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Pattern of chromosomal changes in 'beta' *Anolis* (Norops group) (Squamata: Polychrotidae) depicted by an ancestral state analysis

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Abstract

Background: Neotropical lizards, genus *Anolis* (Polychrotidae), with nearly 380 species, are members of one of the most diversified genera among amniotes. Herein, we present an overview of chromosomal evolution in 'beta' *Anolis* (Norops group) as a baseline for future studies of the karyotypic evolution of anoles. We evaluated all available information concerning karyotypes of Norops, including original data on a recently described species, *Anolis unilobatus*. We used the phylogeny of Norops based on DNA sequence data to infer the main pattern of chromosomal evolution by means of an ancestral state analysis (ASR).

Results: We identified 11 different karyotypes, of which 9 in the species had so far been used in molecular studies. The ASR indicated that a change in the number of microchromosomes was the first evolutionary step, followed by an increase in chromosome numbers, likely due to centric fissions of macrochromosomes. The ASR also showed that in nine species, heteromorphic sex chromosomes most probably originated from six independent events.

Conclusions: We observed an overall good correspondence of some characteristics of karyotypes and species relationships. Moreover, the clade seems prone to sex chromosome diversification, and the origins of five of these heteromorphic sex chromosome variants seem to be recent as they appear at the tip nodes in the ancestral character reconstruction. Karyotypic diversification in Norops provides an opportunity to test the chromosomal speciation models and is expected to be useful in studying relationships among anole species and in identifying cryptic taxa.

Keywords: Anole; *Anolis*; Chromosomal evolution; Cytogenetics; Norops; Sex chromosomes; Systematics

Background

The genus *Anolis* includes nearly 380 species of neotropical lizards and is the most species-rich amniote genus (Uetz 2013). *Anolis* was recently the subject of a systematic revision that resulted in it being split into eight different genera (Nicholson et al. 2012). However, this systematic arrangement has been vigorously criticized (e.g., Poe 2013), and in this paper, we adopted the classical view that considers anole lizards as being comprised of a single genus (Losos 2009; Uetz 2013). Anole species are distributed throughout the Caribbean islands

and mainland Americas from Texas to tropical South America (reviewed in Losos 2009), where they exhibit a wide array of ecological and morphological specializations (Etheridge 1959; Williams 1983; Losos et al. 1998; Köhler 2000). Anoles have been thoroughly investigated in various ecological, behavioral, and evolutionary studies (reviewed in Losos 2009). One species, the green anole lizard *Anolis carolinensis*, was the first reptile genome to be sequenced. This achievement may considerably advance research outcomes on various biological aspects of anoles (Alföldi et al. 2011).

Based on a single osteological character, i.e., the presence/absence of caudal transverse processes, Etheridge (1959) divided *Anolis* into major sections: 'alpha' anoles, which lack this morphological trait, and 'beta' anoles

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(Norops), which carry the trait. In a recent revision and in a previous report, based on molecular phylogenetics (Poe 2004; Nicholson et al. 2012), Norops was recognized as a monophyletic group that includes 169 species.

Karyological studies in anoles were very intensive in the 1970s and 1980s, especially studies that were conducted by Gorman and coworkers who defined karyotypes of numerous species (Gorman 1973). Since then, scientific interest has shifted to other fields of study, and the chromosomal evolution of anoles has not been further investigated. Indeed, only a few papers in this regard were published from 1980 to the present (Blake 1986; Brandley et al. 2006; Castiglia et al. 2010). However, anoles exhibit remarkable inter- and intraspecific variations in chromosome number and morphology as well as in the presence/absence of heteromorphic sex chromosomes. For this reason, investigating speciation of anoles as driven by chromosomal changes remains challenging. Moreover, chromosomal variability is expected to be useful in studying relationships among anole species and in identifying cryptic taxa.

Herein, we present an overview of the chromosomal evolution in Norops as a baseline for future studies of the karyotypic evolution of anoles. Previous phylogenetic reconstructions of anoles, which also included chromosomal data, applied them in the same matrix together with molecular and morphological characters (Poe 2004; Brandley et al. 2006). In contrast, our approach consists of inferring the pattern of chromosomal evolution in a phylogeny purely based on molecular data. A similar approach was recently used for an ancestral state reconstruction (ASR) of chromosomal characters in some other reptilian clades, such as the Phrynosomatidae (genus *Sceloporus*) (Leaché and Sites 2009), Gekkonidae (Gamble 2010), and Serpentes (Oguiura et al. 2009). For this study, we reviewed all available data on karyotypes of Norops and also included original data on one recently described species, *A. unilobatus* (Köhler and Vesely 2010). We used the phylogeny of Norops based on DNA sequence data to infer the main pattern of chromosomal evolution by means of an ASR. ASR analysis allowed us to test whether karyotypic diversity matched phylogenetic relationships among species and enabled us to track the direction of chromosomal change. We also tested a hypothesis of an ancient common origin of heteromorphic sex chromosomes vs. their independent origin in different species.

Methods

Materials

We examined published karyotypes of Norops species for the shape of macrochromosomes, the number of microchromosomes, the presence/absence of sex chromosomes, and their size and shape (Table 1). One additional species,

A. unilobatus, was newly studied and included in this survey. One male and three female *A. unilobatus* were collected in dry tropical forest located in the Reserva da Biosfera Sepultura, Chiapas State, Mexico. Field numbers cited in the text (RCMX) belong to Riccardo Castiglia. Animals were managed following all applicable institutional animal care guidelines, and all required state and federal permits were obtained.

Femurs were taken from individuals after an intraperitoneal injection of vinblastine sulfate, Velbe (Lilly, Italy) (1:1,000) for 1 h. Samples were then processed following procedures described in Castiglia et al. (2010). Pictures of metaphases were collected using a Photometrics Sensys 1600 digital camera (Roper Scientific Photometrics, Tucson, AZ, USA).

DNA was extracted from tissues preserved in 100% ethanol using the QIAamp tissue extraction kit (Qiagen, Hilden, Germany). The mitochondrial ND2 gene, five transfer (t) RNAs, the origin of light-strand replication, and a portion of the cytochrome oxidase subunit 1 (CO1) gene were amplified with primers designed by Macey et al. (1999) as follows: L4160 ND1 5'-CGATTCCGATATGACCARCT-3', H4980 ND2 5'-ATTTTTCGTAGTTGGGTTTGRTT-3', L4437 tRNA-Met 5'-AAGCTTTCGGGCCCATACC-3', and H5934a CO1 5'-AGRGTGCCAATGTCTTTGTGRTT-3'.

We used a Bayesian algorithm to construct a phylogeny based on mitochondrial DNA sequences (NDH2, 5 tRNAs, the origin of light-strand replication, and a portion of the CO1 gene) that included all 62 species for which mtDNA sequences were available in GenBank (mainly published by Nicholson et al. 2005), plus *A. unilobatus*. The final alignment consisted of 1,406 bp.

