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Original Article

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Karyotype stasis but species-specific repetitive DNA patterns in *Anguis* lizards (Squamata: Anguidae), in the evolutionary framework of Anguiformes

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ABSTRACT

Karyotype divergence may strongly affect the degree of hybridization between species. Western Palearctic slow worms (*Anguis*) are legless lizards forming different types of secondary contact zones. To identify the level of chromosomal variation in slow worms, we examined karyotype in multiple populations of all species except one and *Pseudopus apodus* as an outgroup. We applied conventional and molecular cytogenetic methods and whole-chromosome painting using macrochromosome probes from *Varanus komodoensis* to interpret results within the evolutionary framework of the common clade Anguiformes. All *Anguis* species and *P. apodus* have conserved karyotype structures composed of 44 chromosomes. Despite the conserved chromosome morphology, the phylogenetically oldest *Anguis cephallonica* living in partial sympatry with *Anguis graeca*, and parapatric *Anguis colchica* vs. *Anguis fragilis* exhibit distinct patterns of constitutive heterochromatin distribution and telomeric repeat accumulation. In contrast, the sister species *A. colchica* and *A. graeca* living in allopatry display highly similar karyotype features. Our findings thus indicate karyotype stasis in *Anguis* and *Pseudopus* for > 20 Myr, with fixed species-specific differences present in sympatric and parapatric species. These differences in repetitive DNA patterns may play a role as intrinsic factors co-maintaining species divergence. They may also be used as cytotaxonomic markers to identify slow worm species in practice.

Keywords: Anguimorpha; chromosome painting; chromosomal rearrangements; heterochromatinization; karyotype conservation; rDNA; sex chromosomes; squamate reptiles; telomeres; Zoo-FISH.

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INTRODUCTION

Assessing global biodiversity is often challenging due to frequent cryptic speciation events, where the high similarity or uniformity at the morphological level hampers the identification of diverging taxa. Instead, molecular and cytogenetic analyses have been repeatedly applied to address this issue (e.g. Pereira *et al.* 2013, Vasconcelos *et al.* 2016, Cioffi *et al.* 2018). Cytogenetic studies, in particular, have helped with species delimitation in different vertebrate groups such as fishes, amphibians, reptiles, and mammals (e.g. Ruedi *et al.* 1990, Tymowska 1991, Oliver *et al.* 2009, Ferreira *et al.* 2017, Knytl *et al.* 2023). On the other hand, cytogenetics fails in providing relevant data in groups with highly conserved diploid chromosome number ($2n$) and karyotype structure during phylogenetic divergence. This so-called karyotype stasis has been documented in several lineages of plants (Mandáková *et al.* 2010, Bomfleur *et al.* 2014), amphibians (Aprea *et al.* 2004), fishes (e.g. Barby *et al.* 2019, Motta-Neto *et al.* 2019), birds (Ellegren 2010), and also in certain mammals (Tian *et al.* 2004).

Among reptiles, several groups show a high degree of karyotypic variation (Gorman 1973, Olmo and Signorino 2005) driven by various types of inter- and intrachromosomal rearrangements detectable even among closely related taxa (e.g. Olmo 2005, 2008, Johnson Pokorná *et al.* 2015, Rovatsos *et al.* 2017). Cytogenetic approaches may thus significantly complement taxonomic and evolutionary understanding of reptile species complexes and morphologically conserved groups.

Slow worms, the semifossorial legless lizards of the genus *Anguis* Linnaeus, 1758 (family Anguidae), represent such an example. They are morphologically quite uniform or difficult to distinguish by external characters (Benkovský *et al.* 2021, Jablonski *et al.* 2021) but have been shown to represent genetically relatively deeply divergent lineages harbouring five currently recognized species (Gvoždík *et al.* 2010, 2013, 2023, Jablonski *et al.* 2016).

All five slow worm species live in parapatry. The sole exception is the partial sympatry of *Anguis cephallonica* Werner, 1894 and *Anguis graeca* Bedriaga, 1881 in the southernmost Balkans, the Peloponnese Peninsula. These two species, which are morphologically quite distinct, probably do not hybridize or only rarely (Thanou *et al.* 2021). Phylogenetic reconstruction using genome-wide markers showed that *A. cephallonica*, endemic to the Peloponnese and nearby islands, is the oldest lineage within the extant taxa, which diverged around 12 Mya (Gvoždík *et al.* 2023). The remaining four species represent the *A. fragilis* species complex, which began to diversify around 6.7 Mya, although it has retained relatively conserved morphology. The complex represents two clades, each comprising one widely distributed species and one being geographically more restricted. *Anguis fragilis* Linnaeus, 1758 is mainly distributed in western Europe, whereas its sister species *Anguis veronensis* Pollini, 1818 (divergence 2.7 Mya) is near-endemic to the Italian Peninsula. *Anguis colchica* (Nordmann, 1840) is widespread in eastern Europe (*A. colchica incerta* Krynicki, 1837) and western Asia, and its sister species *A. graeca* (divergence 4.4 Mya) is endemic to the southern Balkans. Phylogenetic controversy exists for *A. veronensis*, which, according to mtDNA, represents a sister lineage to *A. cephallonica*, but this is likely the result of ancient mitochondrial capture (Gvoždík *et al.* 2023). Some degree of hybridization has been documented between *A. fragilis* and *A. colchica* (Szabó and Vörös 2014, Gvoždík *et al.* 2015, Benkovský *et al.* 2021), *A. fragilis* and *A. graeca* (Mikulíček *et al.* 2018), and *A. fragilis* and *A. veronensis* (Gvoždík *et al.* 2013, Dufresnes *et al.* 2023). Therefore, the question arose as to whether and to what extent the speciation events were associated with karyotype changes and how much their karyotypes may be differentiated while still allowing gene flow.

The genus *Pseudopus*, with the single extant species *Pseudopus apodus* (Pallas, 1775), presumably represents a sister lineage to *Anguis*, which probably diverged around 21–25 Mya (Lavin and Girman 2019, Gvoždík *et al.* 2023). It is distributed in the more southern and eastern regions of the western Palearctic. Some doubts exist about the close phylogenetic relationship of *Anguis* and *Pseudopus*, with some studies showing the two genera as non-sister lineages within the subfamily Anguinae, and with the genus *Ophisaurus* possibly closely related either to *Anguis* (Klembara *et al.* 2014, 2019) or *Pseudopus* (species tree in Lavin and Girman 2019). However, most studies have recovered *Pseudopus* as a sister lineage to *Anguis* (Macey *et al.* 1999, Pyron *et al.* 2013, Lavin and Girman 2019, Gvoždík *et al.* 2023).

