Novel X-Linked Genes Revealed by Quantitative Polymerase Chain Reaction in the Green Anole, Anolis carolinensis

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ABSTRACT The green anole, Anolis carolinensis (ACA), is the model reptile for a vast array of biological disciplines. It was the first nonavian reptile to have its genome fully sequenced. During the genome project, the XX/XY system of sex chromosomes homologous to chicken chromosome 15 (GGA15) was revealed, and 106 X-linked genes were identified. We selected 38 genes located on eight scaffolds in ACA and having orthologs located on GGA15, then tested their linkage to ACA X chromosome by using comparative quantitative fluorescent real-time polymerase chain reaction applied to male and female genomic DNA. All tested genes appeared to be X-specific and not present on the Y chromosome. Assuming that all genes located on these scaffolds should be localized to the ACA X chromosome, we more than doubled the number of known X-linked genes in ACA, from 106 to 250. While demonstrating that the gene content of chromosome X in ACA and GGA15 is largely conserved, we nevertheless showed that numerous interchromosomal rearrangements had occurred since the splitting of the chicken and anole evolutionary lineages. The presence of many ACA X-specific genes localized to distinct contigs indicates that the ACA Y chromosome should be highly degenerated, having lost a large amount of its original gene content during evolution. The identification of novel genes linked to the X chromosome and absent on the Y chromosome in the model lizard species contributes to ongoing research as to the evolution of sex determination in reptiles and provides important information for future comparative and functional genomics.
remained unidentified. During the whole-genome sequencing, it was proven that ACA possesses the XX/XY system of sex chromosomes. The X chromosome (ACAX) was identified at that time using fluorescent in situ hybridization of 11 bacterial artificial chromosomes (BACs) containing loci from two contigs. These BACs hybridized to the p arms of two microchromosomes in females but only to the p arm of a single microchromosome in males (Alföldi et al. 2011), thus suggesting that these sequences are specific to the X and absent on the Y chromosome. The Y chromosome has not yet been identified, but it is assumed to be another microchromosome (Alföldi et al. 2011). The known X-linked region of ACA includes approximately 106 genes (National Center for Biotechnology Information) with orthologs linked to chromosome 15 of the chicken (Gallus gallus, GGA). Alföldi et al. (2011) speculated that additional X-linked genes might be present on unanchored scaffolds in the AnoCar 2.0 assembly.

Recent studies have demonstrated that the ACA X-linked region is X-linked not only among anoles (Gamble et al. 2014; Rovatsos et al. 2014a) but also across most phylogenetic lineages of iguanas (Pleurodonta; Rovatsos et al. 2014b). The origin of sex chromosomes in iguanas can in fact be traced back to the basal splitting of this group that occurred during the Cretaceous period, and their age can therefore be comparable with the age of sex chromosomes in birds or viviparous mammals. Taking into account that female heterogamety and environmental sex determination have been reported from dragon lizards and chameleons (Acrodonta), the closest outgroup of iguanas (Ezaz et al. 2005; Pokorná and Kratochvíl 2009; Young et al. 2013), it seems that these XX/XY sex chromosomes constitute a synapomorphy of iguanas.

In contrast to mammals and birds (Ferguson-Smith and Trifonov 2007; Griffin et al. 2007), our knowledge about ancestral karyotypes and rates of chromosomal evolution in the majority of the other

