→
MT	*Oryzias latipes*	Medaka	Ensembl+NCBI	Ory_MT	MT	MT	Known protein coding	Provisional	ENSORLT00000019509	ENSORLG00000015580	Gene ID: 100049397	(370-60)	6	3	25+66+92	2	89+229	23,291,204-23,291,891	→
MT1F	*Takifugu rubripes*	Fugu	Ensembl+NCBI	Tak_MT1F	MT1F	MT-A-like	Known by_projection protein_coding	Model	ENSTRUT00000022487	ENSTRUG00000008907	Gene ID: 101067922	(318-60)	?	3	25+66+92	2	90+162	313,177-313,746	←
MT	*Tetraodon*	Tetraodon	Ensembl	Tet_a_MT	MT	-	Novel protein coding	-	ENSTNIT00000011862	ENSTNIG00000008823	not annotated	(353-60)	13	3	25+66+92	2	82+250	1,418,484-1,419,168	→
MT	*nigroviridis*		Ensembl	Tet_b_MT	MT	-	Novel protein coding	-	ENSTNIT00000011863	ENSTNIG00000008824	not annotated	(373-69)	13	3	25+66+120	2	87+84	1,407,286-1,407,834	←

Supplementary Material 2 - List of sequences removed, edited or replaced after checking them on the Ensembl and NCBI genomic databases.

1) One *Rattus norvegicus* sequence (Rat, ENSRNOG00000028841) was removed due to an annotation reference to unknown protein translation as “hypothetical protein”, to the lack of introns, and for being in a different chromosome from all other known functional Rat MTs.

2) Four sequences (Rat, ENSRNOG00000038047; Rat, ENSRNOG00000038624; Chimpanzee, ENSPTRG00000042268; Chimpanzee, ENSPTRG00000042180) from *Rattus rattus* and *Pan troglodytes* were removed for being annotated as “pseudogene”.

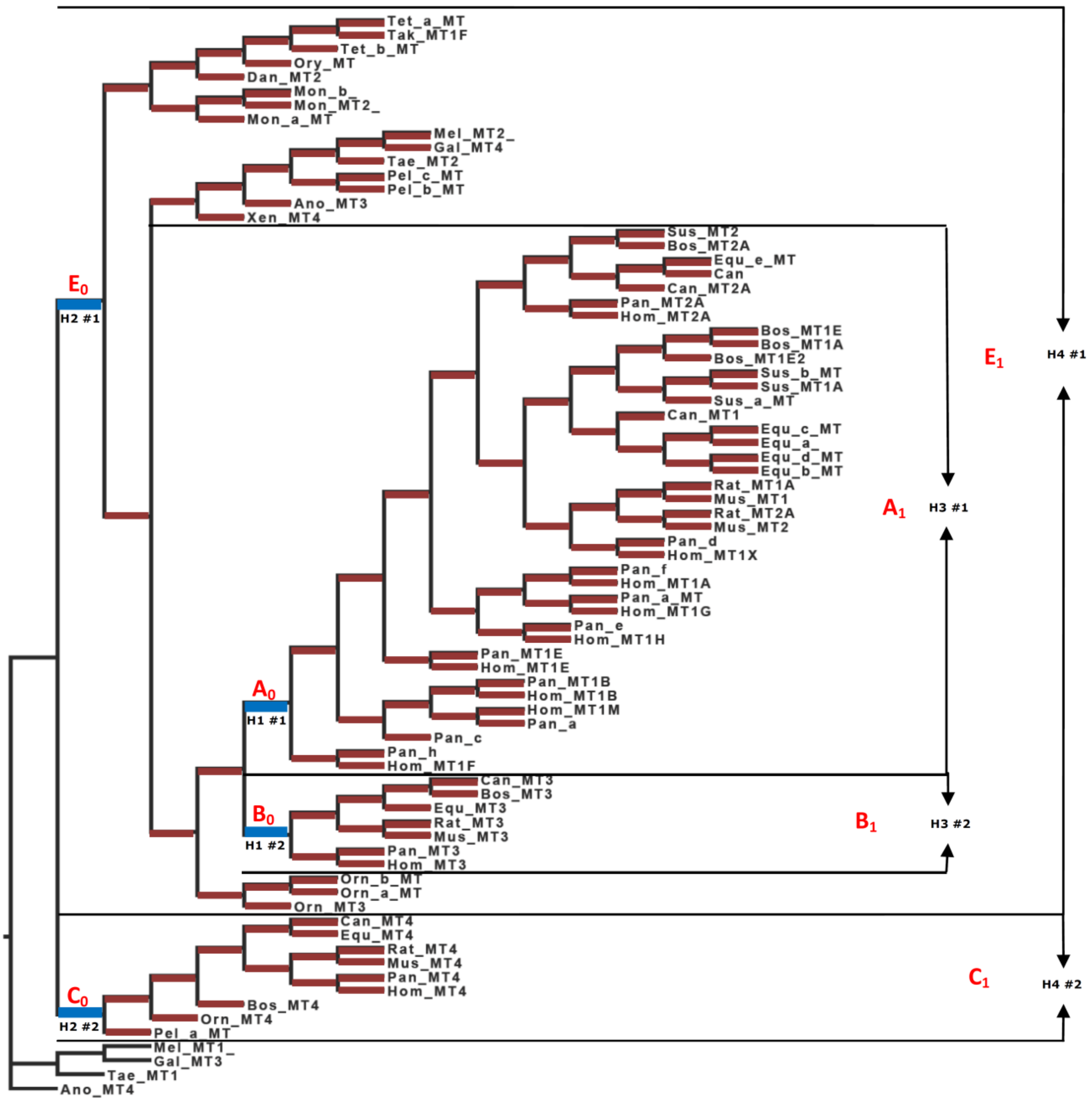
3) One *Sus scrofa* sequence (Pig, ENSSSCG00000024305) was removed for having associated uncommonly large annotated intron sequences (40x longer than the average number).

