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REVIEW

Molecular sexing applicable in 4000 species of lizards and snakes? From dream to real possibility

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Summary

1. While the stability of sex chromosomes is widely accepted in viviparous mammals and birds, ectothermic vertebrates are still largely viewed as having frequent turnovers in sex-determining systems. Frequent changes in sex-determining systems in ectotherms could be problematic for field ecological studies as well as for breeding programs, as molecular sexing across a phylogenetically widespread spectrum of ectothermic vertebrates would not be possible. However, we recently documented that sex-determining systems in three important reptile lineages (caenophidian snakes, iguanas and lacertid lizards) are in fact highly conserved.

2. We applied a new molecular procedure to identify sex within each of these three lineages (encompassing altogether around 4000 species, i.e. nearly 50% of the recent species of reptiles). This technique uses quantitative PCR (qPCR) to compare copy numbers of genes specific for their respective Z (in caenophidian snakes and lacertids) and X (in iguanas) chromosomes between male and female genomes. The DNA samples required can be collected relatively non-invasively. Unlike molecular sexing based on repetitive elements, this technique can be easily applied to previously unstudied species of these lineages, as the number of copies of protein-coding genes linked to the differentiated sex chromosomes is evolutionarily highly conserved in each.

3. We suggest that qPCR-based molecular sexing using the comparison of gene copy number is a practical choice for non-model species of caenophidian snakes, iguanas and lacertids. Moreover, it should also soon be available for other reptile lineages with differentiated sex chromosomes.

Key-words: lizards, molecular sexing, reptiles, sex determination, snakes

Molecular sexing is an attractive option for the accurate identification of the sex of adults in monomorphic or slightly sexually dimorphic species, and it is by far the most superior, and often, only method to identify the sex of embryos and juveniles before the development of primary and secondary sexual characteristics. Therefore, molecular sexing is widely used in evolutionary and ecological research as well as in conservation and breeding. As sex chromosomes are conserved in birds and in the overwhelming majority of viviparous mammals, developed molecular sexing techniques are often applied throughout this group (Fridolfsson & Ellegren 1999; Fernando & Melnick 2001; Morin *et al*. 2005; Brubaker *et al*. 2011; Morinha, Cabral & Bastos 2012; Morinha *et al*. 2015). However, no such widely applicable technique was thought possible for non-avian reptiles as they possess variability in sex-determining systems (Ezaz *et al*. 2009; Pokorná & Kratochvíl 2009; Gamble 2010; Sarre, Ezaz & Georges 2011; Gamble *et al*. 2015; Johnson Pokorná & Kratochvíl 2016) (note: the term 'reptiles' is further used here for the paraphyletic taxon, i.e. it is understood as the synonym for non-avian sauropsids).

Squamates, the ancient lineage with >10 000 known extant species of lizards and snakes (Uetz & Hosek 2016), possess the greatest variability in sex determination in reptiles (Pokorná & Kratochvíl 2009; Gamble 2010) with systems ranging from environmental sex determination (ESD) to genotypic sex determination (GSD) with well-differentiated ZZ/ZW or XX/XY sex chromosomes (Pokorná & Kratochvíl 2009; Gamble 2010; Bachtrog *et al*. 2014; Gamble *et al*. 2015). As typical ectothermic vertebrates, and hence equally to fish and amphibians, squamates were believed to possess frequent turnovers in sex-determining systems and unstable sex chromosomes (Grossen, Neuenschwander & Perrin 2011; Kikuchi & Hamaguchi 2013). Any developed molecular sexing technique in GSD reptiles would therefore only be very limited, restricted to a specific narrow lineage with homologous sex chromosomes.

