Extensive Sex Chromosome Polymorphism of *Microtus thomasi*/*Microtus atticus* Species Complex Associated with Cryptic Chromosomal Rearrangements and Independent Accumulation of Heterochromatin

Michail T. Rovatsos^a Juan A. Marchal^b Ismael Romero-Fernández^b
Maria Arroyo^b Eva B. Athanasopoulou^c Antonio Sánchez^b

^aDepartment of Ecology, Faculty of Science, Charles University in Prague, Praha, Czech Republic; ^bDepartamento de Biología Experimental, Facultad de Ciencias Experimentales, Universidad de Jaén, Campus Las Lagunillas, Jaén, Spain; ^cSection of Animal Biology, Department of Biology, University of Patras, Rion, Greece

Keywords

Chromosome painting · *Microtus* · Polymorphism · Sex chromosome heterochromatin

Abstract

The sibling species *Microtus thomasi* and *M. atticus* represent probably the highest karyotypic diversity within the genus *Microtus* and are an interesting model for chromosomal evolution studies. In addition to variation in autosomes, they show a high intraspecific variation in the size and morphology of both sex chromosomes. We analyzed individuals with different sex chromosome constitutions using 3 painting probes, 2 from Y chromosome variants and 1 from the small arm of the submetacentric X chromosome. Our comparative painting approach uncovered 12 variants of Y and 14 variants of X chromosomes, which demonstrates that the polymorphism of sex chromosomes is substantially larger than previously reported. We suggest that 2 main processes are responsible for this sex chromosome polymorphism: change of morphology from acrocentric to submetacentric or metacentric chromosomes and increase in size due to accumulation of repetitive DNA sequences, generating heterochro-

matic blocks. Strong genetic drift in small and fragmented populations of these 2 species could be related to the origin and maintenance of the large polymorphism of sex chromosomes. We proposed that a similar polymorphism variation combined with random drift fixing the biggest sex chromosomes could have occurred in the origin of some of the actual *Microtus* species with giant sex chromosomes.

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The rodent species of the subfamily Arvicolinae constitute one of the most variable mammalian groups characterized by high karyotypic variation, especially in the genus *Microtus* that is associated with rapid speciation events [Conroy and Cook, 1999; Musser and Carleton, 2005]. This genus includes 65 extant species currently classified into 14 subgenera [Musser and Carleton, 2005; Golenishchev and Malikov, 2006] and presents the most remarkable rate of karyotypic variability and evolution among rodents [Maruyama and Imai, 1981; Lemskaya et al., 2010]. The chromosomal variation ranges from $2n = 18$ in *Microtus oregoni* to $2n = 64$ in *M. longicaudus* [Modi, 1987a; Volobouev et al., 2006]. In relation to the karyo-

Table 1. Additional individuals of *M. thomasi* analyzed with indication of the locality of trapping, the specimen code, the chromosomal race, the chromosome number (2n), the fundamental number (FN), sex, and the variants of sex chromosomes

Locality	Specimen	Chromosomal race ^a	2n	FN	Sex	X-chr	Y-chr
Mpalntouma, Greece	D20	hybrid “thomasi” × “Rb subalpine”	43	44	female	X _{st1} -X ₀	
Mpalntouma, Greece	7889	hybrid “Kastrosikia” × “Rb subalpine”	41	44	male	X _{st1}	Y ₁
Kali, Greece	R27	“Kali”	40	42	female	X ₃ -X ₃	
Aridea, Greece	R152	“Aridea”	38	42	male	X ₄	Y ₂
Veria, Greece	R252	“subalpine”	42	42	male	X ₄	Y ₁
Ducat, Albania	R160	“subalpine”	42	42	female	X ₄ -X ₄	

Data about the rest of individual analyzed is shown in Rovatsos et al. [2014]. ^aAccording to Rovatsos and Giagia-Athanasopoulou [2012].

typic variation, the sex chromosomes are also highly variable in morphology, ranging from an acrocentric to a metacentric shape, and in size due to accumulation of constitutive heterochromatin in both X and Y chromosomes [Modi, 1987a, b; Burgos et al., 1988a, b; Mitsainas et al., 2009; Marchal et al., 2003, 2004a; Acosta et al., 2011]. In some cases, the heterochromatin accumulation led to a notable increase in size of both sex chromosomes, which represent a significant part of the genome and are referred to as ‘giant’ sex chromosomes. Such giant sex chromosomes occur in the species *M. agrestis*, *M. cabreræ*, *M. chrotorrhinus*, *M. rossiaemeridionalis*, and *M. transcaspicus* [Modi, 1987a, b; Nanda et al., 1988; Kalscheuer et al., 1996].