4) One *Pan troglodytes* sequence (Chimpanzee, ENSPTRG00000016106) was removed for not having introns and for being situated in a chromosome different from all remaining chimpanzee MTs.

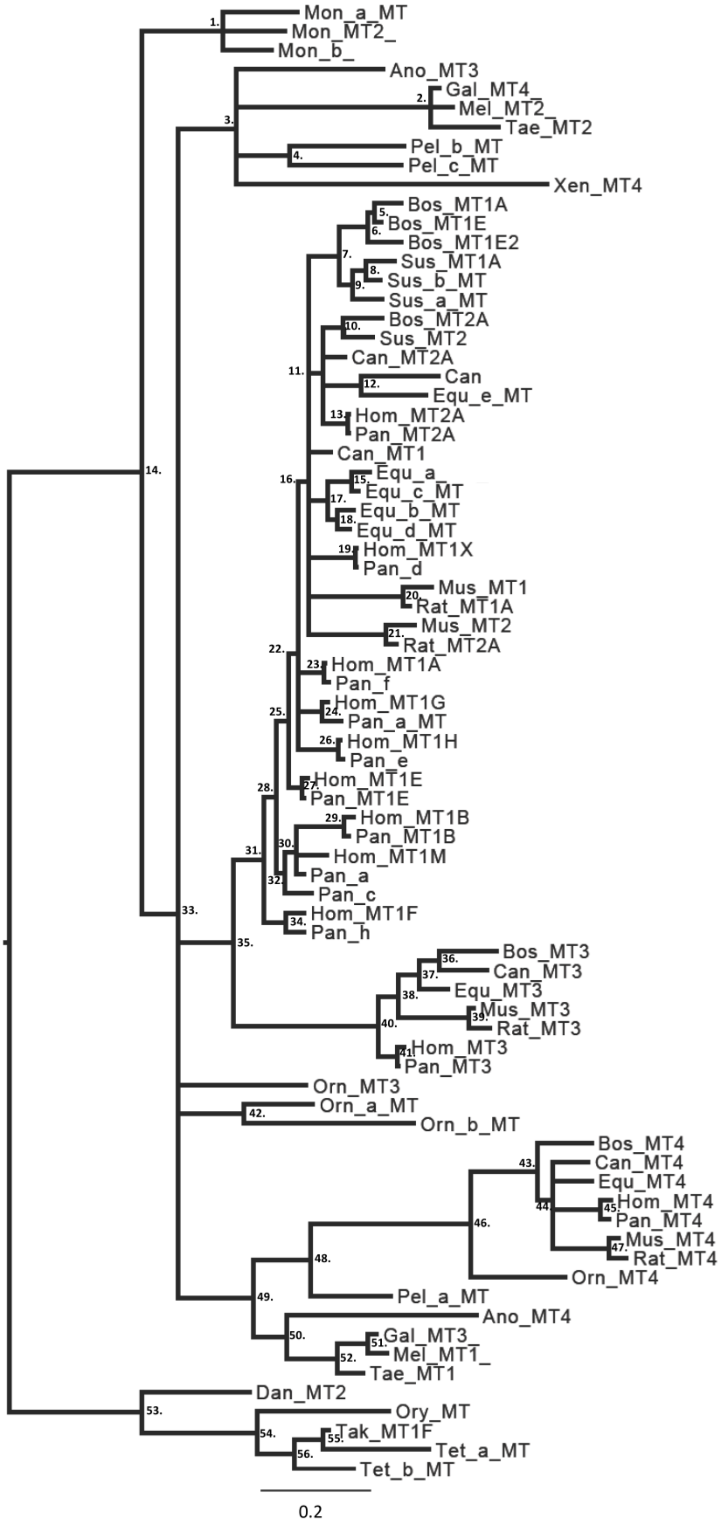
5) Two sequences (chicken, ENSGALT00000023565; turkey, ENSMGAG00000000947) from *Gallus gallus and Meleagris gallopavo,* respectively, were removed and replaced by two different MT genes each, retrieved from NCBI database and genome (chicken, Gene ID: 770592 and Gene ID: 396212; and turkey, Gene ID: 678662; and gene not currently annotated in either database). *G. gallus* MT gene removal was due to a BLAST/BLAT search and posterior comparison of Ensembl gene transcripts and the NCBI *G. gallus* obtained genes (two). The single Ensembl MT transcript for this species seemed most likely to be the result of the exon assemblage of the two MT NCBI genes. These two genes could only be retrieved as one when using a BLAST/BLAT search on the *G. gallus* genome in Ensembl. On the other hand, the single Ensembl gene is recovered as part of the two NCBI genes when using the NCBI BLAST tool. It has also been previously proposed by Villarreal et al. (2006) the existence of two MTs in *G. gallus*. *Meleagris gallopavo* MT gene retrieved from Ensembl seemed to reflect instead an annotation error likely to have been caused by a sequencing gap (NNNNN) of 682 bp length; 468bp of this sequencing gap are an integrative part of a second MT turkey gene, referenced on NCBI non genomic sequences with the access number X62513. Additionally, the second MT turkey gene (“MT1”) was not correctly annotated in any of the databases. This last gene was therefore retrieved directly from the turkey genome in NCBI.