Data were partitioned by the codon position with a separate partition for each gene. The general time reversible substitution model with gamma-distributed rate heterogeneity and the proportion of invariant sites were used for each partition. For the Bayesian analysis, two independent Monte Carlo (MCMC) analyses were run with MrBayes 3.2 (Ronquist et al. 2012). We used 3×10^6 generations, four chains, and a burn-in of 10% of the generated tree. *Anolis cristatellus* (AF528724) and *A. distichus* (AF528725) were used as outgroups (Nicholson et al. 2005).

The consensus tree was pruned so as to eliminate species for which chromosomal data were unknown. The resulting tree, including 31 Norops species, was used for the ASR analysis that was performed with Mesquite 2.74 software (Maddison and Maddison 2011). A maximum likelihood (ML) reconstruction method was used to reconstruct the ancestral states. We used the one-rate MK1 model (Lewis 2001), the only model in Mesquite allowing multistate characters, and proportional likelihood (PL) values were used to determine which state was the most likely. Preliminarily,

Table 1 Karyological data of *Norops* species

Species	Karyotype	Macrochromosomes	Microchromosomes	Sex chromosomes	Source
<i>Anolis mestrei</i>	28	14 M/SM	14	-	Gorman and Atkins (1968)
<i>Anolis homolechis</i>	28	14 M/SM	14	-	Gorman and Atkins (1968)
<i>Anolis quadriocellifer</i>	28	14 M/SM	14	-	Gorman and Atkins (1968)
<i>Anolis rubribarbus</i>	28	14 M/SM	14	-	Gorman and Atkins (1968)
<i>Anolis sagrei</i>	29-30 ^a (type-B)	18 M/SM	Male 11 Female 12 ^a	X ₁ X ₂ Y - X ₁ X ₁ X ₂ X ₂ ^a	De Smet (1981)
<i>Anolis lineatopus</i>	30 (type-A)	14 M/SM	16	-	Gorman (1965, 1969)
<i>Anolis valencienni</i>	30 (type-A)	14 M/SM	16	-	Gorman and Atkins (1968)
<i>Anolis garmani</i>	30 (type-A)	14 M/SM	16	-	Gorman and Atkins (1968)
<i>Anolis lineatus</i>	30 (type-A)	14 M/SM	16	-	Gorman and Atkins (1968)
<i>Anolis nitens</i> = <i>Anolis chrysolepis</i>	30 (type-A)	14 M/SM	16	-	Gorman (1973)
<i>Anolis auratus</i>	30 (type-A)	14 M/SM	16	-	Gorman et al. (1967), De Smet (1981)
<i>Anolis aquaticus</i>	30 (type-A)	14 M/SM	16	-	Gorman unpublished (in Lieb 1981)
<i>Anolis conspersus</i>	30 (type-B)	14 M/SM	16	XY (seventh) (similar sizes)	Gorman and Atkins (1966)
<i>Anolis opalinus</i>	30 (type-B)	14 M/SM	16	XY (seventh) (similar sizes)	Gorman (1969)
<i>Anolis onca</i>	30 (type-B)	14 M/SM	16	XY (seventh) (similar sizes)	Gorman (1969)
<i>Anolis grahami</i>	30 ~ 37	14 M/SM-10 M/SM, 9A, ST	16-18 ^b	-	De Smet (1981)
<i>Anolis biporcatus</i>	29-30 (type-A)	Male: 13 M/SM, 1 A; female: 12 M/SM, 2 A	Male 15, female 16	X ₁ X ₂ Y - X ₁ X ₁ X ₂ X ₂	Gorman and Atkins (1966)
<i>Anolis nebulosus</i>	30 (type-C)	14 M/SM	16	XY (different sizes)	Lieb (1981), Castiglia et al. (2010)
<i>Anolis quercorum</i>	30 (type-C)	14 M/SM	16	XY (different sizes)	Lieb (1981)
<i>Anolis gracilipes</i>	36	8 M/SM; 12 A	16	-	Gorman (1973)
<i>Anolis liogaster</i>	38	6 M/SM; 16 A	16	XY (different sizes)	Lieb (1981)
<i>Anolis omiltemanus</i>	38	6 M/SM; 16 A	16	XY (different sizes)	Lieb (1981)
<i>Anolis gadovii</i>	36	8 M/SM; 12 A	16	XY (different sizes)	Lieb (1981)
<i>Anolis unilobatus</i>	40	20 SM/ST, 4 M	16	XY (different sizes)	Present data
<i>Anolis tropidolepis</i>	40	24 ^c	16	-	Gorman (1973)
<i>Anolis fuscoauratus</i>	40	24 ^c	16	-	Gorman (1973)
<i>Anolis humilis</i>	40	24 ^c	16	-	Gorman (1973)
<i>Anolis cupreus</i>	40	4 M/SM; 20 A	16	-	Gorman (1973)
<i>Anolis polylepis</i>	40	24 ^c	16	-	Gorman (1973)
<i>Anolis tropidonotus</i>	40	24 ^c	16	-	Gorman (1973)
<i>Anolis capito</i>	40	24 ^c	16	-	Gorman (1973)
<i>Anolis limifrons</i>	40	24 ^c	16	-	Gorman (1973)
<i>Anolis lionotus</i>	40	24 ^c	16	-	Gorman (1973)
<i>Anolis tropidogaster</i>	40	4 M/SM; 20 A	16	-	Gorman (1973)
<i>Anolis intermedius</i>	40	24 ^c	16	-	Gorman unpublished (in Lieb 1981)

Table 1 Karyological data of *Norops* species (Continued)

<i>Anolis dunnii</i>	40	4 M/SM; 20 A	16	XY (different sizes)	Lieb (1981)
<i>Anolis taylori</i>	40	4 M/SM; 20 A	16	XY (different sizes)	Lieb (1981)
<i>Anolis nebuloides</i>	42	ND	ND	XY (different sizes)	Lieb (1981)

M, metacentric; SM, submetacentric; A, acrocentric; ST, subtelocentric; ND, no distinction was made between macro- and microchromosomes. ^aInferred from the male karyotype; ^bthe presence of inter-individual variation of microchromosomes; ^cthe morphology of macrochromosomes was not studied.

we also performed an ASR by maximum parsimony (MP) (not shown) and altered the assumption of an equal cost for state changes building different step matrices in Mesquite. The results obtained with the ML and MP methods were highly congruent. Thus, we decided to show only the results obtained with ML because one problem with the parsimony ASR is the fact that supports at nodes are usually represented as unambiguous, often obscuring the actual degree of support for alternative character states (Cunningham 1999).