Moreover, in many cases, the suggested molecular sexing methods for reptiles were based on the comparison of sex-specific markers with high rates of molecular evolution such as microsatellites, other repetitive sequences and non-coding regions, which typically do not allow their application to wider phylogenetic spectra. Indeed, the Y-specific marker in *Anolis carolinensis* does not work for molecular sexing in more distantly related species of the genus (Gamble & Zarkower 2014). Similarly, in our experience, the sex-specific marker in Komodo dragons (Halverson & Spelman 2002; Sulandari *et al*. 2014) is not applicable to two other species of monitor lizards (M. Rovatsos, L. Kratochvil, unpublished data). It is also likely that other molecular sexing techniques in reptiles

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based on microsatellite markers, such as the X-specific marker in the skinks *Tiliqua rugosa* (Cooper, Bull & Gardner 1997) and *Egernia cunninghami* (Stow *et al*. 2001), the uncharacterized Y-specific marker in the skink *Bassiana duperreyi* (Quinn *et al*. 2009) and the uncharacterized, probably non-coding W-specific marker in the bearded dragon (*Pogona vitticeps*; Quinn *et al*. 2010), would have equally narrow applicability. The same may be true for the anonymous sex-specific markers recently identified by digest RAD-sequencing for various species of geckos (Gamble *et al*. 2015). Furthermore, geckos are an old, highly radiated group possessing extraordinarily numerous transitions among sex-determining systems, which by itself precludes finding a general molecular sexing technique (Pokorná *et al*. 2014; Gamble *et al*. 2015).

Other molecular sexing techniques in reptiles are not based on the detection of the presence/absence of a sex-linked marker by PCR or the detection of a sex-specific allele, but on the comparison of the number of repetitive sequences linked to sex chromosomes measurable by quantitative PCR (qPCR). For example, females in the thick-tailed gecko (*Underwoodisaurus milii*) possess large accumulations of telomeric-like repeats on the W chromosome. Using qPCR, we estimated that their genome includes c. 50% more telomeric-like repeats than the male (Pokorná *et al*. 2014), a difference large enough to be used for molecular sexing. Sizeable accumulations of telomeric-like sequences were also found on the W chromosomes in other lizards (e.g. in the sand lizard, *Lacerta agilis*) and snakes (e.g. in the dragon snake, *Xenodermus javanicus*) (Matsubara *et al*. 2015; Rovatsos, Johnson Pokorná & Kratochvíl 2015), and so the comparison of telomeric-like repeats should be likely usable for molecular sexing in these species as well. Similarly, Literman, Badenhorst & Valenzuela (2014) demonstrated the applicability of molecular sexing in the spiny softshell turtle (*Apalone spinifera*) using qPCR comparison of the number of rDNA genes, as these are accumulated much more on the W chromosome. Nevertheless, repetitive elements on the degenerated Y and W sex chromosomes likely represent one of the most evolutionary dynamic parts of the genome (Rovatsos *et al*. 2011, 2015a; Altmanová *et al*. 2016; Johnson Pokorná *et al*. 2016; Matsubara *et al*. 2016), and therefore it is expected that molecular sexing methods based on such markers would provide reliable results only in a narrow phylogenetic range.

Recently, on the basis of molecular evidence, we documented that the gene content of sex chromosomes in certain squamate lineages is highly conserved and therefore Z- or X-linked genes would be suitable markers for a widely applicable technique of molecular sexing. We observed that all tested lineages of lacertid lizards (the lineage currently including >320 species) share homologous differentiated ZZ/ZW sex chromosomes for about 70 MYA (Rovatsos, Vukić & Kratochvíl 2016; Rovatsos *et al*. 2016). The age estimations presented here represent the reconstructed dates of the basal splitting of each lineage with confirmed homologous sex chromosomes and thus represent the minimal estimated age of these sex chromosomes; the time estimations are largely taken from the TimeTree data base (Hedges *et al*. 2015). Homologous and highly differentiated ZZ/ZW sex chromosomes can be traced back to the common ancestor of advanced snakes (Caenophidia) living ca. 60 MYA (Hsiang *et al*. 2015) and it can be seen that members of all families of this widely radiated clade (>3000 living species) still share these sex chromosomes (Rovatsos *et al*. 2015b). Similarly, XX/XY sex chromosomes are conserved across the majority of the iguanas with around 900 species (Rovatsos *et al*. 2014a, b), including the highly radiated genus *Anolis* (Gamble & Zarkower 2014; Rovatsos *et al*. 2014a) as well as within the Madagascan family Opluridae (Altmanová *et al*. 2016). These differentiated sex chromosomes were already present in the common ancestor of iguanas living ca. 123 MYA (Rovatsos *et al*. 2014b). For comparison, there are about 10 000 current species of birds and their sex chromosomes can be dated back to about 70 MYA (Mank & Ellegren 2007) and about 5500 species of viviparous mammals with the age of sex chromosomes going back to at least 166 MYA (Veyrunes *et al*. 2008).