6) One *Pan troglodytes* sequence (Chimpanzee, ENSPTRG00000008137) was replaced by the equivalent gene sequence annotated on NCBI (Gene ID: 739074), since the last one is situated in the same chromosome as the other annotated *Pan* sequences and not in an unplaced scaffold as in Ensembl database.

Supplementary Material 3 - Branches tested in different hypotheses (H1-H4) for PAML codeml analysis. Different rates for each hypothesis represented by #1 and #2; branch after duplication represented by A_0, B_0, C_0 and E_0 in blue; main clades and all internal branches as A_1, B_1, C_1 and E_1 in brown.



Supplementary Material 4 - Bootstrap and posterior probability support values for all phylogenetic analyses represented on the unrooted Bayesian consensus tree (50% majority-rule, one model of evolution) topology. Numbers on the tree refer to nodes. Bootstrap for ML (in % and only when above 60%) and Posterior Probability (pp only for values above 0.70) values are given for each node (indicated here by the numbers in bold) in the following order: ML nucleotide/ML amino acid/Bayesian nucleotide no partition/Bayesian nucleotide partition/Bayesian amino acid. Values replaced by "-" when below 60% bootstrap or 0.70 pp. Values not shown when clade is not recovered by a specific analysis are indicated with "#". **1.** 96/81/1/1/0.97; **2.** 100/65/1/1/1; **3.** -/#/-/0.75/#; **4.** 75/-/0.88/-/#; **5.** -/-/-/#/0.71; **6.** 89/-/0.99/1/-; **7.** -/-/0.97/0.97/1; **8.** 68/74/0.98/0.96/1; **9.** 71/-/0.88/0.99/#; **10.** -/#/0.73/0.86/#; **11.** #/76/-/0.71/#; **12.** -/-/0.91/0.99/#; **13.** 100/75/0.98/1/0.95; **14.** #/#/1/1/#; **15.** #/#/0.74/-/#; **16.** 87/89/1/1/0.97; **17.** -/-/0.97/0.99/0.75; **18.** -/-/0.74/0.82/1; **19.** 100/98/1/1/0.97; **20.** 100/98/1/1/1; **21** 100/98/1/1/1; **22.** #/#/-/0.91/#; **23.** 100/97/1/1/1; **24.** 95/-/1/1/99; **25.** #/#/0.75/0.72/#; **26** 95/99/1/1/1; **27.** 99/-/0.99/0.96/#; **28.** 78/#/0.90/0.85/#; **29.** 100/94/1/1/1; **30.** -/-/-/0.95/0.96; **31** 78/-/0.79/1/-; **32.** -/-/-/-/#; **33.** #/#/0.85/#/#; **34.** 83/79/0.94/1/1; **35.** #/#/0.85/0.96/#;**36** 74/#/0.94/-/#; **37.** 76/#/0.94/0.79/#; **38.** #/#/-/#/#; **39.** 98/96/1/1/1; **40.** 99/94/1/1/1; **41** 98/92/0.89/0.99/0.99; **42.** 79/-/1/1/0.96; **43.** 91/#/1/#/#; **44** -/#/0.66/#/#; **45.** 98/92/1/1/0.85; **46.** 99/100/1/1/1; **47.** 99/79/1/1/0.92; **48.** 39/#/-/-/#; **49.** 62/#/0.85/0.71/0.99; **50.** -/-/-/-/#; **51.** 81/84/0.98/0.96/1; **52.** 87/84/0.98/1/1; **53.** 93/99/1/1/1 **54.** 65/-/1/#/#; **55.** -/-/0.99/0.88/#; **56.** -/#/-/#/#.