We coded different karyotypic characteristics as categorical characters (as for Serpentes in Oguiura et al. 2009). In fact, both the chromosomal number (2n) and the number of chromosomal arms (FN) may discretely change by several simultaneous chromosomal rearrangements. The presence/absence of sex chromosomes can be used as a straightforward categorical character.

The choice of an outgroup is a crucial step in the ASR. According to Nicholson et al. (2005), the sister group of *Norops* is the clade of anoles composed of species that belong to the 'bimaculatus', 'crystalinus', and 'distichus' groups. Unfortunately, there is uncertainty about the identity of the basal karyotype in this highly diversified clade (Olmo and Signorino 2005; Brandley et al. 2006). All of these species are characterized by a heteromorphic XY or an $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system. Therefore, the outgroup was considered uncertain with the exception of the constitution of sex chromosomes. We first performed an ASR by considering the karyotype as a single character with nine different states corresponding to different karyotypes.

We then separately considered some karyotype characteristics. First, we coded the different karyotypes in *Norops* as a two-state character: state 0, karyotypes with 7 ~ 9 pairs of macrochromosomes ($28 < 2n < 30$), and state 1, karyotypes with 12 pairs of macrochromosomes ($2n = 40$). The karyotype of *A. nebuloides* ($2n = 42$) was coded as state 1, even though it does not show a distinction between macro- and microchromosomes, because the diploid number is similar to that observed in species with a high number of macrochromosomes. The polymorphic karyotype in *A. grahami* ($30 < 2n < 37$) was coded as state 1 since polymorphic states are not allowed under the likelihood ASR in Mesquite. However, in the preliminary analysis with the parsimony ASR, *A. grahami* was coded as polymorphic.

The number of microchromosomes was then coded as a five-state character: state 0, 14 microchromosomes; state 1, 12 microchromosomes (female condition in *A. sagrei*); state 2, 16 microchromosomes; state 3, 18 microchromosomes; and state 4, the absence of microchromosomes (only observed in *A. nebuloides*). For micro- and macrochromosomes, the outgroup was coded as uncertain due to the observed variability in the number of chromosomes in the outgroup species (Olmo and Signorino 2005; Brandley et al. 2006).

Finally, we analyzed the emergence of heteromorphic sex chromosomes. We identified three different patterns of sex chromosomes in *Norops* (Table 1) and coded them in four states: state 0, the absence of heteromorphic sex chromosomes; state 1, heteromorphism at macrochromosome pair 7 (same size); state 2, multiple sex chromosomes $X_1X_1X_2X_2/X_1X_2Y$; and state 3, heteromorphism at one macrochromosome pair (different sizes). Character states 2 and 3 included species with slightly different heteromorphic sex chromosomes. Additional separation using more states was not feasible. We also coded 11 species as uncertain in view of the fact that the macrochromosomes in those species have not been studied in sufficient detail. The outgroup was coded as state 2 because of the presence of multiple sex chromosomes in the outgroup species.

Results and discussion

Results

The karyotype of *A. unilobatus*

Karyotypes were made from metaphase chromosomes of three females (RCMX70, RCMX71, and RCMX91) and one male (RCMX90) of *A. unilobatus*. All samples showed $2n = 40$ with 24 macrochromosomes and 6 microchromosomes, so they are designated because they markedly differed in size (Figure 1).

The macrochromosomes of *A. unilobatus* included two pairs of metacentric, three pairs of submetacentric, and seven pairs of subtelocentric/acrocentric chromosomes (Figure 1). The shape of the microchromosomes was not easily discernible, but some banded elements were observed in more elongated metaphases. In the male, the smallest metacentric chromosomes (pair 11) were heteromorphic in size and likely corresponded to sex chromosomes of the XY type.

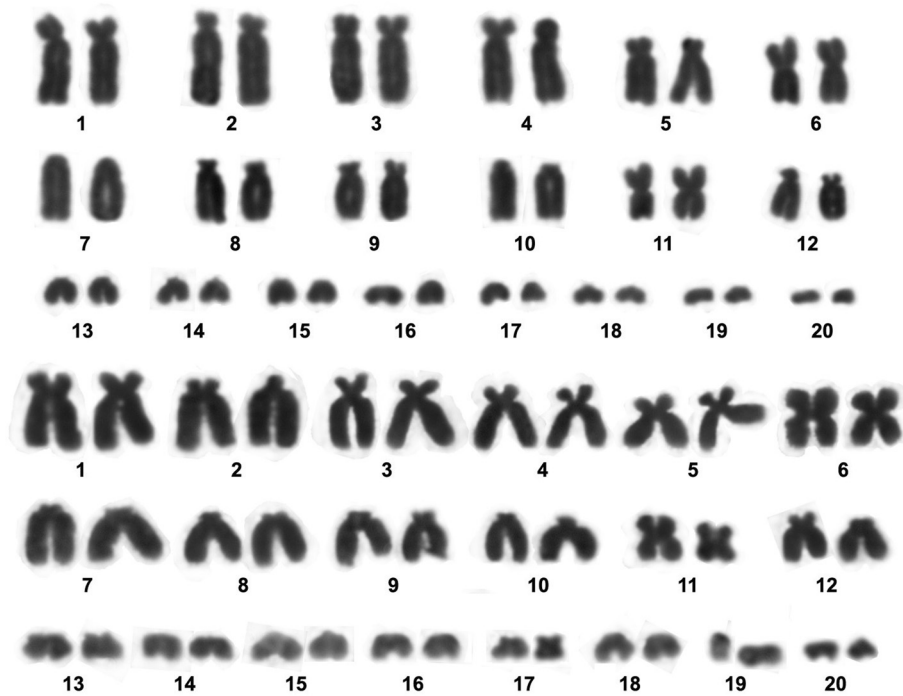


Figure 1 Karyotype of *Anolis unilobatus* ($2n = 40$). Female (above) and male (below). Note the heteromorphic pair 11 in the male karyotype.