The sibling voles *M. thomasi* and *M. atticus* are interesting models for studies on chromosomal evolution as they demonstrate probably the highest rate of karyotypic diversity among the *Microtus* species. Nine chromosomal races in *M. thomasi* and 2 in *M. atticus* were described [Giagia-Athanasopoulou and Stamatopoulos, 1997; Mitsainas et al., 2009; Rovatsos et al., 2011a, 2014; Rovatsos and Giagia-Athanasopoulou, 2012]. The karyotype of the chromosomal race assigned as *M. thomasi* ‘*thomasi*’ with all chromosomes being acrocentric (2n = 44, FN = 44) was considered to represent a likely ancestral karyotype for this species complex [Giagia-Athanasopoulou and Stamatopoulos, 1997]. The variation in chromosome number among chromosomal races can be probably attributed to Robertsonian and tandem fusions [Giagia-Athanasopoulou et al., 1995; Giagia and Ondrias, 1973; Giagia, 1985; Rovatsos et al., 2011a, 2014; Rovatsos and Giagia-Athanasopoulou, 2012].

In addition to variation in autosomes, *M. thomasi* and *M. atticus* show a high intraspecific variation in size and morphology of both sex chromosomes. C-banding tech-

nique demonstrated a significant variation in the amount of pericentromeric heterochromatin and in the size and position of heterochromatic bands or blocks. Nine different X chromosomes have been described with either acrocentric (X₀-X₅) or subtelocentric (X_{st0}-X_{st2}) morphology and variable amounts of heterochromatin [Acosta et al., 2009; Mitsainas et al., 2009; Rovatsos et al., 2011a, 2014]. Similarly, 8 Y chromosome variants exist, either acrocentric (Y₀-Y₄) or metacentric (Y_m and 2 Y_{m1}); all of them are almost completely heterochromatic and also highly variable in size [Acosta et al., 2009; Mitsainas et al., 2009; Rovatsos et al., 2011a; Rovatsos and Giagia-Athanasopoulou, 2012].

Several studies have shed light on the molecular composition and the evolution of sex chromosomes in arvicolids. These investigations have characterized different families of complex repeated DNAs, satellite DNAs, retroelements, and pseudogenes as components of the sex chromosome heterochromatin [Marchal et al., 2003, 2004b, 2006, 2008; Acosta et al., 2008, 2009; Rovatsos et al., 2014]. Many of these sequences are significantly accumulated in the heterochromatic blocks of the giant sex chromosomes and display patterns of distribution, organization, and amplification that are highly variable within species. Also, comparative painting with the giant X and Y chromosomes of *M. cabreræ* and *M. agrestis* revealed the rapid and independent evolution of the sequences forming the heterochromatic blocks and the conservation of the euchromatic X content [Marchal et al., 2004a; Sitnikova et al., 2007].

In particular, 4 repetitive DNA sequences located on the pericentromeric chromosomal regions have been characterized in *M. thomasi*: Msat-160 [Acosta et al., 2010], Mth-Alu900 [Acosta et al., 2009], Mth-Alu2.2 [Rovatsos et al., 2014], and interstitial telomeric sequences

Fig. 1. Examples of painting on chromosomes of different individuals with the painting probes Y_0 (a–c), Y_{m1} (d–f), and X_{st1p} (g–i). Notation of sex chromosomes includes the classification of C-banding $X_\#$ or $Y_\#$ according to Rovatsos et al. [2014], and in brackets the variant number of Figures 2 and 3 identified by painting are shown.