Knowledge about slow worm genome organization is scarce. Only two species, *A. fragilis* and *A. veronensis*, have been analysed at least at the conventional cytogenetic level, providing a simple karyotype description (Dalcq 1920a, b, 1921, Matthey 1931, Margot 1946, Gigantino *et al.* 2002, Mezzasalma *et al.* 2013). Comparative molecular cytogenetic studies in reptiles, including *A. fragilis,* have provided information on the distribution of telomeric repeats (Rovatsos *et al.* 2015) and revealed the homeology of several chromosomes using whole-chromosome painting with selected chicken probes (Pokorná *et al.* 2011). Those *Anguis* species show $2n = 44$ chromosomes, with a karyotype composed of 20 macrochromosomes and 24 microchromosomes. The same karyotype composition was found in *P. apodus* (Matthey 1931), pointing to possible karyotype conservatism within the genus *Anguis*.

In this study, we primarily examined species of the anguid genus *Anguis* using cytogenetics and comparatively assessed observed patterns in the context of deeper karyotype evolutionary history. We follow Burbrink *et al.* (2020) for higher phylogenetic relationships, dating, and terminology. The family Anguidae consists of two subfamilies, the predominantly Old World and strictly legless Anguinae and the strictly New World and limbed Gerrhonotinae. Two other lineages were sometimes considered subfamilies of the Anguidae (e.g. Vitt and Caldwell 2009), but are now usually considered two distinct families, both from the New World, the legless Anniellidae and the limbed Diploglossidae. Together with the family Xenosauridae, they form the superfamily Anguioidea, which is part of the common clade Anguiformes (earlier Anguimorpha), along with, for example, helodermatids and varanids. Anguiformes, together with Iguania (iguanian lizards: e.g. agamas, chameleons, iguanas) and Serpentes (snakes), form the common clade Toxicofera, which originated in the Middle Jurassic about 166–172 Mya. Anguiform lizards began to diversify in the Early Cretaceous about 128–134 Mya. This timespan also applies to the divergence between Anguidae and Varanidae (see below). Anguiforms represent a cytogenetically diverse group with $2n$

ranging from 20 to 48 chromosomes and exhibit a continuum in karyotype shape from completely biarmed macrochromosomes [e.g. in *Caribicus warreni* (Schwartz, 1970), previously *Celestus warreni*; Diploglossidae] to exclusively acrocentric chromosomes (e.g. *Gerrhonotus liocephalus* Wiegmann, 1828; Anguidae, Gerrhonotinae) (Augstenová *et al.* 2021; and references therein). Despite considerable karyotype reshuffling during the evolution of anguiforms, some genera, such as the species-rich *Varanus* (Varanidae), represent striking karyotype stasis (King and King 1975) confirmed by chromosome painting with a set of whole-chromosome probes obtained from *Varanus komodoensis* Ouwens, 1912 (Iannucci *et al.* 2019a, b).

Squamate reptiles exhibit a wide variety of sex determination mechanisms, including genetic and/or temperature sex-determining factors (reviewed in Straková *et al.* 2020, Kratochvíl *et al.* 2021a, Mezzasalma *et al.* 2021). In *Anguis*, none of the previous conventional cytogenetic studies have revealed sex chromosomes (Margot 1946, Mezzasalma *et al.* 2013), although other anguiforms such as helodermatids, varanids, or the New World anguid *Abronia lythrochila* Smith & Alvarez del Toro, 1963 (Gerrhonotinae) possess the ZZ/ZW system of well-differentiated sex chromosomes where the W chromosome is heterochromatic (Johnson Pokorná *et al.* 2014, 2016, Iannucci *et al.* 2019b, Augstenová *et al.* 2021). Moreover, these sex chromosomes share gene content (at least part of it), suggesting an ancient origin dating back to around 130 Mya (Iannucci *et al.* 2019b, Rovatsos *et al.* 2019a; dating sensu Burbrink *et al.* 2020). Although the New World genus *Abronia* also shares this ZZ/ZW sex chromosome system (Rovatsos *et al.* 2019a; but see Augstenová *et al.* 2021 for potential exceptions), this is not the case for the Old World *Anguis fragilis*, raising the question of whether a novel sex-determining system has evolved in this genus (Rovatsos *et al.* 2019a).

Here, we investigate the genome organization of the morphologically difficult-to-distinguish but genetically divergent species of *Anguis* (at the multi-population scale) and its putative sister taxon, the European glass lizard, *Pseudopus apodus*, using conventional and molecular cytogenetic methods, including cross-species chromosome painting (Zoo-FISH) with *Varanus* macrochromosome probes. We aim to assess whether: (i) *Anguis* species diversity is related to karyotype differentiation at the interspecific and interpopulation levels, and whether karyotype differences might be useful for species delimitation, and hence for identification of hybrids; (ii) *Anguis* lizards possess differentiated sex chromosomes; and (iii) the evolutionary dynamics of *Anguis* karyotype differs from that of other anguiform lizards and toxicoferan reptiles.

MATERIAL AND METHODS

Sampling and species identification

In total, we cytogenetically investigated 34 individuals of five slow worm species, with the majority of species from multiple sites, including geographically distant localities: *A. cephallonica* (two females, two males; Greece: Kefalonia Island and Peloponnese), *A. colchica incerta* (four females, six males; Czech Republic, Bulgaria), *A. fragilis* (seven females, five males; Czech Republic, Bulgaria), *A. graeca* (three females, four males; Albania, Greece), and *A. veronensis* (one juvenile of unknown sex; Italy). Additionally, one individual of unknown sex of *P. apodus* from Albania was used as a comparative outgroup. Supporting Information Table S1 provides an overview of material, including geographic origin and methods applied per individual.

Species identification was based on a combination of morphological characters and distribution (Gvoždík *et al.* 2015, Benkovský *et al.* 2021, Jablonski *et al.* 2021), and DNA barcoding using a fragment of mitochondrial DNA [mtDNA; the *NADH dehydrogenase 2* (*ND2*) gene] according to previously published protocols (e.g. Gvoždík *et al.* 2010, Jablonski *et al.* 2016). Obtained nucleotide sequences were deposited in GenBank (acc. nos OR352076–OR352110). Individuals of *A. colchica* and *A. fragilis* from near the hybrid zone in the Czech Republic and Bulgaria were also genetically tested using diagnostic single nucleotide polymorphisms of nuclear DNA (V. Gvoždík *et al.*, unpublished data), which always validated previous mtDNA-based identification (i.e. no hybrids or individuals with introgressed mtDNA were included in the sampling). Sex was identified based on external morphology and the presence of hemipenes, which males usually evert during handling.