1.
Mon_a_MT
Mon_MT2_
Mon_b_
Ano_MT3
2.
Gal_MT4_
Mel_MT2_
Tae_MT2
3.
4.
Pel_b_MT
Pel_c_MT
Xen_MT4
5.
Bos_MT1A
6.
Bos_MT1E
Bos_MT1E2
7.
8.
Sus_MT1A
9.
Sus_b_MT
Sus_a_MT
10.
Bos_MT2A
Sus_MT2
11.
Can_MT2A
12.
Can
Equ_e_MT
13.
Hom_MT2A
Pan_MT2A
Can_MT1
14.
16.
15.
Equ_a_
Equ_c_MT
17.
18.
Equ_b_MT
Equ_d_MT
19.
Hom_MT1X
Pan_d
20.
Mus_MT1
Rat_MT1A
21.
Mus_MT2
Rat_MT2A
22.
23.
Hom_MT1A
Pan_f
25.
24.
Hom_MT1G
Pan_a_MT
26.
Hom_MT1H
Pan_e
28.
27.
Hom_MT1E
Pan_MT1E
29.
Hom_MT1B
Pan_MT1B
30.
31.
Hom_MT1M
32.
Pan_a
Pan_c
33.
34.
Hom_MT1F
Pan_h
35.
36.
Bos_MT3
37.
Can_MT3
38.
Equ_MT3
39.
Mus_MT3
Rat_MT3
40.
41.
Hom_MT3
Pan_MT3
Orn_MT3
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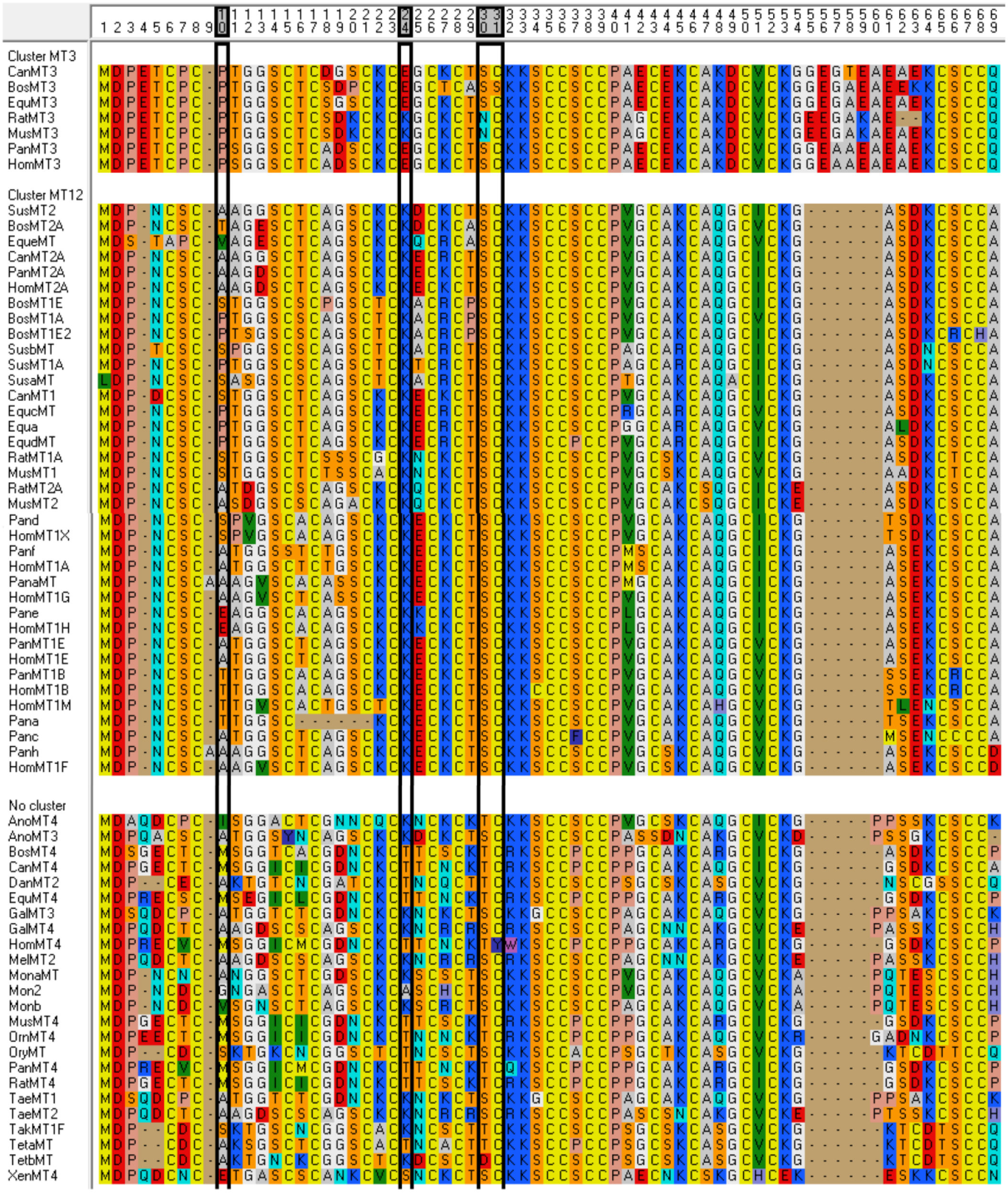
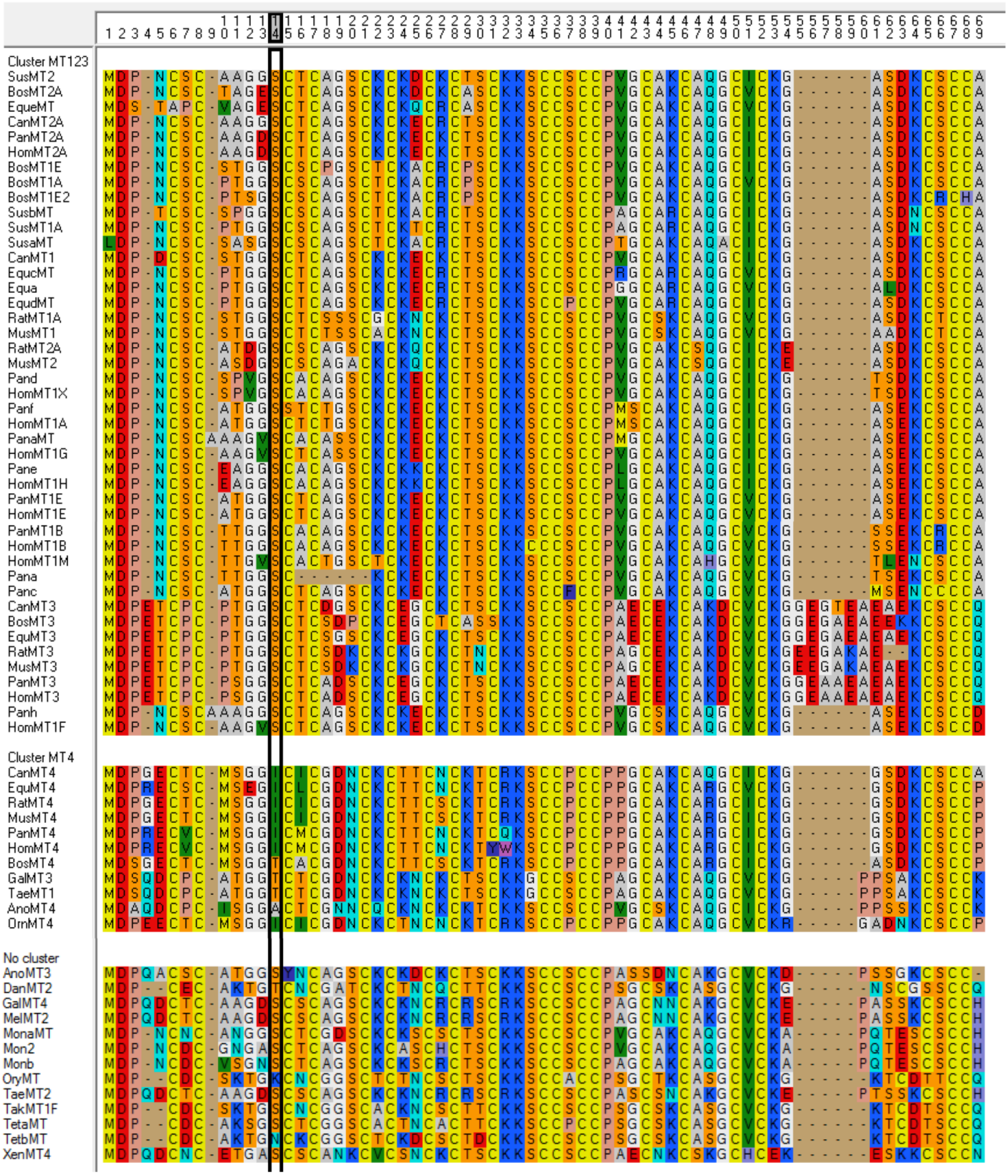
Orn_a_MT
Orn_b_MT
43.
Bos_MT4
Can_MT4
Equ_MT4
44.
45.
Hom_MT4
Pan_MT4
46.
47.
Mus_MT4
Rat_MT4
48.
Orn_MT4
49.
Pel_a_MT
Ano_MT4
50.
51.
Gal_MT3_
52.
Mel_MT1_
Tae_MT1
Dan_MT2
53.
Ory_MT
54.
55.
Tak_MT1F
56.
Tet_a_MT
Tet_b_MT
0.2