Our proposed widely applicable molecular sexing methodology uses qPCR to compare copy numbers in genes specific to Z and X chromosomes, that is, genes missing on the degenerated parts of the W and Y chromosomes, respectively, between male and female genomes. In genes hemizygous in heterogametic sex, qPCR can be reliably used to determine the number of gene copies present in genomic DNA, that is, we can expect twice as many copies in XX or ZZ individuals than in XY and ZW individuals relative to an autosomal control gene. For qPCR, primers should be designed to amplify 150–200 bp fragments of exons of the control autosomal, ideally single copy gene and X- or Z-specific genes. The primers should be tested by PCR to prevent amplification of secondary products, etc. For iguanas, lacertids and caenophidian snakes, we have already found several reliable primers applicable to a wide array of species (Table S1, Supporting Information). For our molecular sexing approach, it is necessary to have DNA samples from one male and one female individual of the same species, in addition to the tested samples of unknown sex, obtained using standard DNA isolation and quality control. Furthermore, for accurate sex identification, we suggest using qPCR to amplify a minimum of five distinct primer pairs: two for the normalization gene, one for the autosomal gene (control) and two for sex-linked loci (please see Table S2 for lacertids, Table S3 for advanced snakes, Table S4 for iguanas).

The qPCR was carried out in a LightCycler II 480 thermal cycler (Roche Diagnostics GmbH, Mannheim, Germany), running all samples in triplicate in a 15 μL reaction volume containing 2 ng of genomic DNA, 0·3 μL of each of the forward and reverse primers (stock solution 10 pmol/μL) and 7·5 μL SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan) using the same cycling conditions: 95 °C for 3 min, followed by 44 amplification cycles of 95 °C for 15 s, 56 °C for 30 s, 72 °C for 30 s, ending with a melting curve analysis to monitor for potential non-specific products. The melting curve test includes an initial denaturation at 94 °C for 15 s and subsequent fluorescent measurements every 0·1 °C from 65 °C to 95 °C. Quantification cycle values (crossing point–Cp) were calculated with LightCycler 480 software (version 1.5.0), using the second-derivative maximum algorithm.

Subsequently, the gene dosage of each target gene is determined from the crossing point values and normalized to the dose of the autosomal reference gene with stable gene copy numbers from the same DNA sample. The single copy gene *eef1a1* (eukaryotic translation elongation factor 1 alpha 1) is usually used; however, in the case of a mutation in the primer binding sites or instability in its copy number in unstudied species, any autosomal gene with stable copy numbers could be used for normalization. The target-to-reference gene dosage ratio (R) is calculated by the equation: $R = E_{eef1a1}^{\text{Cp } eef1a1}/E_{\text{gene}}^{\text{Cp gene}}$ using default amplification efficiencies (E) of 2. Ideally, the amplification efficiency (E) of each primer pair should be calculated in each species based on the standard curve analysis performed with genomic DNA dilution series; however, in our experience, the expected value of $E = 2$ (e.g. Cawthon 2002) is usually suitable for detecting sexual differences in gene copy numbers (Rovatsos *et al.* 2014a). Finally, the relative gene dosage ratio (r) between males and females of each species for each target gene was obtained by dividing the gene dosage in a male by the gene dosage in a female of the same species, as $r = R_{\text{male}}/R_{\text{female}}$. The relative gene dosage ratios between sexes (r) should be 0·5 for X-specific (e.g. in iguanas), 2·0 for Z-specific (e.g. in lacertids and caenophidian snakes) and 1·0 for (pseudo-)autosomal genes. The sex of unidentified individuals of the same species can be determined using the same procedure calculating the ratio between an individual with unknown sex and using the male and the female from the pair as a standard (Fig. 1).