Chromosome preparations and differential staining

Chromosome spreads were prepared by the leukocyte cultivation of peripheral full blood following the protocol of Johnson Pokorná *et al.* (2016) with minor modifications described in the Supporting Information. Chromosomes stained in 5% Giemsa solution (Merck, Darmstadt, Germany) were used for karyotype description and karyogram construction. The distribution of GC-rich (GC+) and AT-rich (AT+) regions was revealed by staining with Chromomycin A_3 (CMA_3) and 4',6-diamidino-2-phenylindole (DAPI), respectively, following Sola *et al.* (1992). In several individuals, the CMA_3/DAPI analysis was followed by a C-banding procedure for visualization of constitutive heterochromatin on the same slides. For this purpose, the CMA_3/DAPI staining was removed by three washes in distilled water for 10 min each and soaked in 70% ethanol for 5 min, air-drying, and then used for C-banding to determine the GC+ or AT+ character of the constitutive heterochromatin. The C-banding method (Sumner 1972) protocol is given in Supporting Information. In selected individuals, the activity of rDNA genes was confirmed by silver-staining of nucleolar organizer regions following the protocol of Howell and Black (1980).

Fluorescence *in situ* hybridization (FISH) with telomeric and 18S rDNA probes

To reveal the distribution of the telomeric repeats $(TTAGGG)_n$, including interstitial telomeric repeats (ITRs), the commercial DAKO telomere PNA kit/Cy3 was applied following the protocol supplied by the manufacturer (Agilent Technologies, Santa Clara, CA, USA) with hybridization prolonged to 1 h. The slides were mounted and chromosomes counterstained with Vectashield Antifade Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI).

The 18S rDNA specific probe was prepared from DNA of *Anguis fragilis* by nick-translation labelling of the PCR product containing the 18S rRNA gene partial sequence (Cioffi *et al.* 2009). The detailed protocol of PCR amplification,Supporting

Information Table S2 cloning, plasmid DNA extraction, and verification of final sequences and probe preparation is provided in Supporting Information.

The biotin-dUTP-labelled probe was denatured for 6 min at 85 °C and chilled on ice for 10 min prior to the hybridization. The slides with chromosomal spreads were aged for 1 h at 60 °C and went through RNase A, pepsin, and 1% formaldehyde treatment. The chromosomes were denatured in 70% formamide/2 × SSC for 3 min at 75 °C. A total of 300 ng of labelled DNA in 15 µL of hybridization mixture was hybridized on chromosome spreads overnight at 37 °C per slide. Blocking reagent (Roche, Basel, Switzerland) was applied for 30 min at 37 °C to minimize the unspecific fluorochrome binding. The probe signal was detected using Fluorescein Avidin D (Vector Laboratories, Burlingame, CA, USA) and a Biotinylated Anti-Avidin D (Vector Laboratories) antibodies complex. Finally, the slides were mounted and stained with Vectashield Antifade Medium containing DAPI. For the detailed protocol, see Supporting Information.

Male/female comparative genomic hybridization (CGH)

To test for the presence of sex chromosomes, we used the CGH method, which detects sex-specific or repeats-enriched DNA regions, presumably on the non-recombining part of sex-limited sex chromosomes. At least two independent experiments were performed for each *Anguis* species (DNA and chromosomes originating from individuals are specified in Supporting Information Table S1). The only exception was *A. cephallonica*, where only two males and one female were tested due to the low amount of chromosome material. For each test, 1 µg of male gDNA and 1 µg of female gDNA were used for the probe preparation. Whole gDNA was directly labelled during nick translation using a commercial kit: male DNA was labelled with a Fluorescein NT Labeling Kit, and female DNA was labelled with Cy3 NT Labeling Kit (both Jena Bioscience, Jena, Germany). After nick translation, male and female products were co-precipitated together overnight at -20 °C. The obtained probe was denatured for 6 min at 75 °C and chilled on ice prior to hybridization. Slides with chromosomal spreads were prepared for hybridization as described in the rDNA FISH section. On each slide, 500 ng of male and 500 ng of female labelled DNA re-dissolved in 15 µL of hybridization mixture was hybridized on chromosome spreads for 3 days at 37 °C. Chromosomes were counterstained by Vectashield Antifade Mounting Medium with DAPI. The entire protocol is given in Supporting Information.

Chromosome painting with *Varanus komodoensis* macrochromosome probes

To assess the degree of conserved synteny between *Varanus*, *Anguis*, and *Pseudopus* macrochromosomes, we performed Zoo-FISH with probes derived from flow-sorted chromosomes of the Komodo dragon *Varanus komodoensis* (Iannucci *et al.* 2019a), which were already applied for interspecific painting experiments (Iannucci *et al.* 2019b) and to achieve chromosome-level genome assembly (Lind *et al.* 2019). The probes (referred to as 'VKO' and the specific chromosome number from which they were derived) were prepared by two rounds of degenerate oligonucleotide-primed PCR (DOP-PCR). The first DOP-PCR amplified the genetic material of flow-sorted chromosomes, in which each peak contained one macrochromosome; only two peaks (H and I, comprising VKO 6 and VKO 8, respectively) also contained an additional macrochromosome (VKO 7). During the second round of DOP-PCR, where the product of the first DOP-PCR was used as template, DNA was labelled by incorporation of biotin-16-dUTP (Roche, Mannheim, Germany) or digoxygenin-dUTP (Roche, Mannheim, Germany). All PCR conditions followed the protocol described in Iannucci *et al.* (2019b). Another DOP-PCR using genomic DNA of *V. komodoensis* and *A. fragilis* as a template was used to prepare competitors blocking nonspecific hybridization. Each probe cocktail contained 15 µL of biotin-labelled PCR product and/or 15 µL of digoxygenin-labelled PCR product co-precipitated with 5 + 5 µg of *Anguis* and *Varanus* competitors, all finally dissolved together in 20 µL of hybridization mixture.

FISH was performed according to Iannucci *et al.* (2019b) with the only modifications being probe denaturation (10 min at 90 °C and pre-hybridization for 30–45 min at 37 °C) and hybridization duration (5 days at 37 °C).