Supplementary Material 5 - Functional divergence alignments (Type I divergence). Vertical bar represents amino acid site indicated as functionally divergent among the indicated clades for a cut-off value = 0.9

1) MT1/MT2 vs MT3 functional divergence alignments



2) MT1/MT2/MT3 vs MT4 functional divergence alignments

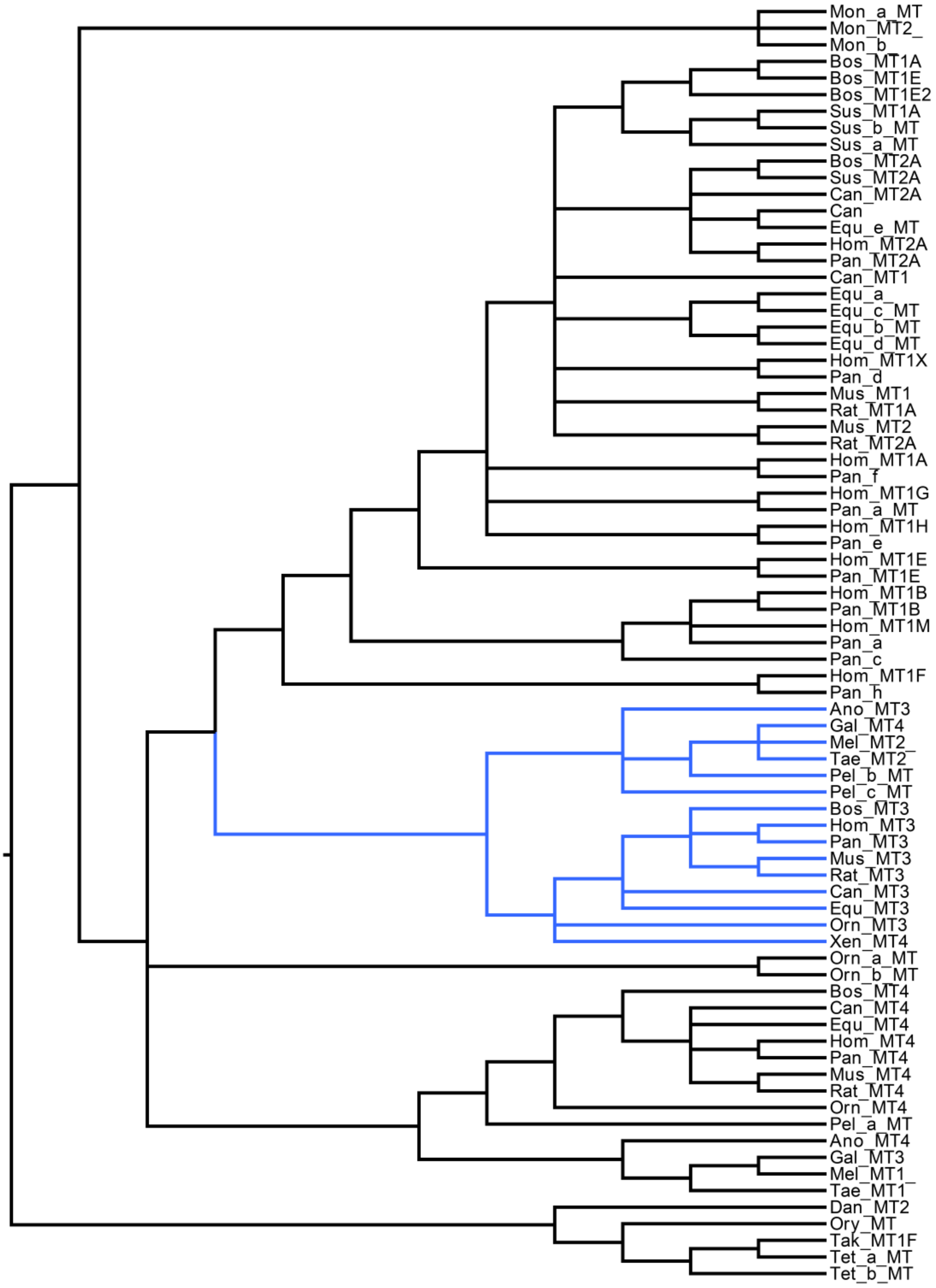


```
            1        10        20        30        40        50        60       69
Cluster MT123
SusMT2      MDP-NCSC-AAGGSCTCAGSCKCKDCKCTSCKKSCCSCCPVGCAKCAQGCICKG------ASDKCSCCA
BosMT2A     MDP-NCSC-TAGESCTCAGSCKCKDCKCASCKKSCCSCCPVGCAKCAQGCVCKG------ASDKCSCCA
EqueMT      MDS-TAPC-VAGESCTCAGSCKCKQCRCASCKKSCCSCCPVGCAKCAQGCVCKG------ASDKCSCCA
CanMT2A     MDP-NCSC-AAGGSCTCAGSCKCKECRCTSCKKSCCSCCPVGCAKCAQGCICKG------ASDKCSCCA
PanMT2A     MDP-NCSC-AAGDSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKG------ASDKCSCCA
HomMT2A     MDP-NCSC-AAGDSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKG------ASDKCSCCA
BosMT1E     MDP-NCSC-STGGSCSCPGSCTCKACRCPSCKKSCCSCCPVGCAKCAQGCICKG------ASDKCSCCA
BosMT1A     MDP-NCSC-PTGGSCSCAGSCTCKACRCPSCKKSCCSCCPVGCAKCAQGCVCKG------ASDKCSCCA
BosMT1E2    MDP-NCSC-PTSGSCSCAGSCTCKACRCPSCKKSCCSCCPVGCAKCAQGCICKG------ASDKCRCHA
SusbMT      MDP-TCSC-SPGGSCSCAGSCTCKACRCTSCKKSCCSCCPAGCARCAQGCICKG------ASDNCSCCA
SusMT1A     MDP-NCSC-PTGGSCSCAGSCTCKTCRCTSCKKSCCSCCPAGCARCAQGCICKG------ASDNCSCCA
SusaMT      LDP-NCSC-SASGSCSCAGSCTCKACRCTSCKKSCCSCCPTGCAKCAQACICKG------ASDKCSCCA
CanMT1      MDP-DCSC-STGGSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKG------ASDKCSCCA
EqucMT      MDP-NCSC-PTGGSCTCAGSCKCKECRCTSCKKSCCSCCPRGCARCAQGCVCKG------ASDKCSCCA
Equa        MDP-NCSC-PTGGSCTCAGSCKCKECRCTSCKKSCCSCCPGGCARCAQGCVCKG------ALDKCSCCA
EqudMT      MDP-NCSC-PTGGSCTCAGSCKCKECRCTSCKKSCCPCCPVGCARCAQGCICKG------ASDKCSCCA
RatMT1A     MDP-NCSC-STGGSCTCSSSCGCKNCKCTSCKKSCCSCCPVGCSKCAQGCVCKG------ASDKCTCCA
MusMT1      MDP-NCSC-STGGSCTCTSSCACKNCKCTSCKKSCCSCCPVGCSKCAQGCVCKG------AADKCTCCA
RatMT2A     MDP-NCSC-ATDGSCSCAGSCKCKQCKCTSCKKSCCSCCPVGCAKCSQGCICKE------ASDKCSCCA