A literature survey of Norops karyotypes

We found 11 different karyotypes, of which 9 were from species previously used in molecular studies. They are briefly described below. Ideograms are shown for the eight karyotypes for which the morphology of the macrochromosomes was studied in detail and molecular data were available (Figure 2). The karyological information for each species is summarized in Table 1:

- $2n = 28$. This karyotype is composed of seven pairs of biarmed macrochromosomes and seven pairs of microchromosomes. The morphology of the macrochromosomes seemed to be conserved throughout the group, even if we could not exclude small differences in the arm ratio. The chromosomes were all meta-submetacentric and unevenly decreasing in size. The shape of the microchromosomes was not classifiable in major published karyotypes. However, in *A. rubribarbus* and *A. mestrei*, one or two pairs of microchromosomes were distinctly metacentric (Gorman and Atkins 1968). The $2n = 28$ karyotype was found in four species: *A. mestrei*, *A. homolechis*, *A. quadriocellifer*, and *A. rubribarbus* (Table 1).
- $2n = 30$ (type-A). This karyotype is composed of seven pairs of biarmed macrochromosomes and eight pairs of microchromosomes. Examining published karyotypes (Gorman and Atkins 1968; Gorman 1969), we can say that the macrochromosomes were identical to those observed in the $2n = 28$ karyotype. The only difference between the $2n = 28$ and $2n = 30$ (type-A) karyotypes was the presence of one additional pair of microchromosomes. The $2n = 30$ (type-A) karyotype was found in seven species (Table 1).
- $2n = 30$ (type-B). Three species, *A. opalinus*, *A. conspersus*, and *A. onca*, had a karyotype identical to the $2n = 30$ (type-A), except for the smallest pair of macrochromosomes (number 7) which was in a heteromorphic condition (submetacentric and acrocentric homologues were of similar size), thus matching the XY sex chromosome system. Unfortunately, no females of these species were studied.
- $2n = 29-30$ (type-A). *Anolis biporcatus* had a karyotype that resembled the $2n = 30$ (type-A) but showed an $X_1X_1X_2X_2/X_1X_2Y$ multiple sex chromosome system ($2n = 29$ in males, $2n = 30$ in females; Gorman and Atkins 1966). X_1 is an acrocentric chromosome and X_2 is a microchromosome. The Y chromosome is metacentric and is similar in size to X_1 .
- $2n = 29-30$ (type-B). Males in *A. sagrei* showed a peculiar karyotype composed of 9 pairs of macrochromosomes and 11 pairs of

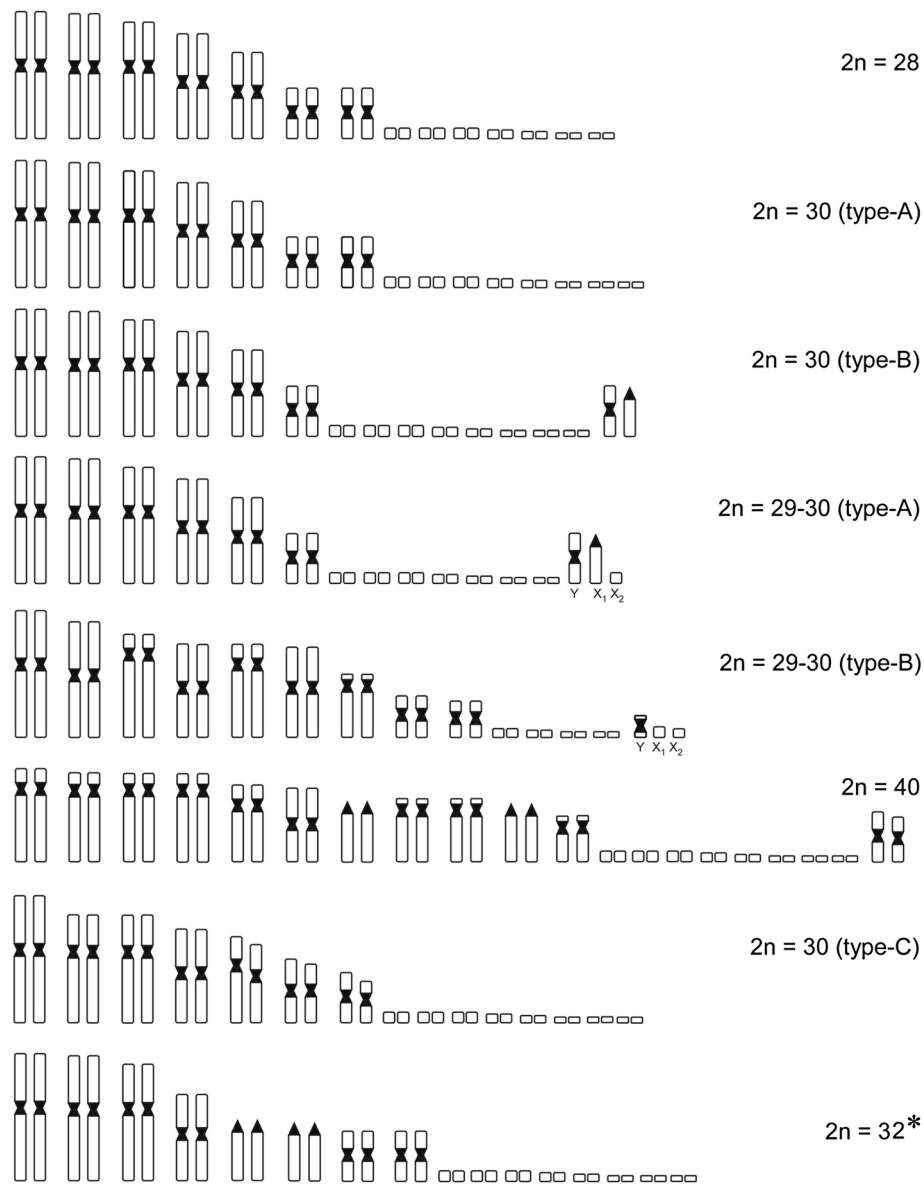


Figure 2 Ideograms of eight different karyotypes found in Norops males. Dark areas indicate centromeric regions. Asterisks denote that the karyotype with $2n = 32$ is one of the karyotypes found in *A. grahami*, characterized by a $2n$ variation. See text for explanation.

microchromosomes (De Smet 1981). The shape of the macrochromosomes, however, differed from both the $2n = 28$ and $2n = 30$ karyotypes. In addition, there were two X chromosomes and one Y chromosome (X_1X_2Y) in males, all of which were microchromosomes. The female karyotype has not been studied yet, but it is expected to be $2n = 30$ with four X chromosomes ($X_1X_1X_2X_2$).

- $2n = 40$. This karyotype has 12 pairs of macrochromosomes and 8 pairs of microchromosomes. Unfortunately, there were

insufficient data to discuss variations of macrochromosomal complements within the $2n = 40$ karyotype because the morphology of the macrochromosomes so far has been studied in only three species: *A. dumni*, *A. taylori* (in Lieb 1981), and *A. unilobatus* (this work). The first two species shared a very similar $2n = 40$ karyotype with one large metacentric autosomal pair. However, they have not been studied by molecular methods, and therefore, it is not possible to place them in a phylogenetic context. In addition, Gorman (1973)

reported a similar, although haploid, karyotype for *A. tropidogaster*. Heteromorphic X and Y (different-sized) chromosomes were described for *A. dunni*, *A. taylori*, and *A. unilobatus*.