Image analysis and processing

All results were analysed using an Axio Imager Z2 microscope (Zeiss, Oberkochen, Germany) equipped with a MetaferMSearch automatic scanning platform and a CoolCube 1 b/w digital camera (MetaSystems, Altlussheim, Germany). The karyograms were arranged in Ikaros karyotyping software (MetaSystems). Morphometric measurements were performed for all macrochromosomes in 10 metaphase spreads per species using ImageJ software (Schneider *et al.* 2012), with the LEVAN plugin (Sakamoto and Zacaro 2009). Only chromosome plates exhibiting high-quality spreading and showing no apparent damage or chromosome shrinkage were included in the analysis. Whenever possible, several individuals of both sexes were considered to ensure representative measurements. Finally, the arm ratio and centromeric index were calculated for each chromosome, and chromosome morphology was classified following Levan *et al.* (1964); Supporting Information Table S3for detailed methodology and calculation, see the Supporting Information. The results of FISH experiments were visualized by superimposing black and white digital images captured using appropriate fluorescence filters and pseudocolored in the Isis Fluorescence Imaging System (MetaSystems). The photographs were arranged using Adobe Photoshop CS6.

RESULTS

Karyotype description

All studied species of *Anguis* namely *A. cephallonica*, *A. colchica*, *A. fragilis*, *A. graeca*, *A. veronensis*, and the supposed sister taxon *P. apodus* possess $2n = 44$ chromosomes with a karyotype consisting of 10 pairs of macrochromosomes and 12 pairs of microchromosomes (Fig. 1 for males; Supporting Information Fig. S1 for both sexes). Based on visual analysis supported with macrochromosome measurements and calculations of arm ratio and centromeric index, all studied species share a common karyotype structure with certain chromosomal pairs exhibiting specific characteristics. Pair Nos 1 and 5 were found to be metacentric (*m*), pair No. 2 was telocentric (*t*), pairs Nos 9 and 10 were submetacentric (*sm*), while the remaining pairs (Nos 3, 4, 6, 7, and 8) were classified as subtelocentric (*st*).

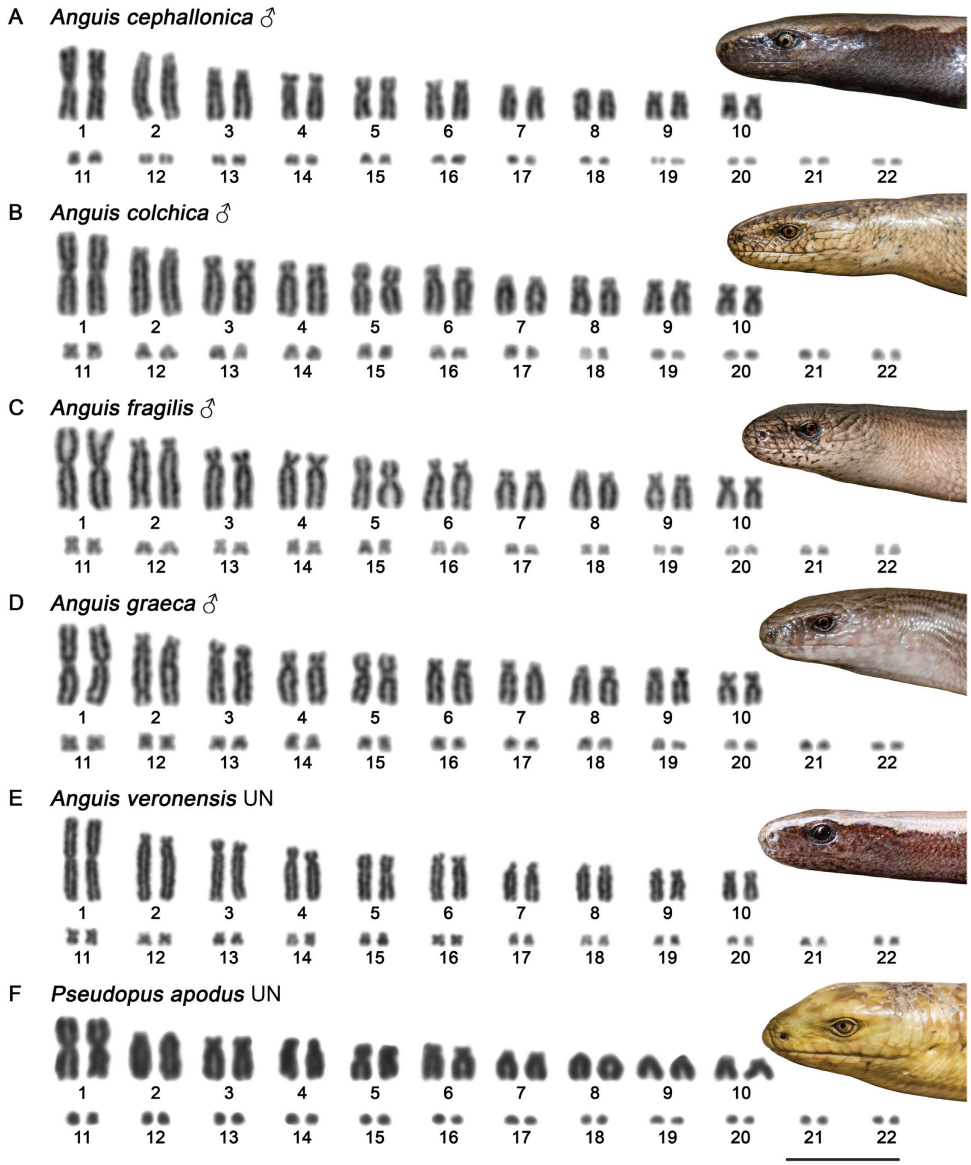

Figure 1. Karyograms of *Anguis* and *Pseudopus*. Male karyograms are shown, the karyograms of *A. veronensis* and *P. apodus* are from a juvenile of unknown sex. All tested individuals including females share the karyotype of $2n = 44$ consisting of 20 macrochromosomes and 24 microchromosomes. Where available, karyograms of both sexes are shown in Supporting Information Figure S1. Scale bar = 10 μm. Photos on the right, not to scale.

However, there were a few exceptions to this general pattern. In *A. cephallonica*, pair No. 8 showed a telocentric morphology. In *A. fragilis*, pair No. 2 displayed a subtelocentric morphology, and in *P. apodus*, pair No. 2 was subtelocentric, and pair No. 9 exhibited a telocentric morphology. All morphometric results are summarized in Supporting Information Tables S4 and S5, and in Supporting Information Figure S2. The karyotype could then be coded as:

- *A. cephallonica*: 4 *m* + 4 *sm* + 8 *st* + 4 *t* + 24 *micro*; *AN* = 52
- *A. colchica*: 4 *m* + 4 *sm* + 10 *st* + 2 *t* + 24 *micro*; *AN* = 52
- *A. fragilis*: 4 *m* + 4 *sm* + 12 *st* + 24 *micro*; *AN* = 52
- *A. veronensis*: 4 *m* + 4 *sm* + 10 *st* + 2 *t* + 24 *micro*; *AN* = 52
- *P. apodus*: 4 *m* + 2 *sm* + 12 *st* + 2 *t* + 24 *micro*; *AN* = 50/52

where *micro* and *AN* stand for microchromosomes and chromosome arm number, respectively. However, in *P. apodus*, pair No. 6 lays on the edge *sm*/*st*, therefore is *AN* = 50/52; for a comparison of intrachromosomal variability and between species, see Supporting Information Figure S2. No sex-specific differences or intraspecific variation (when comparing geographically distant or sea-isolated localities) in chromosome number, size, or shape are present in any of the individuals and/or species under study.