MusMT2      MDP-NCSC-ASDGSCSCAGACKCKQCKCTSCKKSCCSCCPVGCAKCSQGCICKE------ASDKCSCCA
Pand        MDP-NCSC-SPVGSCACAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKG------TSDKCSCCA
HomMT1X     MDP-NCSC-SPVGSCACAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKG------TSDKCSCCA
Panf        MDP-NCSC-ATGGSSTCTGSCKCKECKCTSCKKSCCSCCPMSCAKCAQGCICKG------ASEKCSCCA
HomMT1A     MDP-NCSC-ATGGSCTCTGSCKCKECKCTSCKKSCCSCCPMSCAKCAQGCICKG------ASEKCSCCA
PanaMT      MDP-NCSCAAAGVSCACASSCKCKECKCTSCKKSCCSCCPMGCAKCAQGCICKG------ASEKCSCCA
HomMT1G     MDP-NCSC-AAGVSCTCASSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKG------ASEKCSCCA
Pane        MDP-NCSC-EAGGSCACAGSCKCKKCKCTSCKKSCCSCCPLGCAKCAQGCICKG------ASEKCSCCA
HomMT1H     MDP-NCSC-EAGGSCACAGSCKCKKCKCTSCKKSCCSCCPLGCAKCAQGCICKG------ASEKCSCCA
PanMT1E     MDP-NCSC-ATGGSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCVCKG------ASEKCSCCA
HomMT1E     MDP-NCSC-ATGGSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCVCKG------ASEKCSCCA
PanMT1B     MDP-NCSC-TTGGSCACAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCVCKG------SSEKCRCCA
HomMT1B     MDP-NCSC-TTGGSCACAGSCKCKECKCTSCKKCCCSCCPVGCAKCAQGCVCKG------SSEKCRCCA
HomMT1M     MDP-NCSC-TTGVSCACTGSCTCKECKCTSCKKSCCSCCPVGCAKCAHGCVCKG------TLENCSCCA
Pana        MDP-NCSC-TTGGSC------KCKECKCTSCKKSCCSCCPVGCAKCAQGCVCKG------TSEKCSCCA
Panc        MDP-NCSC-ATGGSCTCAGSCKCKECKCTSCKKSCCFCCPVGCAKCAQGCVCKG------MSENCCCCA
CanMT3      MDPETCPC-PTGGSCTCDGSCKCEGCKCTSCKKSCCSCCPAECEKCAKDCVCKGGEGTEAEAEKCSCCQ
BosMT3      MDPETCPC-PTGGSCTCSDPCKCEGCTCASSKKSCCSCCPAECEKCAKDCVCKGGEGAEAEEKKCSCCQ
EquMT3      MDPETCPC-PTGGSCTCSGSCKCEGCKCTSCKKSCCSCCPAECEKCAKDCVCKGGEGAEAEAEKCSCCQ
RatMT3      MDPETCPC-PTGGSCTCSDKCKCKGCKCTNCKKSCCSCCPAGCEKCAKDCVCKGEEGAKAE--KCSCCQ
MusMT3      MDPETCPC-PTGGSCTCSDKCKCKGCKCTNCKKSCCSCCPAGCEKCAKDCVCKGEEGAKAEAEKCSCCQ
PanMT3      MDPETCPC-PSGGSCTCADSCKCEGCKCTSCKKSCCSCCPAECEKCAKDCVCKGGEAAEAEAEKCSCCQ
HomMT3      MDPETCPC-PSGGSCTCADSCKCEGCKCTSCKKSCCSCCPAECEKCAKDCVCKGGEAAEAEAEKCSCCQ
Panh        MDP-NCSCAAAGGSCTCAGSCKCKECKCTSCKKSCCSCCPVGCSKCAQGCVCKG------ASEKCSCCD