The large conservation of sex chromosomes in caenophidian snakes, iguanas and lacertid lizards would imply that within each of these groups, the technique of molecular sexing should be applicable in about 4000 species of squamate reptiles, that is, nearly half of the total species richness (Uetz & Hosek 2016). As sex chromosomes in these three lineages are non-homologous, that is, they evolved from different putatively autosomal ancestral chromosomes (Pokorná & Kratochvíl 2009; Johnson Pokorná & Kratochvíl 2016), different genes should be applied for molecular sexing in each. Within each of these lineages, the procedure works very well; it was successfully applied in 78 squamate species, including 18 lacertids, 28 species of caenophidian snakes (covering all families) and 32 iguanas (from seven families) (Table S1).

One of the advantages of this technique is that it is also easily applicable to previously unstudied species of these lineages using the same standard protocol and primers. Based on our experience, the primers listed in Tables S2–S4 are highly conserved across phylogeny. Moreover, as this technique provides information about differences in the gene copy numbers between males and females, rather direct evidence for sex-specific genome differences among the sexes of the studied species is also obtained. In addition, the technique was found to work on both fresh and preserved samples, and on DNA samples of different condition (fresh, frozen, preserved in ethanol in museum collections) and origin (blood, muscle, tip of tail, saliva collected by cotton swabs), providing that the isolated DNA is of adequate quality and quantity (concentration more than 20 ng/μL, measured in NanoDrop; Thermo Fisher Scientific – NanoDrop Products, Wilmington, DE, USA).

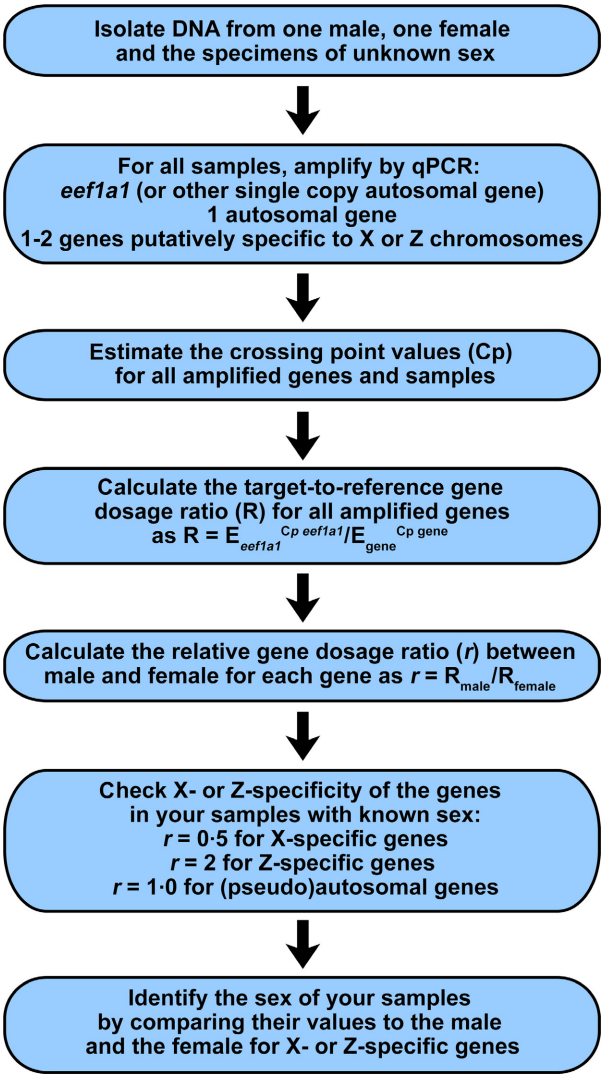

Fig. 1. Schematic depiction of the pipeline for molecular sexing by qPCR.

At the same time, we would like to stress that molecular sexing using qPCR has inherited limitations and disadvantages. For example, it cannot be used for lineages with poorly differentiated sex chromosomes. Another disadvantage is that when used as a routine technique for sexing in a species, it is more costly in comparison to PCR-based techniques. However, it should be taken into account that the initial material and laboratory time costs are much lower, as it is not usually necessary to laboriously optimize the procedure for each new species in a lineage. When applied to a new species, it should be tested in a pair or pairs of reliably sexed animals, as is the case for all other molecular sexing techniques. It is also possible that the primers might not work in all cases, especially in species phylogenetically distantly related to the species used for primer design. This limitation can be overcome by selecting other primer pairs from our list (Tables S2–S4), or by designing new ones for other genes present in the Z- or X-specific regions.