Distribution of heterochromatin and GC-rich regions

The constitutive heterochromatin displays a similar pattern in all studied species, with a visible accumulation in the distal part of the p-arm of pair No. 2 (Fig. 2; Supporting Information Fig. S2, first columns). Remarkable differences in heterochromatinization are present in the centromeric region of pair No. 2, which appeared C-positive in *A. fragilis*, *A. veronensis*, and *A. cephallonica*,

whereas it is C-negative in *A. graeca* and *A. colchica* as well as in *P. apodus*. Different levels of heterochromatinization are also detected in microchromosomes, where *A. graeca* displays more microchromosomes with heterochromatic regions than the other species.

The chromosomes of *Anguis* and *Pseudopus* present a rather uniform GC/AT composition with slightly GC-richer microchromosomes in comparison with macrochromosomes (Fig. 2; Supporting Information Fig. S2, second columns). Other apparent GC+ regions occur in the telomeric region of the first chromosome in *Pseudopus* and a relatively diffused GC+ pattern in the distal part of pair No. 2 in *Anguis*.

Distribution of telomeric sequences and 18S rDNA clusters

FISH with the telomeric probe visualizes the standard pattern at the ends of all chromosomes in all species (Fig. 2; Supporting Information Fig. S2, third columns). In addition, it reveals almost species-specific patterns of distribution of ITRs. These telomeric-like sequence accumulations are detected in the pericentromeric area of chromosome pair Nos 1, 2, 4, 5, 7, and 9 in *A. cephallonica*. In *A. colchica* and *A. graeca*, ITRs occur only in the pericentromeric region of chromosome pair No. 4. *Anguis fragilis* possesses ITRs in pair Nos 2–4 and 7, which agrees with the results reported by Rovatsos *et al.* (2015). *Anguis veronensis* displays ITRs in chromosome pair Nos 1–5, 7, and 9. Finally, in *P. apodus*, ITRs are only detected in the pericentromeric region of the first chromosome pair. In all species, ITRs are also present in one or two pairs of microchromosomes (Fig. 2; Supporting Information Fig. S2, third columns), but due to the small size of these chromosomes, some ITRs can be overlooked.

18S rDNA clusters are present exclusively on the first chromosome pair in *Pseudopus*, whereas in the species of *Anguis*, they reside on three pairs of microchromosomes (in *A. colchica*, *A. fragilis*, *A. veronensis*, and *A. graeca*) and an additional weak signal on another microchromosome pair in *A. cephallonica* (Fig. 2; Supporting Information Fig. S2, fourth columns).

Sex chromosome detection by CGH

The CGH method reveals no sex-specific differences in *Anguis* (Fig. 3). The probes strongly label the heterochromatic regions of pair No. 2, although the detected signal is mostly yellow, indicating a balance between the co-hybridizing male- and female-specific probes. In *A. fragilis* and *A. graeca*, four microchromosomes possess reddish and greenish accumulations, respectively. These signals probably arose due to intraspecific variability in the amount of repetitive content accumulated on these chromosomes, and since they are always detected on two pairs in both sexes, we do not consider them to be sex-specific.

Chromosome painting with *Varanus komodoensis* macrochromosome probes

All control reverse-FISH experiments conducted on chromosomes of *V. komodoensis* (i.e. VKO probes on VKO chromosomes) hybridized specifically (results not shown). Cross-species chromosome painting (Zoo-FISH) reveals striking conservation of large blocks of *Varanus komodoensis* and *Anguis fragilis* (AFR) macrochromosomes (Fig. 4). The *Varanus* probes VKO 1, 2, and 3 each label two *Anguis* chromosome pairs: AFR 2 and 8, AFR 3 and 4, and AFR 9 and 10, respectively. VKO 4 labels the chromosome pair AFR 5. Contrary, the probes VKO 6 and 8 both hybridize on the same chromosome, AFR 1, each on a different arm. For VKO 7, whose DNA is contained in the probes VKO 6 and 8, the synteny remains rather speculative in *Anguis* because the overlapping hybridization signal of the probes was too weak and difficult to detect. However, cross-specific experiments with these probes show clear separate signals on the *Pseudopus apodus* (PAP) chromosome pair PAP 1 and overlapping hybridization on PAP 7. The probe VKO 5 hybridizes inconsistently on *Anguis* and *Pseudopus* (results not shown); therefore, given the large conservatism of the VKO and AFR macrochromosomes, we tentatively assign VKO 5 to AFR 6, but their synteny needs to be confirmed in the future. The chromosome painting results are summarized in Table 1.

DISCUSSION

Conserved karyotypes

Our cytogenetic analysis revealed that all five currently recognized slow worm species share the same chromosome number and similar or nearly identical karyotype structure, with the $2n = 44$ being composed of 20 macrochromosomes and 24 microchromosomes. The diversification of extant *Anguis* taxa is dated to approximately 12 Mya (Gvoždík *et al.* 2023). However, the presumed sister genus *Pseudopus*, with the single extant species *P. apodus*, also has comparable karyotype characteristics (Matthey 1931; our results). The only other anguinine species with a known karyotype, the North American *Ophisaurus ventralis* (Linnaeus, 1766), has $2n = 30$ (with 20 macrochromosomes, 10 microchromosomes; Matthey 1931). Our confirmation of the karyotype of *Pseudopus*, which is similar to *Anguis*, thus supports the sister relationship of *Pseudopus* and *Anguis*. The existence of fossil representatives of both genera, *Anguis* and *Pseudopus*, approximately 20 Mya (Klembara and Rummel 2018, Villa and Delfino 2019) suggests that the most recent common ancestor (MRCA) of the two genera had to exist more than 20 Mya with phylogenetic hypotheses suggesting that the *Anguis–Pseudopus* lineage diverged probably around 25–28 Mya (Lavin and Girman 2019, Gvoždík *et al.* 2023). If we assume that the karyotype consisting of 44 chromosomes evolved in the common ancestor of *Anguis* and *Pseudopus*, then the genome architecture at the chromosomal level has been conserved for at least 21 Myr.