HomMT1F     MDP-NCSC-AAGVSCTCAGSCKCKECKCTSCKKSCCSCCPVGCSKCAQGCVCKG------ASEKCSCCD

Cluster MT4
CanMT4      MDPGECTC-MSGGICICGDNCKCTTCNCKTCRKSCCPCCPPGCAKCAQGCICKG------GSDKCSCCA
EquMT4      MDPRECSC-MSEGICLCGDNCKCTTCNCKTCRKSCCPCCPPGCAKCARGCVCKG------GSDKCSCCP
RatMT4      MDPGECTC-MSGGICICGDNCKCTTCSCKTCRKSCCPCCPPGCAKCARGCICKG------GSDKCSCCP
MusMT4      MDPGECTC-MSGGICICGDNCKCTTCSCKTCRKSCCPCCPPGCAKCARGCICKG------GSDKCSCCP
PanMT4      MDPRECVC-MSGGICMCGDNCKCTTCNCKTCQKSCCPCCPPGCAKCARGCICKG------GSDKCSCCP
HomMT4      MDPRECVC-MSGGICMCGDNCKCTTCNCKTYWKSCCPCCPPGCAKCARGCICKG------GSDKCSCCP
BosMT4      MDSGECTC-MSGGTCACGDNCKCTTCSCKTCRKSCCPCCPPGCAKCARGCICKG------ASDKCSCCP
GalMT3      MDSQDCPC-ATGGTCTCGDNCKCKNCKCTSCKKGCCSCCPAGCAKCAQGCVCKG-----PPSAKCSCCK
TaeMT1      MDSQDCPC-ATGGTCTCGDNCKCKNCKCTSCKKGCCSCCPAGCAKCAQGCVCKG-----PPSAKCSCCK
AnoMT4      MDAQDCPC-ISGGACTCGNNCQCKNCKCKTCKKSCCSCCPVGCSKCAQGCICKG-----PPSSKCSCCK
OrnMT4      MDPEECTC-MSGGICICGDNCKCTNCNCKTCRKSCCPCCPPGCAKCAQGCVCKR-----GADNKCSCCP

No cluster
AnoMT3      MDPQACSC-ATGGSYNCAGSCKCKDCKCTSCKKSCCSCCPASSDNCAKGCVCKD-----PSSGKCSCC-
DanMT2      MDP--CEC-AKTGTCNCGATCKCTNCQCTTCKKSCCSCCPSGCSKCASGCVCKG------NSCGSSCCQ
GalMT4      MDPQDCTC-AAGDSCSCAGSCKCKNCRCRSCRKSCCSCCPAGCNNCAKGCVCKE-----PASSKCSCCH
MelMT2      MDPQDCTC-AAGDSCSCAGSCKCKNCRCRSCRKSCCSCCPAGCNNCAKGCVCKE-----PASSKCSCCH
MonaMT      MDP-NCNC-ANGGSCTCGDSCKCKSCSCTSCKKSCCSCCPVGCAKCAQGCVCKA-----PQTESCSCCH
Mon2        MDP-NCDC-GNGASCTCAGSCKCASCHCTSCKKSCCSCCPVGCAKCAQGCVCKA-----PQTESCSCCH
Monb        MDP-NCDC-VSGNSCTCAGSCKCKSCRCTSCKKSCCSCCPAGCAKCAQGCVCKA-----PQTESCSCCH
OryMT       MDP--CDC-SKTGKCNCGGSCTCTNCSCTSCKKSCCACCPSGCTKCASGCVCKG------KTCDTTCCQ
TaeMT2      MDPQDCTC-AAGDSCSCAGSCKCKNCRCRSCRKSCCSCCPASCSNCAKGCVCKE-----PTSSKCSCCH
TakMT1F     MDP--CDC-SKTGSCNCGGSCACKNCSCTTCKKSCCSCCPSGCSKCASGCVCKG------KTCDTSCCQ
TetaMT      MDP--CDC-AKSGSCTCGGSCACTNCACTTCKKSCCPCCPSGCSKCASGCVCKG------KTCDTSCCQ
TetbMT      MDP--CDC-AKTGNCKCGGSCTCKDCSCTDCKKSCCSCCPSGCSKCASGCVCKG------KTCDTSCCQ
XenMT4      MDPQDCNC-ETGASCSCANKCVCSNCKCTSCKKSCCSCCPAECNKCSKGCHCEK------ESKKCSCCN
```