[Rovatsos et al., 2011b]. In sex chromosomes, the highly repetitive satellite sequence Mth-Alu900 was located in the pericentromeric heterochromatin but not in the proximal or interstitial C-bands of the acrocentric X chromosome in this species. However, this satellite DNA is located also in the submetacentric X_{st1} chromosome in the adjacent heterochromatic blocks. Only the large, completely heterochromatic Y_2 chromosome variant presents this satellite DNA in its pericentromeric heterochromatin [Acosta et al., 2009]. In addition, the Mth-Alu2.2 is located in the heterochromatic block of X_{st1} and in the ter-

minal or interstitial small bands in the other X or Y chromosome variants [Rovatsos et al., 2014]. The telomeric-like repetitive sequences are located in the heterochromatin of 2 X chromosome variants analyzed by Rovatsos et al. [2011b] as well.

The sex chromosome system in *M. thomasi*/*M. atticus* has been studied extensively only by C-banding. The location of Mth-Alu900 and Mth-Alu2.2 repetitive sequences in sex chromosomes suggested that the polymorphism detected by classical cytogenetic techniques could be even more pronounced at the molecular level [Acosta

Fig. 2. Representative Y chromosome variants showing the painting pattern with the painting probes Y_0 , Y_{m1} , and X_{st1p} , and the C-banding. Notation of sex chromosomes includes the order number according to painting identification (pY#) and the classification with C-banding $Y_{\#}$ according to Rovatsos et al. [2014].

et al., 2009; Rovatsos et al., 2014]. A detailed analysis by molecular cytogenetics was required in order to better characterize this polymorphism. Here, we present the analysis of most of the sex chromosome variants described in *M. thomasi*/*M. atticus* based on comparative chromosome painting with 3 probes derived from 2 Y chromosome variants (Y_0 and Y_{m1}) and the small arm of the submetacentric X_{st1} chromosome.

Material and Methods

Individuals Analyzed and Chromosome Preparations

For this study, we used chromosome preparations from 26 individuals of different chromosomal races of 2 species from the subgenus *Terricola* of the genus *Microtus*: *M. thomasi* and *M. atticus* belonging to different chromosomal races from Greece, Albania, and Montenegro. The data about most of the specimens utilized in

this work is shown in Rovatsos et al. [2014]. In addition, we included additional 6 specimens of different chromosomal races of the species *M. thomasi* (Table 1).

Chromosomes were prepared from bone marrow using a modified version of the Hsu and Patton [1969] method. The karyotypes of the individuals have different sex chromosome constitutions and were previously analyzed by C-banding [Mitsainas et al., 2009; Rovatsos et al., 2011a].

Chromosome Painting

Three painting probes were prepared from the whole small acrocentric Y_0 chromosome (*M. thomasi* specimen R32), the biggest metacentric Y_{m1} chromosome (*M. thomasi* specimen R94), and the small heterochromatic p arm of the submetacentric X_{st1} chromosome (specimen R32). For probe preparation and painting, we followed the protocol previously described by our group [Marchal et al., 2004a]. Briefly, microdissection was performed using an inverted microscope (Zeiss Axiovert S100) with a sterile glass needle attached to a mechanical micromanipulator (Eppendorf Transfer-Man NK2). A total of 10–15 microdissected chromosomes were

Fig. 3. Representative X chromosome variants showing the painting pattern with the painting probes Y₀, Y_{m1}, and X_{st1p}, and the C-banding. Notation of sex chromosomes includes the order number according to painting identification (pX#) and the classification with C-banding X_# according to Rovatsos et al. [2014].

used in an initial DOP-PCR amplification, and a second labelling DOP-PCR using Spectrum-Orange dUTP (Abbott) was performed to obtain the painting probe. Approximately 200 ng of probe were used for every FISH analysis. The images were captured using a fluorescence microscope (Olympus BX51) equipped with a CCD camera (Olympus DP70).