- *2n = 30 (type-C)*. *Anolis nebulosus* showed one more $2n = 30$ karyotype, but the chromosome arm ratios in the macrochromosomal complement differed with respect to the abovementioned 'ordinary' $2n = 30$ (type-A) and (type-B). In some specimens, there were at least two pairs of chromosomes in a heterozygous condition of an unknown nature (Castiglia et al. 2010). A similar karyotype may be that found in *A. quercorum*. These two karyotypes share an apparently similar macrochromosomal constitution, and both have XY chromosomes that are similar in shape and size (Lieb 1981; Castiglia et al. 2010).
- *2n = 30 ~ 37*. *Anolis grahami* was characterized by extensive chromosomal polymorphism with chromosome numbers ($2n$) ranging 30 ~ 37 due to multiple fission events within the macrochromosomal complement as well as by eight or nine pairs of microchromosomes (Blake 1986). Only the karyotype with $2n = 32$ is shown as an ideogram in Figure 2.
- *2n = 42 (not figured)*. *Anolis nebuloides* showed a very distinctive karyotype with the highest diploid number among Norops (Lieb 1981). Except for the four large meta-submetacentric chromosomes, the autosomes had an uncertain morphology. It is worth noting that this karyotype was unique among Norops because chromosomes could not be divided into macro- and microchromosomes. Moreover, the heteromorphic XY chromosomes were of very different sizes. The X was a large metacentric chromosome, and the Y was a medium-sized subtelocentric one.
- *2n = 36 (not figured)*. *Anolis gracilipes* had a $2n = 36$ karyotype with four pairs of metacentric and six pairs of acrocentric macrochromosomes and 16 pairs of microchromosomes (reported by Gorman (1973) as 'Gorman unpublished'). This karyotype is very similar (or identical) to that observed in *A. gadovii* (Lieb 1981). In that species, Lieb (1981) observed heteromorphic sex chromosomes as two metacentrics of different sizes. Molecular data are lacking for these $2n = 36$ species, so it is impossible to properly place them in a phylogenetic context.
- *2n = 38 (not figured)*. *Anolis liogaster* and *A. omiltemanus* (Lieb 1981) had a karyotype similar to that found in *A. taylori* ($2n = 40$) but with two pairs of large biarmed chromosomes instead of one. No molecular data are available for these species.

Phylogenetic tree

The phylogenetic tree based on mtDNA genes including all Norops species was fully congruent with previously published phylogenies (Nicholson et al. 2012). The phylogenetic analysis placed *A. unilobatus* (GenBank accession number KF796613) close to *A. sericeus* and *A. isthmicus* (with 100% Bayesian posterior probabilities). The latter two species were not included in the tree (Figure 3) because their karyotypes were not yet available. The affinities found with the phylogenetic analysis concurred with those using morphological data. In fact, *A. unilobatus* was only recently distinguished from *A. sericeus* and currently belongs to the same 'species complex' sensu Köhler and Vesely (2010). The resulting topology, once pruned by eliminating species the karyotypes of which were not available, is depicted in Figure 3, 4, 5, and 6. Some basal nodes were numbered from 1 (the ancestor of all Norops) to 5 to facilitate the description of ancestral states.

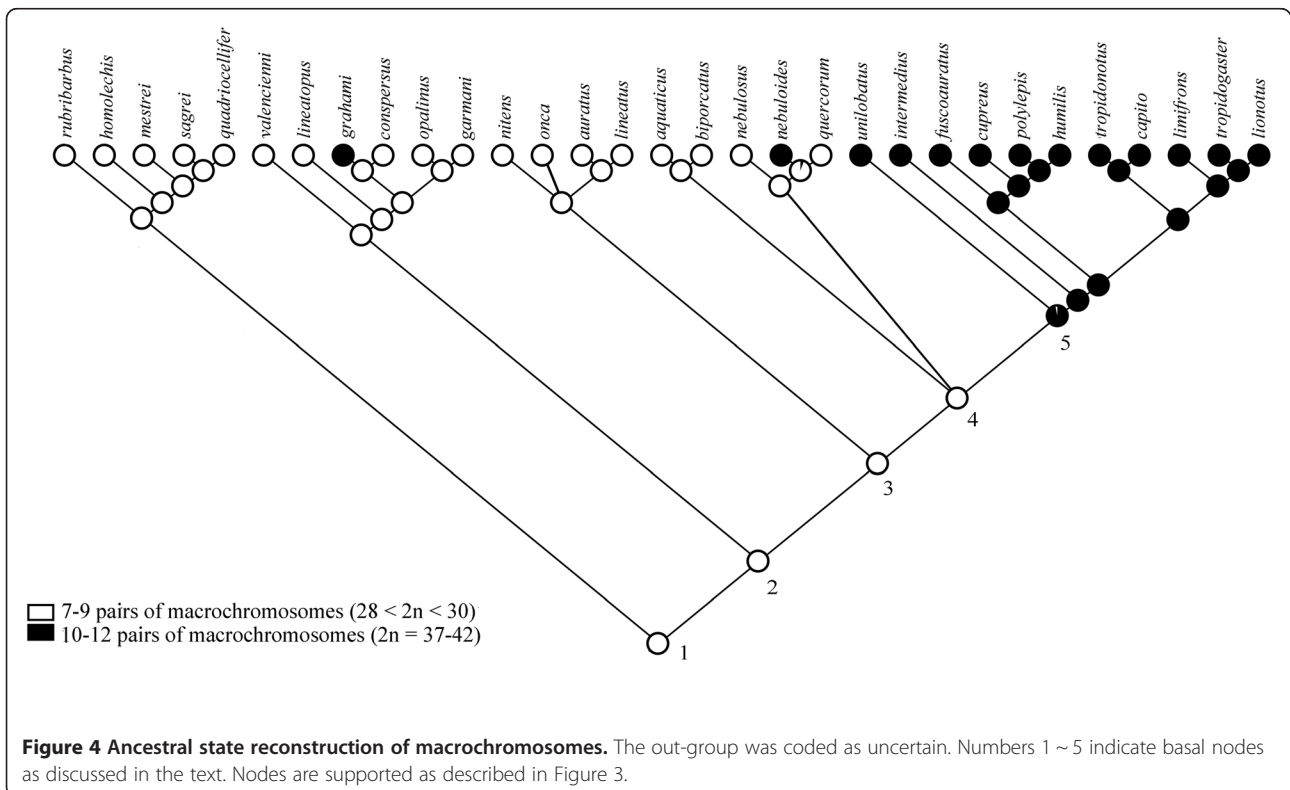
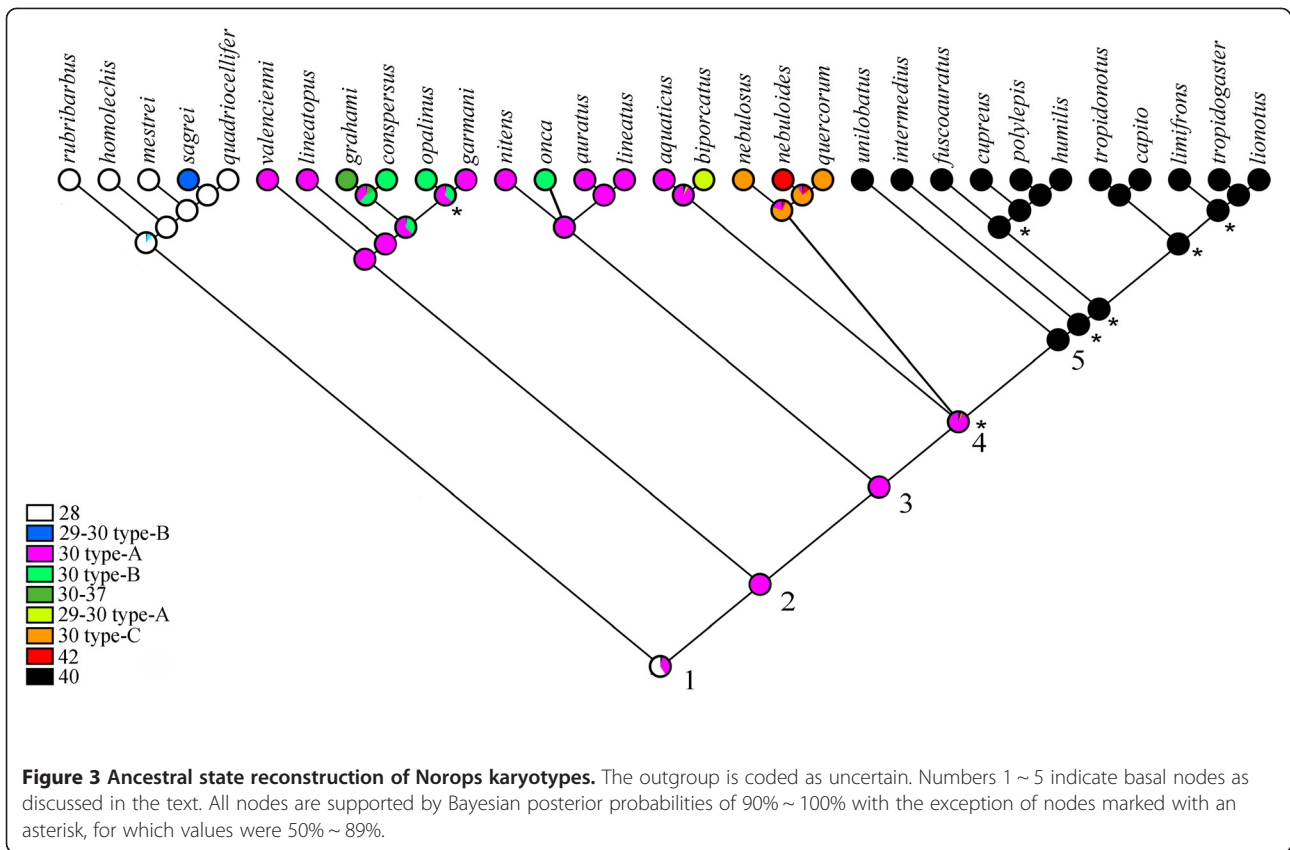
Ancestral state reconstruction