Although highly conserved, we found differences in the morphology of a few macrochromosomes in *A. cephallonica* (pair 8 telocentric), *A. fragilis* (pair 2 subtelocentric), and *P. apodus* (pair 2 subtelocentric and pair 9 telocentric). In addition to these changes in chromosome categories, we also observed a slight shift in the centromeres of several other macrochromosomes (Supporting Information, Fig. S2). The centromeres shifts can be attributed to several mechanisms, e.g. (i) reciprocal chromosomal translocation (which is rather not the case here, based on a conserved karyotype supported by the chromosome painting); (ii) unbalanced loss/gain of repetitive DNA on one of the chromosome arms; (iii) centromere repositioning, i.e. establishment of a new centromere in a different position and the loss of function of the original centromere

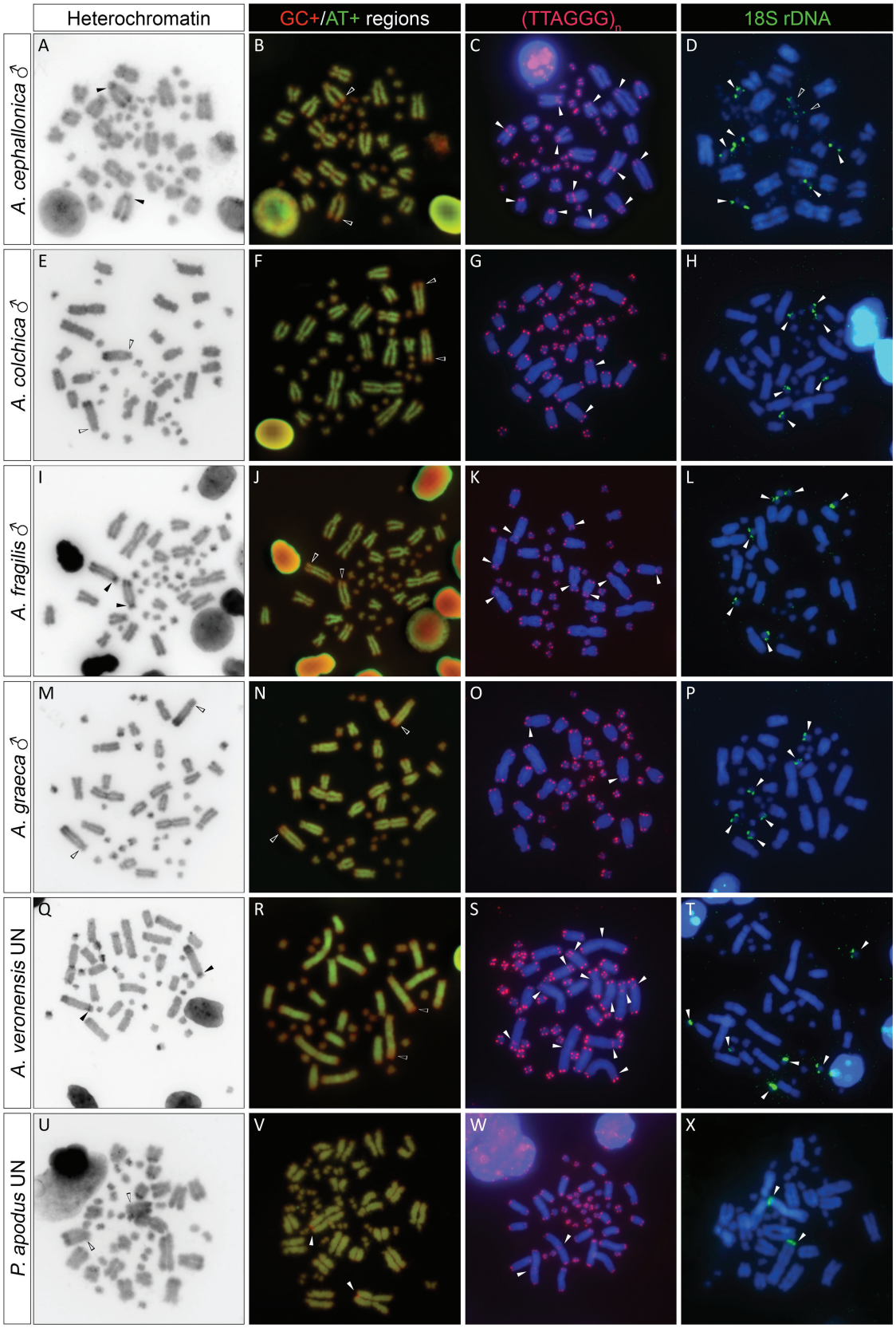

Figure 2. Distribution of constitutive heterochromatin (first column from the left side), GC/AT-positive regions (second column), telomeres and ITRs (third column), and 18S rDNA gene clusters (fourth column) in *Anguis* and *Pseudopus* (males, UN for unknown sex). First column (A, E, I, M, Q, U): presence (full arrowhead) and absence (empty arrowhead) of centromeric heterochromatin in chromosome pair No. 2. Second column (B, F, J, N, R, V): diffused GC+ pattern in distal part of pair No. 2 (empty arrowhead) or strong signal (full arrowhead) in the telomeric region of pair No. 1. Third column (C, G, K, O, S, W): ITRs (full arrowhead). Fourth column (D, H, L, P, T, X): hybridization of 18S rDNA on three pairs of microchromosomes (full arrowheads) and additional weak signal on another microchromosome pair (empty arrowheads). Where available, females do not differ from males and are shown in Supporting Information (Fig. S3).