Results

The 3 painting probes (Y₀, Y_{m1}, and X_{st1p}) hybridized as expected to the entire corresponding chromosome or chromosome region from which they were prepared (Fig. 1a, d, g), but also to various parts of the Y chromosome in different Y variants, most of the X chromosome variants, and depending on each particular karyotype, a

variable number of signals was observed in autosomes either in pericentromeric heterochromatin and in interstitial euchromatic bands (Fig. 1–3). Based on the size, C-banding, and the current painting results, we were able to identify 12 Y chromosome variants (5 small acrocentric and 4 big acrocentric and 3 metacentric chromosomes of variable size) (Fig. 2) and 14 X chromosome variants (12 acrocentrics with variable size and 2 submetacentric chromosomes) (Fig. 3). Below we refer to the different variants of the X and Y chromosomes identified by painting as pX# and pY#, where # is the order number of each chromosome in Figures 2 and 3, while we refer to the corresponding variant described by C-banding with the previously used notation (X chromosomes: X₀–X₅, X_{st0}–X_{st2}; Y chromosomes: Y₀–Y₄, Y_m and Y_{m1}).

Painting with the Y₀ Probe

As expected, this probe hybridized to the complete Y₀ chromosome (pY1) (Fig. 1a) but gave different signals on the other Y chromosome variants (Fig. 1a–c, Fig. 2). In the 5 small acrocentric Y chromosomes (pY1–pY5), the staining occupies the entire chromosome or 1 or 2 terminal bands. In the 4 large acrocentric Y chromosomes (pY6–pY9), the signal was located to 2 main bands, 1 of them always in the telomeric region of the long arm, and the other close to the centromere in the long arm or in the pericentromeric region of the small (pY6 and pY7) or as an interstitial band in the long arm in the big chromosomes (pY8 and pY9). In the metacentric Y chromosomes this probe stained the entire small Y metacentric chromosome (pY10), while in the 2 big metacentrics (pY11 and pY12) only 2 bands were stained, 1 in the telomeric region of the small arm and another in the pericentromeric region (Fig. 2).

The Y₀ probe also hybridized to most of the X chromosome variants with the exception of the small one (pX1) (Fig. 1a–c, Fig. 3). In the acrocentric X chromosomes (pX2–pX12), the signal was diverse in its intensity in the entire euchromatic region, and in some of them the probe also hybridized to some heterochromatic bands and pericentromeric regions (Fig. 3). In the same way, the probe hybridized to the entire euchromatic part of the long arm of the submetacentric X chromosomes (pX13 and pX14) but faintly to X_{st1} (pX13) and intensely to X_{st2} chromosomes (pX14). In addition, in both chromosomes it stained a terminal position in the heterochromatic small arm, which was big and more intense in X_{st1} (pX13) (Fig. 3).

Painting with the Y_{m1} Probe

This probe hybridized as expected to the complete Y_{m1} chromosome (pY12) with the exception of the pericentromeric region (Fig. 1d) and also gave different signals in all Y chromosome variants (Fig. 1d–f, Fig. 2). In the 5 small acrocentric Y chromosomes (pY1–pY5), the probe stained almost entirely the variant pY1 and one intense band in the telomeric region of the long arm in the variants pY2–pY5. In addition, the variant pY3 showed a signal in the pericentromeric region. In the large acrocentric Y chromosomes (pY6–pY9) it stained almost the entire heterochromatic long arm with the exception of a region variable in size close to the centromeric region and of an interstitial band in one of them (pY8). In the metacentric Y chromosomes (pY10–pY12) the probe stained the entire chromosome with the exception of the pericentromeric region in the largest of them (pY12).

The Y_{m1} probe stained the heterochromatic block/band of the acrocentric X chromosomes, with the exception of pX3. As this probe did not paint the pericentromeric heterochromatin, no signal was found in the variants pX1 that lacked heterochromatic bands (Fig. 3). Despite practically identical morphology, this probe permitted the clear differentiation of the 2 submetacentric X chromosomes (X_{st1} and X_{st2}). In X_{st1} (pX13) the probe faintly stained the euchromatic region of the long arm and a terminal band in the small heterochromatic arm, while in X_{st2} (pX14) it stained exclusively and intensely the heterochromatic block of the long arm proximal to the centromere (Fig. 3).