| Gene Symbol | GenBank Gene ID | Topology in Anolis carolinensis | Relative gene dosage Between Sexes |
|-------------|----------------|--------------------------------|-----------------------------------|
| EF1a        | 100566578      | NW_003338888.1                 |                                   |
| FBXW7       | 100554110      | Chromosome 5; NC_014780.1      | 1.08                              |
| NHP2L1      | 100552266      | Chromosome 5; NC_014780.1      | 1.08                              |
| ADARB2      | 100560912      | Chromosome 6; NC_014781.1      | 1.03                              |
| ACAD10      | 100557969      |                                   | 0.53                              |
| CMKLIR1     | 100559738      |                                   | 0.48                              |
| SNAP29      | 100554635      | NW_003338829.1                 | 0.38                              |
| SDF2L1      | 100562697      |                                   | 0.47                              |
| HIRA        | 100556463      |                                   | 0.62                              |
| SEPT5       | 100563096      |                                   | 0.48                              |
| MLEC        | 100557313      |                                   | 0.31                              |
| TBC1D10A    | 100558296      |                                   | 0.38                              |
| CIT         | 100553665      |                                   | 0.44                              |
| DTX1        | 100559683      | NW_00333885.1                  | 0.53                              |
| DDX54       | 100560281      |                                   | 0.49                              |
| IQCD        | 100560479      |                                   | 0.51                              |
| PLBD2       | 100561071      |                                   | 0.51                              |
| LHX5        | 100555029      |                                   | 0.64                              |
| PUS1        | 100562702      |                                   | 0.48                              |
| EP400       | 100562901      |                                   | 0.52                              |
| FBRS1       | 100563489      |                                   | 0.52                              |
| GOLGA3      | 100563881      | NW_003338911.1                | 0.46                              |
| ZDHHC8      | 100561780      |                                   | 0.48                              |
| TRMT2A      | 100564671      |                                   | 0.53                              |
| DGC8        | 100561976      |                                   | 0.48                              |
| GAS2L1      | 100567612      |                                   | 0.32                              |
| SMTN        | 100553012      | NW_003338964.1                | 0.33                              |
| SLC7A4      | 100554971      |                                   | 0.47                              |
| B3GNT4      | 10056391      |                                   | 0.45                              |
| CLIP1       | 100566771      | NW_003338970.1               | 0.63                              |
| KNTC1       | 100567355      |                                   | 0.52                              |
| KDM2B       | 100557128      |                                   | 0.61                              |
| ORAI1       | 100556544      |                                   | 0.53                              |
| WDR66       | 100557915      | NW_003339097.1                | 0.50                              |
| LRRC43      | 100558501      |                                   | 0.54                              |
| MLXIP       | 100558305      |                                   | 0.54                              |
| FICD        | 100557652      |                                   | 0.38                              |
| SART3       | 100557456      | NW_003339461.1              | 0.53                              |
| TMEM119     | 100557060      |                                   | 0.54                              |
| BCR         | 100554393      |                                   | 0.44                              |
| SPECC1L     | 100554592      | NW_003339495.1              | 0.32                              |
| ADORA2A     | 100554785      |                                   | 0.37                              |

Gene names and chromosomal position data follow GenBank database (http://www.ncbi.nlm.nih.gov/genbank).
lineages of amniotes is still limited. Only recent analyses based on gene mapping, chromosomal painting, and whole-genome sequencing have shown that the slow rate of interchromosomal rearrangements is likely to be characteristic for all sauropsids and not just for birds, which constitute their inner group (Matsuda et al. 2005; Srikulnath et al. 2009; Alföldi et al. 2011; Pokorná et al. 2011, 2012; Uno et al. 2012; Srikulnath et al. 2013; Young et al. 2013). All X-linked genes in ACA known before the current study have orthologs on chicken chromosome 15 (GGA15). We therefore can assume that the entire X-chromosome in ACA is homologous to ACAX. Nevertheless, although sauropsids possess a relatively low rate of interchromosomal rearrangement, it has been shown in birds that their intrachromosomal rearrangements occur rather frequently (Völker et al. 2013). We tested whether all these scaffolds are X-linked in ACA and how frequent were intrachromosomal rearrangements of this chromosome during the independent evolutionary histories of chicken and green anole.

MATERIALS AND METHODS
Total genomic DNA was isolated using the DNeasy Blood & Tissue kit (QIAGEN) from the blood of a male and a female of ACA. Primer pairs were designed on Primer3 software (Ye et al. 2012) according to ACA sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank; Benson et al. 2013) for amplifying putative X-linked loci on scaffolds with homology to GGA15 and the known X-linked regions of ACA (Alföldi et al. 2011). Additional primer pairs were designed for autosomal genes localized to ACA chromosome 6, chromosome 5, and for the single-copy gene EF1a, which was used for normalization of the gene dosages in the qPCR analyses (Table 1 and Supporting Information, Table S1).