Supplementary Material 6 - Input tree for additional analyses on variation of selective rates. A tetrapod MT3 clade is highlighted.



Supplementary Material 7 - Table with accession number and name of species retrieved for alignment and primer design of endogenous control genes.

18S	β-Actin	rpL8	GAPDH	ANXA2	eef1a1
Pseudacris regilla JQ511838	*Bufo gargarizans* EU661596	*Rana clamitans* HQ699897	*Bufo gargarizans* FJ617545	*Xenopus laevis* BC046669	*Bufo japonicus* AB066590
Cochranella sp. EF376119	*Physalamus pustulosus* AY226144	*Xenopus tropicalis* NM_203594	*Pelophylax ridibundus* AY072703	*Xenopus tropicalis* NM_203590	*Hyla japonica* AB199910
Scinax boesemani EF376108	*Xenopus laevis* AF079161	*Xenopus laevis* U00920	*Polypedates maculatus* JN681267	*Bombina maxima* GU597351	*Xenopus laevis* NM_001087442
Osteocephalus oophagus EF376098	*Boa constrictor* FJ645273	*Gallus gallus* NM_001277728	*Hoplobatrachus tigerinus* FJ769259	*Rana catesbeiana* AB286846	*Xenopus tropicalis* NM_001016692.2
Hyla sp. EF376092	*Gallus gallus* NM_205518		*Xenopus tropicalis* BC075438	*Gallus gallus* NM_205351	*Gallus gallus* L00677
Rana boylii JQ511853			*Pleurodeles waltl* AF482996		
Rana pipiens JQ511820					