Painting with the X_{st1}p Probe

The pattern of painting with this probe was very similar to the pattern obtained with the Y₀ probe in variants of both X and Y chromosomes (Fig. 1g–i, Fig. 2, Fig. 3). However, some of the euchromatic regions of the X chromosome variants showed less intense signals than with the Y₀ probe (Fig. 3). Also, the large Y acrocentric chromosomes variants (pY8 and pY9) were stained with this probe between the interstitial band of the long arm and the centromere, which is mainly absent with the Y₀ probe (Fig. 2). These results demonstrated that the X_{st1} short arm shared repetitive sequences with the Y₀ chromosome responsible for the similar painting pattern, but in addition it contains also other repetitive sequences not located on the Y₀ chromosome.

Discussion

For the first time we applied the chromosome painting technique to analyze the sex chromosome polymorphism in *M. thomasi* and *M. atticus*, where 9 variants of X chromosomes and 8 variants of Y chromosomes were previously described based on C-banding pattern, size, and morphology [Acosta et al., 2009; Mitsainas et al., 2009; Rovatsos et al., 2011a; Rovatsos and Giagia-Athanasopoulou, 2012; Rovatsos et al., 2014]. Our result demonstrated that this polymorphism is even more extensive than previously shown. On the basis of the painting pattern with the 3 chromosomal probes, we were able to differentiate 14 X chromosome variants (pX1–pX14) and 12 Y chromosome variants (pY1–pY12) (Fig. 2, 3). Moreover, this polymorphism could be even larger, as for example the previously characterized submetacentric X_{st0} chromosome is not included in this study.

Evolutionary Dynamics of Y Chromosomes

We recognized 5 small acrocentric, 4 large acrocentric, and 3 metacentric Y chromosomes. All 5 small acrocentric variants were previously assigned as Y_0 and Y_1 ; however, we found 4 different Y_1 chromosomes (pY2–pY5) on the basis of the painting results. These chromosomes presented subtle variation in size, but the painting pattern suggests that they share some regions with similar repeated sequences, while other regions do not contain the same sequences. We assume that the smallest chromosome (pY1) is the ancestral one, and the other variants could be derived by amplification of different repeated DNA sequences in the heterochromatin. The 2 big acrocentric chromosomes (pY6 and pY7), assigned as Y_2 and Y_3 , arose from a small acrocentric chromosome by a significant amplification of the heterochromatic block stained with the Y_{m1} probe, leaving the terminal regions of the long arm with sequences of the original Y small chromosome (these regions are stained by the X_{st1p} and Y_0 probes). The 2 largest acrocentric Y chromosomes (pY8 and pY9), assigned as Y_3 and Y_4 , probably arose from one of the other big acrocentrics (pY6 or pY7) by acquisition of additional heterochromatin in the long arm proximal to the centromere. The acquired heterochromatic sequences in pY8 have the same composition than the rest of the arm and the Y_{m1} chromosomes, while in pY9 they have a different composition, although the heterochromatin in both chromosomes also shares some sequences stained with the X_{st1p} probe.

The small metacentric Y chromosome (pY10) arose by a pericentric inversion or by accumulation of heterochromatic sequences on the small arm of some of the small acrocentric chromosomes, probably the variant pY4 or pY5. The other 2 big metacentric Y chromosomes (pY11 and pY12) could have arisen through heterochromatin amplification in both arms of the small metacentric Y or by a pericentric inversion in one of the big acrocentric Ys, probably the variant pY6 or pY7. The biggest one (pY12) additionally contains some different repetitive DNA sequences in the pericentromeric region that are not stained with the Y_{m1} probe.

The proposed origins for the different Y chromosome variants are in agreement with the geographic location of each of them, which is represented in Figure 4.

Evolutionary Dynamics of X Chromosomes

We found 12 acrocentric and 2 submetacentric X chromosomes. Giagia-Athanasopoulou and Stamatoopoulos [1997] suggested that the ancestral X chromosome in *M. thomasi* is the acrocentric one with pericentromeric heterochromatin but without heterochromatic bands.