The qPCR was carried out in a LightCycler II 480 (Roche Diagnostics). All samples were run in triplicates. A 15-μL reaction was performed, containing 2 ng of genomic DNA, 7.5 μL of SYBR Premix Ex Taq II (Takara), and 0.3 mM of each primer. The cycling conditions were: 95°C for 3 min, followed by 44 amplification cycles of 95°C for 15 sec, 56°C for 30 sec, 72°C for 30 sec, and ending with a melting curve analysis to monitor for potential nonspecific products. The quantification cycle values (crossing point, C) were calculated with LightCycler 480 software (v. 1.5.0) according to the second derivative maximum algorithm.

The gene dosage of each studied gene was calculated from C values and subsequently normalized to the gene dosage of the single copy gene EF1a based on the equation: \( R = \frac{2C_{\text{gene}}}{2C_{\text{EF1a}}} \) (Cawthon 2002). Finally, the relative gene dosage ratio \( r \) between sexes was calculated for each gene as \( r = R_{\text{male}}/R_{\text{female}} \). Since ACA has the XX/XY sex determination system, we expected a relative gene dosage ratio \( r \) of 0.5 for X-linked genes missing on the Y chromosome and 1.0 for autosomal or pseudoautosomal genes.

RESULTS
The relative gene dosage ratios between males and females were estimated by qPCR for two genes from chromosome 5 (contig No. NC_014780), one gene from chromosome 6 (NC_014781), six genes were tested by qPCR for genes tested by quantitative polymerase chain reaction in A. carolinensis. Value 1.0 is expected for autosomal or pseudoautosomal genes, while value 0.5 is consistent with X-specific position.

Table 2 List of contigs from the ACA genome project shown to be X-linked

| ACA X-Linked Contigs | Contig Size, bp | Number of Genes | Studied Genes by qPCR | Percentage of Studied Genes per Contig |
|----------------------|----------------|-----------------|-----------------------|---------------------------------------|
| NC_014783            | 3 271 537      | 58              | 5a,b                  | 9                                     |
| NW_003338829         | 1 779 868      | 48              | 9a,b,c                | 19                                    |
| NW_003338885         | 1 258 094      | 45              | 8                     | 18                                    |
| NW_003339111         | 1 083 274      | 17              | 7                     | 41                                    |
| NW_003338964         | 831 895        | 37              | 4a,b                  | 11                                    |
| NW_003338970         | 834 740        | 19              | 6a,b                  | 32                                    |
| NW_003339097         | 526 944        | 11              | 5                     | 45                                    |
| NW_003339461         | 147 151        | 5               | 3                     | 60                                    |
| NW_003339495         | 117 443        | 10              | 3                     | 30                                    |

Presented are the contig size, gene content, and number of genes tested by qPCR per contig. ACA, Anolis carolinensis; qPCR, quantitative polymerase chain reaction.

a Data from Rovatsos et al. 2004a.
b Data from Rovatsos et al. 2014.
c Data from Gamble et al. 2014.
from the known X-linked region of ACA (NW_003338829), and 32 putative X-linked genes assigned to seven unanchored scaffolds (see Figure 1, Table 1, Table 2, and Table S1). In all cases, the values from the gene EF1a were used for normalization.

Our qPCR results confirmed the autosomal position of the genes located in chromosomes 5 and 6 of ACA. As expected for autosomal or pseudoautosomal genes, their relative gene dosage ratios were very close to, and did not differ significantly from, the expected value of 1.0 (t-test, P = 0.08). In addition, the six genes from the known X-linked region of ACA had male to female gene dosage ratios ranging from 0.38 to 0.62 (mean 0.50), which did not differ significantly from the expected value for X-linkage of 0.5 (t-test, P = 0.90). The 32 putative X-linked genes demonstrated ratios varying from 0.31 to 0.64 (mean 0.48), which also did not vary significantly from the expected 0.5 (t-test, P = 0.16). Thus, this leads to the conclusion that these genes are localized to the X chromosome of ACA and are absent from Y. We detected no autosomal or pseudoautosomal genes.