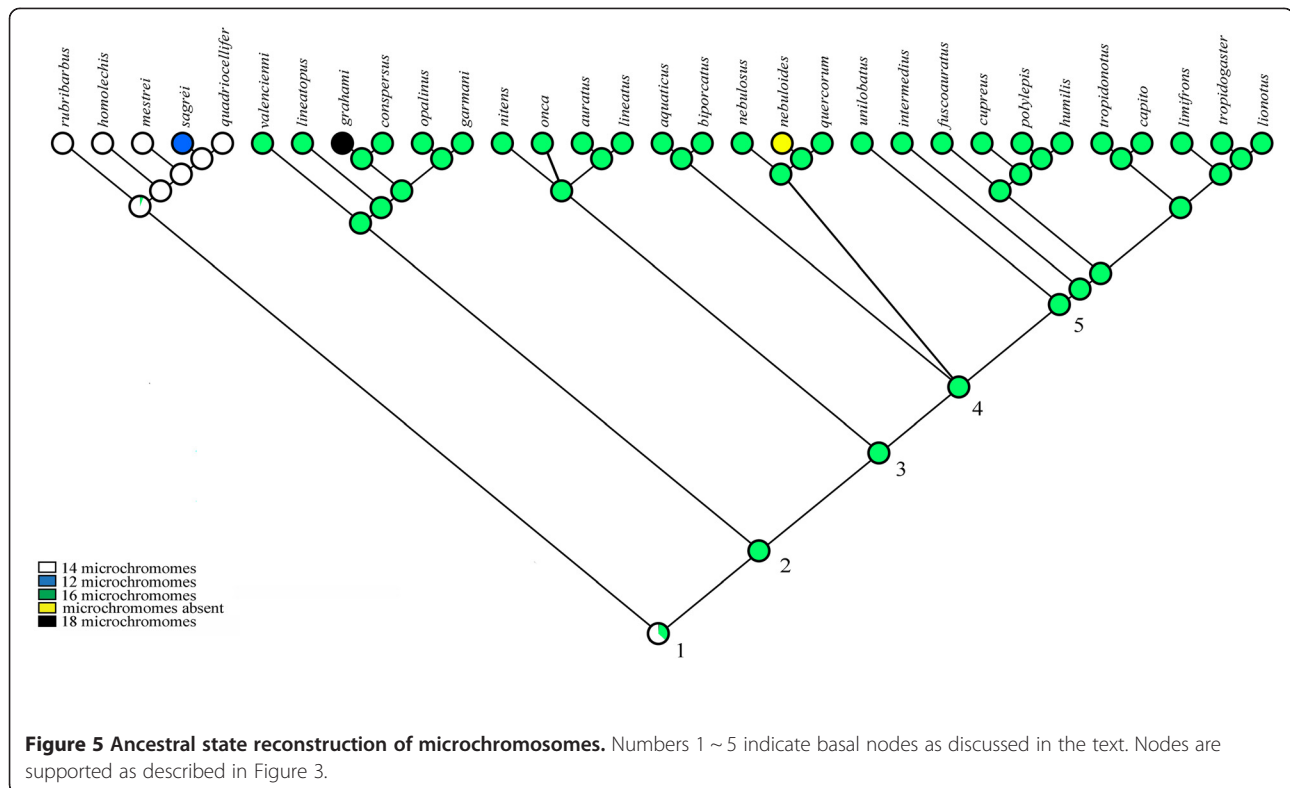
The ASR of anole karyotypes under ML is shown in Figure 3. It shows ambiguity at node 1, corresponding to the basal karyotype of all Norops, either $2n = 28$ (PL = 0.59) or $2n = 30$ (type-A; PL = 0.36) (Figure 3). The $2n = 40$ karyotype was more derived and characterized a monophyletic group of 11 species (node 5).