Fig. 4. Geographic distribution of the different variants of both sex chromosomes showing the painting pattern with the probe Y_{m1} .

According to this scenario, the derived X chromosomes evolved via incorporation of the heterochromatic bands. The variant pX2 (X_1) was the first that incorporated a heterochromatic band which is stained with the Y_{m1} probe. We can suppose that the heterochromatic regions or bands of the other acrocentric X chromosomes (variants pX4–pX12) originated by successive amplification or duplication of the same heterochromatic band, probably accompanied in some cases by paracentromeric inversions which could give rise to the interstitial bands that are not stained with this probe. However, the heterochromatin of the X chromosome variant pX3 (X_2) may have a different origin as its heterochromatic block has a different composition as demonstrated by the lack of the Y_{m1} FISH signal. It is interesting that chromosomes previously assigned to 3 categories by C-banding, X_2 – X_4 included, are in fact 9 variants according to their molecular composition (2 variants for X_2 , 5 for X_3 , and 2 for X_4).

Morphologically, the very similar submetacentric X chromosomes can be clearly differentiated by the painting pattern to 2 variants (pX13 and pX14) with independent origin. The main differences are related to the staining of the small arm and to the heterochromatic block of the long arm. While in the variant pX14 the small arm is slightly stained with the 3 painting probes, there is a subtelomeric band clearly stained in the variant pX13. Also, the heterochromatic region of pX14 is strongly stained with the Y_{m1} probe, while this region is not stained in pX13. The pX13 chromosome probably arose from pX3 by enlargement of the heterochromatic region of the long arm and by acquisition of a larger short arm. However, pX14 could be derived from the variants pX9 or pX10 by enlargement of the short arm. The close geographic distribution of pX3 with pX13 and pX9 or pX10 with pX14 is in agreement with the proposed hypothesis for the origin of pX13 and pX14 (Fig. 4). In both cases, the short arm did not originate by a pericentric inversion as was previously supposed [Giagia-Athanasopoulou and Stamatopoulos, 1997] but probably by amplification of different sequences on the original short arms. Previous results demonstrated that the repetitive DNA sequence Mth-Alu900 is amplified in the heterochromatic long arm but not in the heterochromatin of the short arm of X_{st1} [Acosta et al., 2009].

Heterochromatin Enlargement of Sex Chromosomes in Microtus

Under the assumption that the smaller acrocentric X and Y chromosomes of *M. thomasi* represent the ancestral sex chromosomes [Giagia-Athanasopoulou and Stamatopoulos, 1997], 2 major processes would lead to the polymorphisms: a) change in morphology from acrocentric to submetacentric or metacentric, and b) increase in size due to accumulation of different repeated DNA sequences that generated heterochromatic blocks. In addition, some molecular processes permitting the homogenization of most of the heterochromatic blocks of both sex chromosomes would have to be assumed.

Both sex chromosomes in *Microtus* are able to rapidly accumulate large blocks of constitutive heterochromatin, giving rise to a notable increase in sex chromosome size and leading in some species to the giant sex chromosomes, which present big heterochromatin blocks [Burgos et al., 1990; Modi, 1993; Singh et al., 2000; Marchal et al., 2004a]. In the giant sex chromosomes accumulation of heterochromatin occurs in parallel in both X and Y chromosomes, as it has occurred in the different X and Y variants described in *M. thomasi* and *M. atticus*. How-

ever, the origin, composition, and evolution of these blocks are very different between species and even between both sex chromosomes of the same species. In fact, while the heterochromatic blocks of the X and Y chromosome of *M. agrestis* shared the same sequences, the heterochromatic blocks of the sex chromosomes in *M. cabreræ* have a different composition [Kalscheuer et al., 1996; Marchal et al., 2004a]. The sex heterochromatin in the *Microtus* species is highly heterogeneous within species (with different composition of the X and Y heterochromatic regions in *M. cabreræ*) and between species (as the composition of *M. agrestis* and *M. cabreræ* sex heterochromatin is different) [Marchal et al., 2004a]. The accumulation of heterochromatin in *M. thomasi* and *M. atticus* is similar to *M. agrestis*, where both sex chromosomes share repetitive DNA sequences despite their asynaptic condition [Singh et al., 2000; Rovatsos and Giagia-Athanasopoulou, 2012]. Unequal non-sister chromatid exchanges facilitated by repetitive DNA sequences between the heterochromatic blocks could explain the homogeneity observed in both sex chromosomes of *M. agrestis* [Singh et al., 2000], *M. thomasi*, and *M. atticus*.