Our qPCR approach identified X-specific genes robustly, since the relative gene dosage ratios for the genes located on autosomes (chromosomes 5 and 6) differ significantly from those of the X-linked genes (analysis of variance, P = 0.0004). The two sets are thus clearly distinguishable, without overlap or intermediate values (Figure 2).
two ACA genes (RTDR1 and GNA2) located in a short contig composed from only three genes for relative gene dosage between sexes. RTDR1 displayed relative gene dosage ratios between male and female consistent with an X-specific position. GNA2 yielded equal ratios between sexes, thus suggesting that this gene has a gametolog on the Y chromosome (Gamble and Zarkower 2014). Data from the Genomicus database (http://www.genomicus.biologie.ens.fr/genomicus-75.02/cgi-bin/search.pl; Louis et al. 2013) indicate, however, that some genes from the GGA15 chromosome are localized to autosomes in ACA (e.g., ACACB on ACA chromosome 1 or SF11 on ACA chromosome 3). This probably is due to translocations that occurred during the 250 million years of divergence between ACA and GGA. The test for putative pseudoautosomal position of GNA2 requires further experimental work.

All studied genes were X-specific and not present on the Y chromosome in ACA. We did not detect any evidence for autosomal or pseudoautosomal position of the genes with orthologs linked to GGA15. It is therefore possible that the pseudoautosomal region in ACA is small or absent. Alternatively, a pseudoautosomal region in ACA could be homologous to a chromosome other than GGA15 and, as such, it could not be determined using our approach. These hypotheses should be tested by, for instance, observing the behavior of sex chromosomes during male meiosis.

Comparison of the topology between the X-linked ACA contigs and GGA 15 (Figure 3), as illustrated by the Genomicus database (http://www.genomicus.biologie.ens.fr/genomicus-75.02/cgi-bin/search.pl; Louis et al. 2013), shows that despite high conservation of the chromosome, several intrachromosomal rearrangements, such as inversions, probably occurred at this chromosome after the divergence of ACA and GGA from their common ancestor. Similarly, no interchromosomal but numerous intrachromosomal rearrangements have been documented in the microchromosomes of chicken, turkey, and zebra finch (Lithgow et al. 2014). Several pericentric inversions have been revealed in the chromosomal pairs 1–4 of ACA by in situ hybridization with BACs, but their functional importance remains unclear (Alflöldi et al. 2011). We should keep in mind, however, a recent counterexample based on fluorescent in situ hybridization mapping of 11 markers whereby a high level of synteny was revealed between ACA chromosome 6 and its homologous chromosome in snakes (Z in colubroid snakes) without any detectable large-scale chromosomal rearrangements (Visco et al. 2013).

Reptiles (excluding their inner avian group) usually are considered a group with rapid turnover of sex-determining mechanisms (Sarre et al. 2004; Organ and Janes 2008). In general, they do indeed exhibit large variability in sex-determining systems (Valenzuela and Lance 2004; Pokorná and Kratochvíl 2009; Gamble 2010). It has been suggested that poikilotherms possess more frequent turnovers of sex chromosomes than do homoiotherms, whose effective thermoregulation enables future comparative study on the evolution of sex chromosomes in iguanas. For example, applying the qPCR approach within lineages derived from basal splitting of iguanas may reveal whether highly differentiated X and Y chromosomes described in ACA evolved via a stepwise series of suppressions of recombination along the iguana Y chromosome, i.e., whether some “evolutionary strata” found in mammals and birds (Lahn and Page 1999; Handley et al. 2004; Nam and Ellegren 2008) could be observed also in iguanas.

ACKNOWLEDGMENTS

We express our gratitude to František Marec and Romana Stopková for their valuable guidance on qPCR and to Jan Cervenka for animal handling and care. Deborah Charlesworth and an anonymous referee provided valuable comments. This project was funded by the Czech Science Foundation (GACR 506/10/0718) and the Grant Agency of Charles University (GAUK 591712). This paper represents part 11 of our series “Evolution of sex determining systems in lizards.”

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Communicating editor: B. J. Andrews