The ASR for macrochromosomes (Figure 4) showed a monophyletic group including all species characterized by a high chromosome number ($2n = 40$; node 5). The only exceptions were *A. nebuloides* ($2n = 42$) and *A. grahami* (polymorphic), in which high diploid numbers occurred independently.

For microchromosomes, the ASR retrieved a tree with uncertainty in the ancestral state of all Norops (node 1; Figure 5). Thus, states 0 (14 microchromosomes; PL = 0.63) and 2 (16 microchromosomes; PL = 0.35) were the most likely to have been retrieved.

In the ASR for the origin of heteromorphic sex chromosomes (Figure 6), the ancestral node for all Norops was coded without heteromorphic sex chromosomes (PL = 0.99). Six independent occurrences of heteromorphic sex chromosomes were observed. It is worth noting that the same types of sex chromosomes, the heteromorphism of macrochromosome 7 and multiple sex chromosomes, resulted from independent origins in groups of three (*A. conspersus*, *A. opalinus*, and *A. onca*) and two species (*A. sagrei* and *A. biporcatus*), respectively. Heteromorphic sex chromosomes of different sizes resulted at the origin of the monophyletic group composed of *A. nebuloides*, *A. nebulosus*, and *A. quercorum*. It is not clear when these heteromorphic chromosomes arose. Their formation could have occurred at node 4 (PL = 0.27) but was more likely to have occurred in the





ancestor of the three species (PL = 0.84). Node 5, basal to $2n = 40$ species, was uncertain under ML. Under MP, this node was coded equally as state 0 (absence of heteromorphic XY) or 1 (heteromorphic XY) (not shown).

Discussion

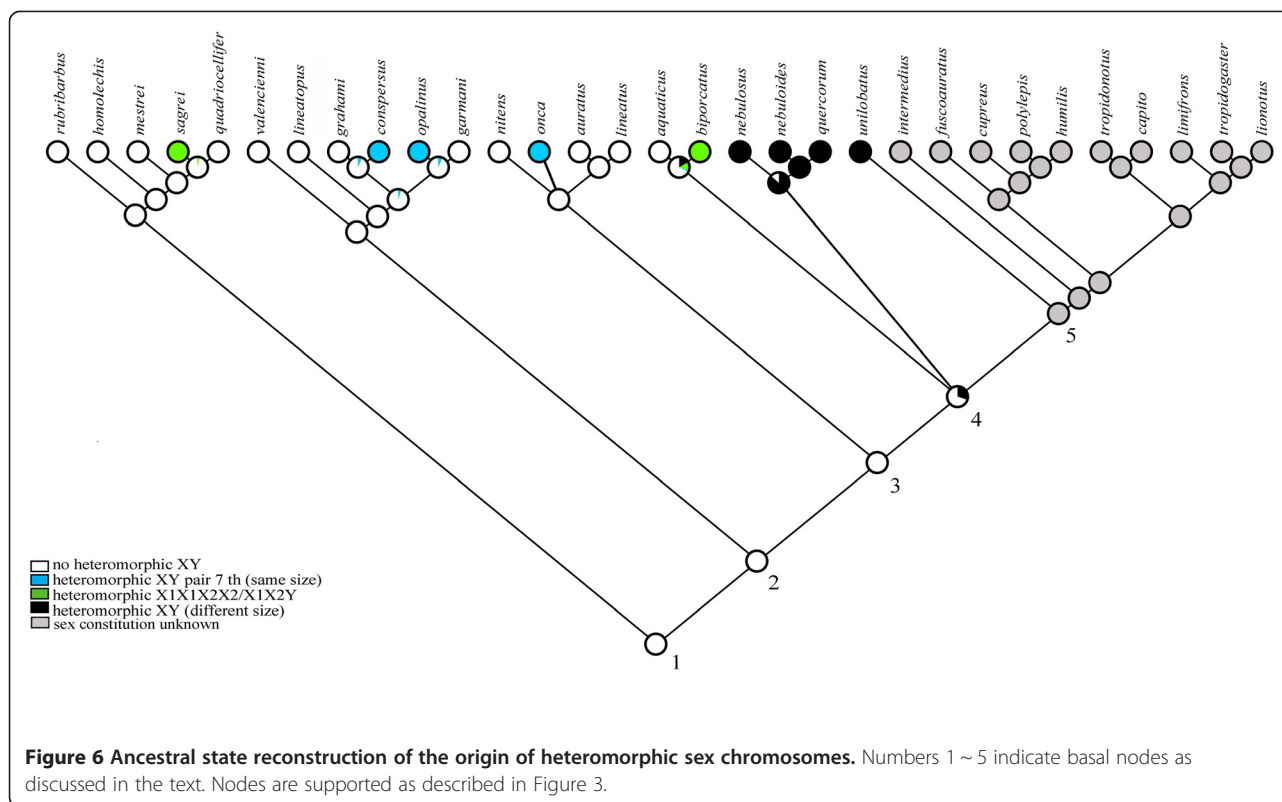
Karyotype evolution in *Norops*

We observed an overall good correspondence of some characteristics of karyotype and species relationships. The state change in the number of macrochromosomes and microchromosomes, which altered the diploid number, marked monophyletic groups of species. This correspondence can most likely be 'predictive' of the phylogenetic position of other species for which molecular data are not available. For example, two of the species with $2n = 40$ studied by Lieb (1981), *A. dunni* and *A. taylori*, can possibly be placed within the monophyletic group that was derived from the ancestor at node 5, characterized by $2n = 40$.

To summarize the results of the ancestral state analysis, the first step during chromosomal evolution of *Norops* consisted of the emergence or disappearance of a pair of microchromosomes (from node 1 to 2). These events were almost equally probable under the ML reconstruction.

The second main event took place between nodes 4 and 5, where an increase in the number of macrochromosomes from 7 ~ 9 to 12 pairs took place,

accomplished by a change in chromosomal numbers from $2n = 30$ to $2n = 40$. The increase in diploid numbers may be explained by a series of five centromeric fissions that occurred in five pairs of metacentric macrochromosomes in the $2n = 30$ chromosome complement, which produced chromosomes of the $2n = 40$ karyotype. This is supported by the comparison of the shape of the macrochromosomes in the two karyotypic forms. Indeed, macrochromosomes are meta-submetacentric in karyotypes with a low diploid number ($2n = 28 \sim 30$), while in karyotypes with a high diploid number ($2n = 40 \sim 42$), macrochromosomes are almost all acro-submetacentric (Table 1). Moreover, some other differences between the $2n = 28 \sim 30$ and $2n = 40$ karyotypes are evident. In fact, the presently studied *A. unilobatus* had several submetacentric chromosomes probably obtained by pericentric inversions that followed chromosomal fissions. Interestingly, one species, *A. grahmi*, showed an extensive chromosome number polymorphism ($2n = 30 \sim 37$), probably due to multiple Robertsonian fission events in metacentric macrochromosomes (Blake 1986). The high chromosomal number found in *A. nebuloides* ($2n = 42$) also seems to be due to fission events (Figure 4). In fact, the common ancestor of *A. nebuloides* and *A. quercorum* had a PL = 0.94 for a low number of macrochromosomes. The present observations concur with the hypothesis that chromosomal fission is a characteristic trait of *Norops*. Other similar



studies should be planned to understand whether chromosomal fission is common throughout the genus *Anolis*. At the moment, it was suggested to occur in at least one more species of anole, *Anolis monticola* (Webster et al. 1972).