The Z- and X-specificity of genes within a lineage with differentiated sex chromosomes seems to be very evolutionary stable (Rovatsos *et al.* 2014a, b, 2015b, 2016; Rovatsos, Vukić & Kratochvíl 2016), definitely much more than non-coding sex-specific alleles or repetitive sequences. Nevertheless, on a wide phylogenetic scale, some exceptions to the pattern can be expected. We identified several such cases in a few genes, which likely reflect translocation of the Z-specific gene to a (pseudo)autosomal position, or less differentiated sex chromosomes with larger pseudoautosomal regions in 'basal' lineages (Rovatsos *et al.* 2015b, 2016). We also found that all tested genes which are X-specific in other iguanas are (pseudo) autosomal in basilisks, although all recent phylogenies agree that basilisks are an inner iguana group (Rovatsos *et al.* 2014b). This situation might reflect a rare case of loss/turnover of already highly differentiated sex chromosomes, as was recently demonstrated in the Madagascan geckos of the genus *Paroedura* (Koubová *et al.* 2014), in dipteran insects (Vicoso & Bachtrog 2013, 2015) and in mammals (Kuroiwa *et al.* 2011; Bagheri-Fam *et al.* 2012). Working on such a large phylogenetic scale, some variability in otherwise conserved sex chromosomes and other such cases could be expected to be uncovered in the future when more data emerge from further studies of different species of lacertids, caenophidian snakes and iguanas.

We suggest that this qPCR-based technique of comparing the number of copies of genes linked to differentiated sex chromosomes is a very promising approach for molecular sexing for at least half of the species diversity of squamate reptiles, a vertebrate group facing diversity crisis (IUCN 2015). As differentiated sex chromosomes are probably conserved in many reptile lineages other than caenophidian snakes, iguanas and lacertids (Pokorná & Kratochvíl 2009; Gamble & Zarkower 2014; Gamble *et al.* 2015; Johnson Pokorná & Kratochvíl 2016), after identification of Z- and X-specific genes it should be a simple matter to find the markers useful for molecular sexing in these lineages as well.

Authors' contribution

Both authors contributed equally to development of the ideas and writing of the manuscript.

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Data accessibility

This paper does not describe any new data. The data used were published in Altmanová *et al.* (2016; Table S3: http://onlinelibrary.wiley.com/wol1/doi/10.1111/bij.12751/suppinfo) and Rovatsos *et al.* (2014a; Table S3: http://onlinelibrary.wiley.com/wol1/doi/10.1111/evo.12357/suppinfo; 2014b; Electronic Supplementary Material S2: http://rsbl.royalsocietypublishing.org/content/10/3/20131093.figures-only; 2015b, Table S3: http://rspb.royalsocietypublishing.org/content/282/1821/20151992.figures-only; 2016; Table S3: http://onlinelibrary.wiley.com/wol1/doi/10.1111/mec.13635/suppinfo).

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Supporting Information

Details of electronic Supporting Information are provided below.

Table S1. List of species of advanced snakes, iguanas and lacertids, where our molecular sexing approach was successfully applied (Rovatsos *et al.* 2014a, b; Rovatsos *et al.* 2015b, Rovatsos, Vukić & Kratochvíl 2016; Rovatsos *et al.* 2016; Altmanová *et al.* 2016).

Table S2. Primers suitable for molecular sexing of lacertids by qPCR. For additional primers see Rovatsos, Vukić & Kratochvíl 2016; Rovatsos *et al.* 2016.

Table S3. Primers suitable for molecular sexing of advanced snakes by qPCR. For additional primers see Rovatsos *et al.* (2015b).

Table S4. Primers suitable for molecular sexing of iguanas by qPCR. For additional primers see Rovatsos *et al.* (2014a, b).