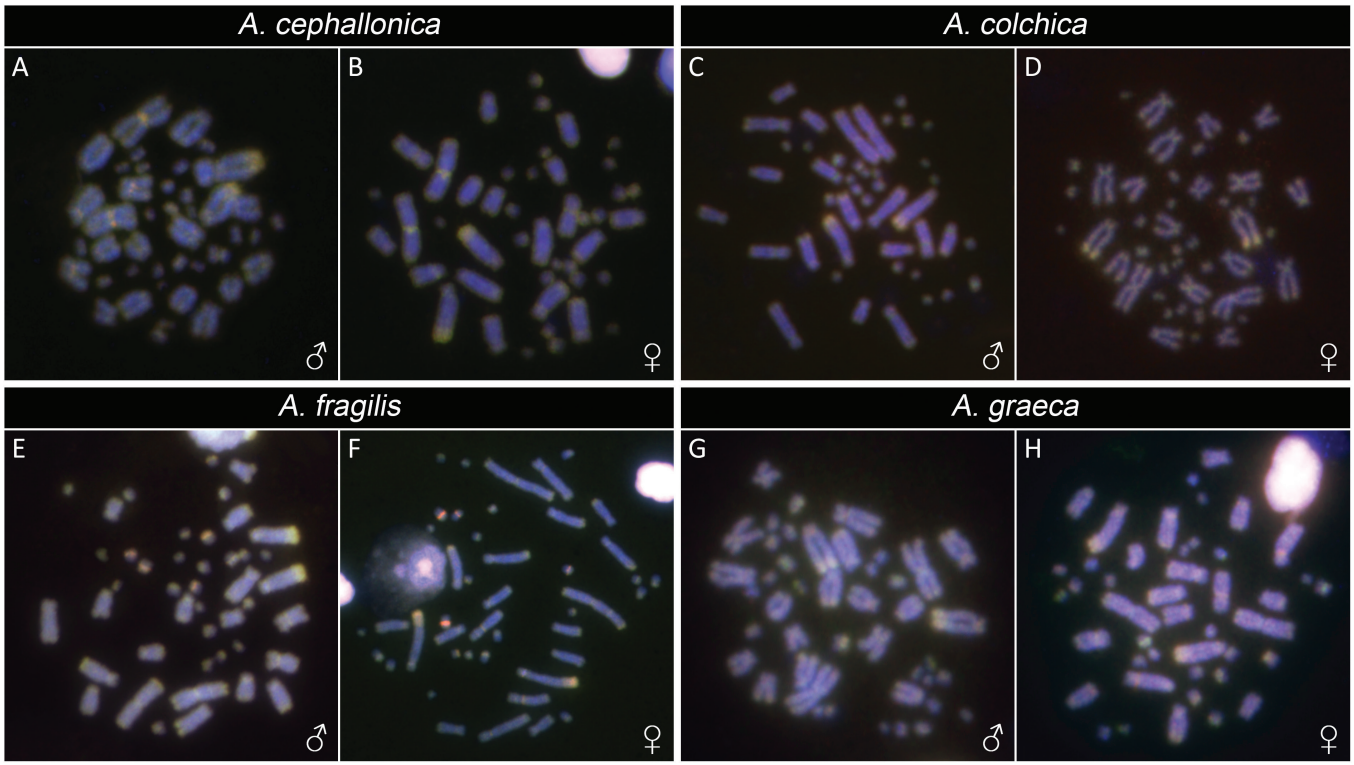

Figure 3. Male (A, C, E, G) and female (B, D, F, H) comparative genomic hybridization in four *Anguis* species. Male-specific DNA is labelled with fluorescein d-UTP (green), and female-specific DNA with Cy3 d-UTP (red). The yellow regions reflect regions of accumulated repetitive elements existing in equilibrium in the male and female genomes. Slightly reddish (E, F) or greenish (G, H) regions indicate certain enrichment of the repetitive fraction in the genome of one of the individuals.

(Rocchi *et al.* 2012, Schubert 2018); or (iv) pericentromeric inversion. Unfortunately, distinguishing between (ii) + (iii) and (iv) is only possible by comparing the gene synteny on the chromosomes, which has not been analysed yet in any species of Anguioidea.

Finally, while it can be helpful for identifying species-specific differences (e.g. Knytl *et al.* 2021), the interpretation of chromosomal morphometrics should be approached with caution, as they are highly correlated with other factors, such as chromosome condensation (reflecting differences between euchromatic and heterochromatic regions) or the placement of chromosomes in the metaphase spread, which can alter a chromosome's elongation (Arefjev and Panov 1984).

Species-specific patterns of karyotype differentiation

The 2*n* and karyotype structures are conserved in all extant *Anguis* and *Pseudopus* species. However, most of the species differ in the level of repetitive DNA accumulation (Fig. 5).

The distribution of ITRs and constitutive heterochromatin accumulation supports the placement of the Peloponnese endemic *A. cephallonica* into an intermediate phylogenetic position between the *A. fragilis* species complex and *Pseudopus*, supporting its early divergence (12 Mya, Gvoždík *et al.* 2023) within slow worms. *Anguis cephallonica* and *P. apodus* share one ITR located on the largest chromosome. On the other hand, *A. cephallonica* already possesses multiple rDNA sites localized in microchromosomes, which is a common feature with the *A. fragilis* species complex, thus probably representing the conserved state in *Anguis*. Contrary to this, *P. apodus* demonstrates the rDNA clusters only on one macrochromosome pair. The karyotypes of *A. colchica* vs. *A. fragilis* (divergence 6.7 Mya) are also diversified by means of repetitive DNA patterns (Figs 2, 5). In contrast, virtually the same distributions of ITRs and heterochromatin support the close relationship of *A. colchica* and *A. graeca*, which corresponds well with molecular phylogenomic reconstructions that date their MRCA to approximately 4.4 Mya (Gvoždík *et al.* 2023). Based on our results, *A. colchica* and *A. graeca* differ only in the heterochromatinization pattern of microchromosomes (Fig. 2; Supporting Information, Fig. S2).

All these species-specific patterns of karyotype differentiation (Fig. 2) are intraspecifically consistent across geography and haplogroups, for example, geographically distant populations of *A. colchica incerta* and *A. fragilis* from the Czech Republic and Bulgaria, or populations of *A. cephallonica* isolated by sea from the island of Kefalonia and the Peloponnese Peninsula (for details on haplogroups, see Jablonski *et al.* 2016, 2017).

In our study, we were able to include only one juvenile of the Italian near-endemic *A. veronensis* in our analyses confirming the previous results of Gigantino *et al.* (2002) and Mezzasalma *et al.* (2013); however, our individual slightly differed in macrochromosome morphology (in categories *st* vs. *t*). These studies revealed heterochromatin blocks in the (peri)centromeric regions of most chromosomes and in the distal part of the 2q arm as well as nucleolus organizer regions (NORs) on three microchromosome pairs using CMA_3-staining and silver impregnation, which are in congruence with our results.