According to Nicholson et al. (2005), mainland species of *Norops* originated from a West Indian ancestor. In our tree, mainland species all arose from node 3. Colonization of the mainland almost certainly was carried out by a $2n = 30$ (type-A) ancestor (PL = 0.99 at node 3, in Figure 3). This colonization was followed by an important change in the chromosomal number and a high level of karyotypic diversification. Moreover, karyotypic diversity within the mainland $2n = 40$ karyotype could possibly have been underestimated because, for many species, macrochromosomes have not yet been studied. One can speculate that chromosomal evolution during adaptive radiation can be driven by adaptation to new environments (the ‘canalization model’ of Bickham and Baker (1979)). However, no empirical data are currently available to support this issue, and more data on island *Norops* species are required to properly assess the rate of karyotypic diversification between continental and island anoles.

Sex chromosome evolution

Sex chromosome heteromorphism was described in nine species of *Norops* included in this analysis: *A.*

biporcatus, *A. conspersus*, *A. nebuloides*, *A. nebulosus*, *A. onca*, *A. opalinus*, *A. quercorum*, *A. sagrei*, and *A. unilobatus*. Furthermore, in *A. carolinensis*, sex microchromosomes (XY) were identified by means of *in situ* hybridization as two identical microchromosomes (Alföldi et al. 2011). Thus, the absence of evident heteromorphic sex chromosomes does not exclude the possibility of genotypic sex determination.

Our survey showed that there are basically three types of heteromorphic sex chromosomes in the *Norops* group. Thus, in males of *A. conspersus*, *A. onca*, and *A. opalinus*, the smallest pair of macrochromosomes (number 7) is heteromorphic. In this pair, one chromosome is metacentric, while its homologue is acrocentric and of similar size. Interestingly, *A. opalinus* and *A. conspersus* are phylogenetically close, as they fall in the same monophyletic group with *A. grahami* and *A. garmani*. However, the ASR indicated likely independent origins of sex chromosome heteromorphism in *A. opalinus* and *A. conspersus* (Figure 6). Furthermore, according to Pokorná and Kratochvíl (2009), sex-determining mechanisms in Squamata evolved by one-way transition, i.e., from the absence to the emergence of sex chromosomes; therefore, heteromorphic sex chromosomes must have been absent in the common ancestor of the four species of the clade. This implies a motivating hypothesis that sex chromosomes arose independently in *A. opalinus* and *A. conspersus*. Considering *A. onca*, which is not

phylogenetically related to the two aforementioned species, the analogous heteromorphism of chromosome pair 7 seems to be another independent event.

The second type of sex chromosomes with X_1X_2Y males and $X_1X_1X_2X_2$ females was described in *A. biporcatus* (Gorman and Atkins 1966) and *A. sagrei* (De Smet 1981). Yet, the sex chromosomes in these two species obviously differ because one of the X chromosomes and the Y chromosome are medium-sized in *A. biporcatus*, while in *A. sagrei*, all sex chromosomes are shaped as microchromosomes (Gorman and Atkins 1966; De Smet 1981). Moreover, *A. sagrei* and *A. biporcatus* are unrelated species (Figure 6), and their sex chromosomes might thus have occurred independently. The presence of multiple sex chromosomes is usually due to autosome-sex chromosome translocations (Ashley 2002). The occurrence of such rearrangements has not been studied in *Anolis*, but our review indicates that the genus may be prone to this kind of rearrangement.

The third sex chromosome constitution was observed in *A. nebulosus*, *A. quercorum*, *A. nebuloides*, and *A. unilobatus*, in which heteromorphic X and Y chromosomes are of different sizes (Lieb 1981; Castiglia et al. 2010). This condition might have easily occurred in the common ancestor of *A. nebulosus*, *A. quercorum*, and *A. nebuloides* (Figure 6). The ancestor at node 5 had an uncertain state because many species have not been studied in detail. Other species of *Norops* with $2n = 40$, albeit not studied phylogenetically, showed sex chromosomes of different sizes (Lieb 1981). Therefore, we cannot exclude that all descendants from node 5 have this type of heteromorphic sex chromosomes. Additional chromosomal studies of $2n = 40$ species are needed to elucidate this issue.

Considering the 31 species of *Norops*, the chromosome morphology and molecular DNA data of which are known, a minimum of six independently originated sex chromosome constitutions are possible. It looks like the clade is prone to sex chromosome diversification. Moreover, the origins of five of these heteromorphic sex chromosome variants seem to be recent as they appear at the tip nodes in the ancestral character reconstruction. The potential predisposition to heteromorphism in sex chromosomes revealed in *Norops* is not unique among reptiles. Actually, many clades at a family level are apparently prone to neo-sex chromosome formation (Ezaz et al. 2009; Gamble 2010), whereas anoles provide an opportunity to study this phenomenon at a low taxonomic scale, thereby facilitating comparative molecular cytogenetic studies of sex chromosome patterns and elucidation of the possible role of chromosomal differentiation in speciation.

Conclusions

The ASR analysis of chromosomal characters in *Norops* allowed us to observe an overall good correspondence of

some characteristics of karyotypes (number of macro- and microchromosomes) and species relationships. However, detailed comparative analysis of the morphology of macrochromosomes is still lacking, and here, we were the first to tentatively categorize karyotypes according to macrochromosome morphology and diploid number. Moreover, the clade seems prone to sex chromosome diversification, and the origins of five heteromorphic sex chromosome variants seem to be recent, as they appear at the tip nodes in the ancestral character reconstruction. The observed karyotypic diversification in *Norops* provides an opportunity to test the chromosomal speciation models and is expected to be useful in studying relationships among anole species and in identifying cryptic taxa.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RC conceived the research and performed the analysis. RC, AMRB, and AM collected the specimens. RC, AMRB, and EG performed the laboratory work. RC and EG wrote the first version of the manuscript; OFV and AMRB critically read the manuscript and made modifications. All authors read and approved the final manuscript.

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