The genus *Microtus* is one of the most karyologically variable rodent groups [Maruyama and Imai, 1981], and sex chromosome variation or polymorphism has been reported in several species. For example, 2 medium-sized X chromosome variants (metacentric and acrocentric) and a small Y chromosome have been described in *M. savii*, while the closely related *M. brachycercus* has a large submetacentric X and a large acrocentric Y chromosome [Galleni et al., 1994; Castiglia et al., 2008]. In *M. guentheri*, 3 X chromosome variants (acrocentric, submetacentric, and metacentric) and 2 Y chromosome variants (small- and medium-sized) have been described [Chasovnikarova et al., 2008; Mitsainas et al., 2010; Zima et al., 2013]. *M. rossiaemeridionalis* has 2 different X chromosomes varying in the size of the heterochromatic block [Fredga et al., 1990]. *M. oregoni* presented differences between 2 populations in both sex chromosomes due to size variation in G-banding regions and in blocks of C-banding constitutive heterochromatin [Libbus and Johnson, 1988]. Also, in *M. cabreræ* polymorphism affecting the giant sex chromosomes with 3 X and 5 Y chromosome variants has been described due to deletion of the heterochromatic blocks [Burgos et al., 1988b]. The sex chromosome polymorphism described here is the largest known up to date in microtids and probably in mammalian species.

The origin and maintaining of this polymorphism could be related to a very limited spatial mobility of sub-

terranean *M. thomasi* and *M. atticus* combined with the rough, mountainous terrain of the Balkan Peninsula. It seems likely that inbreeding and genetic drift in the small and fragmented populations of these 2 species led to the fixation of different sex chromosomes in different regions. In fact, different sex chromosome variants are present in different regions of the distribution area of this species (Fig. 4). It has been suggested that the current geographical distribution of *M. thomasi* and *M. atticus* is a result of a northward colonization from the glacial refuge in southern Greece probably after the last glacial period (10.000–15.000 years ago) [Mitsainas et al., 2009; Rovatsos et al., 2011a]. Hence, this big sex chromosome polymorphism evolved very rapidly from the 'thomasi' chromosomal race through gradual accumulation of heterochromatin in the sex chromosomes in the northward colonization of the species, with the least amount of heterochromatin in the sex chromosomes distributed in the south-east [Mitsainas et al., 2009] (Fig. 4).

In this context, it is possible to imagine a scenario in which some isolated populations could reach enough genetic diversity to become an incipient new species, as probably has occurred previously with *M. atticus* [Rovatsos and Giagia-Athanasopoulou, 2012]. This new species could present one of the existing combinations of sex chromosome variants. If one or both sex chromosomes of the new species are the biggest ones from the existing polymorphism, it could lead into a new species with giant sex chromosomes. In fact, in one of the analyzed individuals (R18; Fig. 1e) we calculated that the X chromosome (pX12) and the Y chromosome (pY9) represent approximately 12% and 11% of the haploid genome. The

giant sex chromosomes in *M. cabreræ*, *M. chrotorrhinus*, and *M. agrestis* account for a significant percentage of their genome [Modi, 1987b; Nanda et al., 1988; Kalscheuer et al., 1996; Marchal et al., 2004a], and the giant X chromosomes of *M. agrestis* and *M. cabreræ* represent nearly 20% and 15% of the genome, respectively [Nanda et al., 1988; Kalscheuer et al., 1996]. Our results on *M. thomasi* and *M. atticus* could help to underline one of the possible origins of giant sex chromosomes. We can suppose that probably a similar polymorphism variation on the sex chromosomes and processes as genetic drift fixation occurred in the origin of some of the actual species with giant sex chromosomes.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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