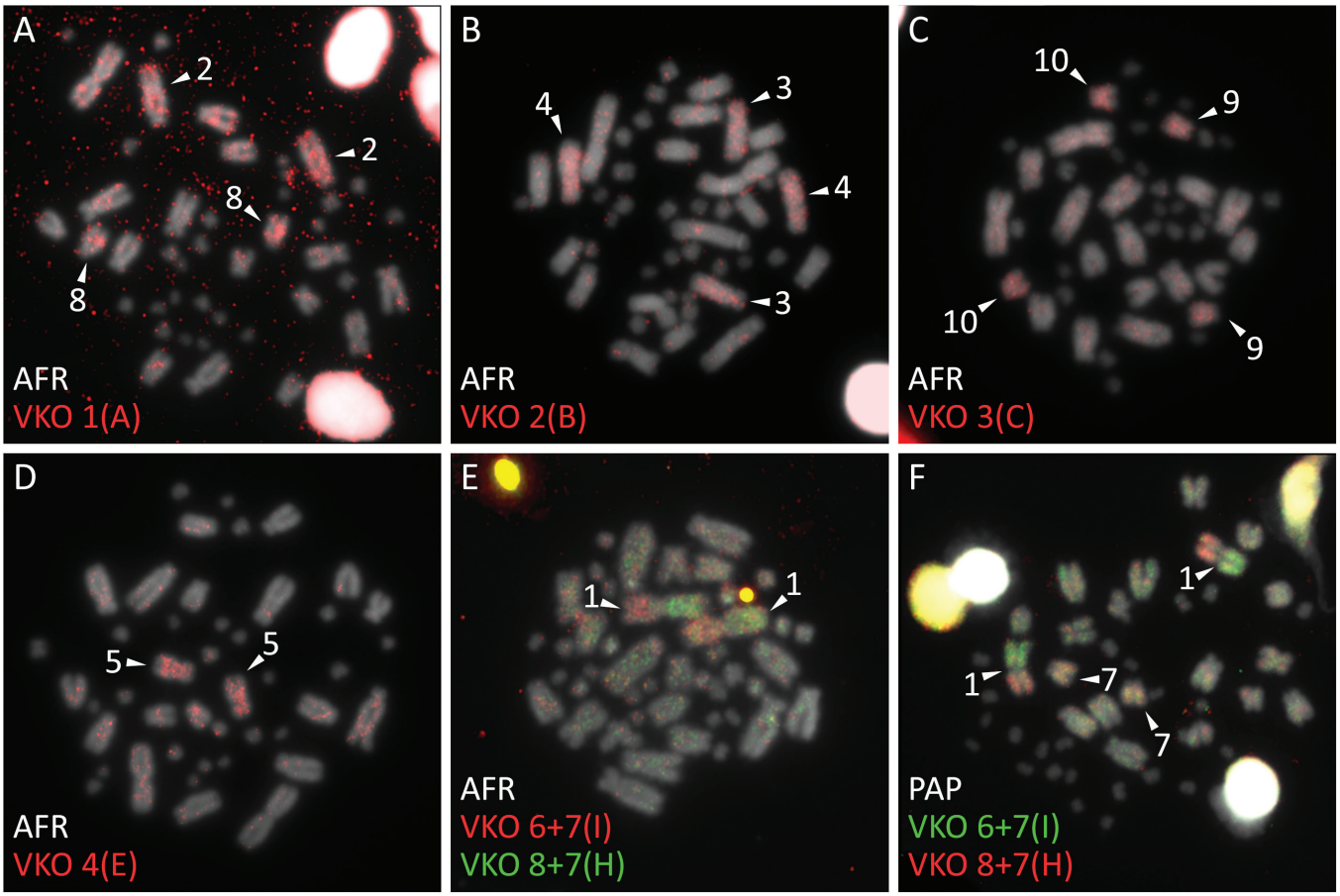

Figure 4. Chromosome painting with *Varanus komodoensis* (VKO) macrochromosome probes on *Anguis fragilis* (AFR) and *Pseudopus apodus* (PAP) chromosomes. The probe identity is indicated (number and letter correspond to VKO chromosome and flow-sorted peak, respectively). Arrowheads mark the hybridization signal on the AFR (A–E) and PAP (F) homeologous chromosomes. Note that each of the probes VKO 1, 2, and 3 marks two different pairs of chromosomes, whereas the probes VKO 6 + 7 and 8 + 7 mark different arms of the same chromosome pair. The hybridization signal of the probes VKO 6 + 7 and 8 + 7 does not clearly overlap in *Anguis*, but it marks chromosome pair No. 7 in *Pseudopus*.

Table 1. Summary of the results of comparative chromosome painting using *V. komodoensis* (VKO) macrochromosome probes on *A. fragilis* (AFR) and *P. apodus* (PAP) chromosomes. The upper index refers to the peak of flow-sorted chromosomes (Iannucci *et al.* 2019b). Note that VKO 7 was not separately flow-sorted but was detected in two peaks (I and H) along with another chromosome pair (VKO 6 and 8, respectively). The probe VKO 5 hybridizes inconclusively on *Anguis* and *Pseudopus*, and therefore the assignment to AFR 6/PAP 6 is tentative and requires future confirmation

Species	Homeology of macrochromosomes						
V. komodoensis	VKO 1^{A}	VKO 2^{B}	VKO 3^{C}	VKO 4^{E}	VKO 5^{D}	VKO 6^{I}, 8^{H}	VKO 7^{IH}
A. fragilis	AFR 2, 8	AFR 3, 4	AFR 9, 10	AFR 5	AFR 6	AFR 1	–
P. apodus	PAP 2, 8	PAP 3, 4	PAP 9, 10	PAP 5	PAP 6	PAP 1	PAP 7

Surprisingly, we found that *A. veronensis* displays a composed ITR pattern of stronger signals common with *A. fragilis* and weaker signals specific for *A. cephallonica* providing support for the hypothesis of past contact between Italian and Peloponnese slow worms (evidenced by ancient mtDNA capture; Gvoždík *et al.* 2023), with repeat DNA remnants of these interactions likely persisting. A closer relationship of *A. veronensis* to *A. fragilis* than to *A. cephallonica* is also implied by the presence of rDNA genes on three microchromosome pairs in the Italian slow worm. This is consistent with the nuclear-genomic phylogenetic reconstruction and thus does not support the mtDNA-based phylogeny. Mezzasalma *et al.* (2013) also pointed out a difference between *A. fragilis* and *A. veronensis* regarding the morphology of chromosome pair No. 10 (i.e. telocentric in *A. fragilis* vs. submetacentric in *A. veronensis*). However, Mezzasalma *et al.* (2013) cited only initial meiotic studies on *A. fragilis*, where the studied individuals were probably originally collected in the vicinity of Brussels (Belgium, Dalcq 1921) and Lausanne (Switzerland, Margot 1946), and thus from localities distant from our collection sites. Moreover, Mezzasalma *et al.* (2013) found a fragile site (i.e. region prone to rearrangements) on chromosome pair No. 10 on several

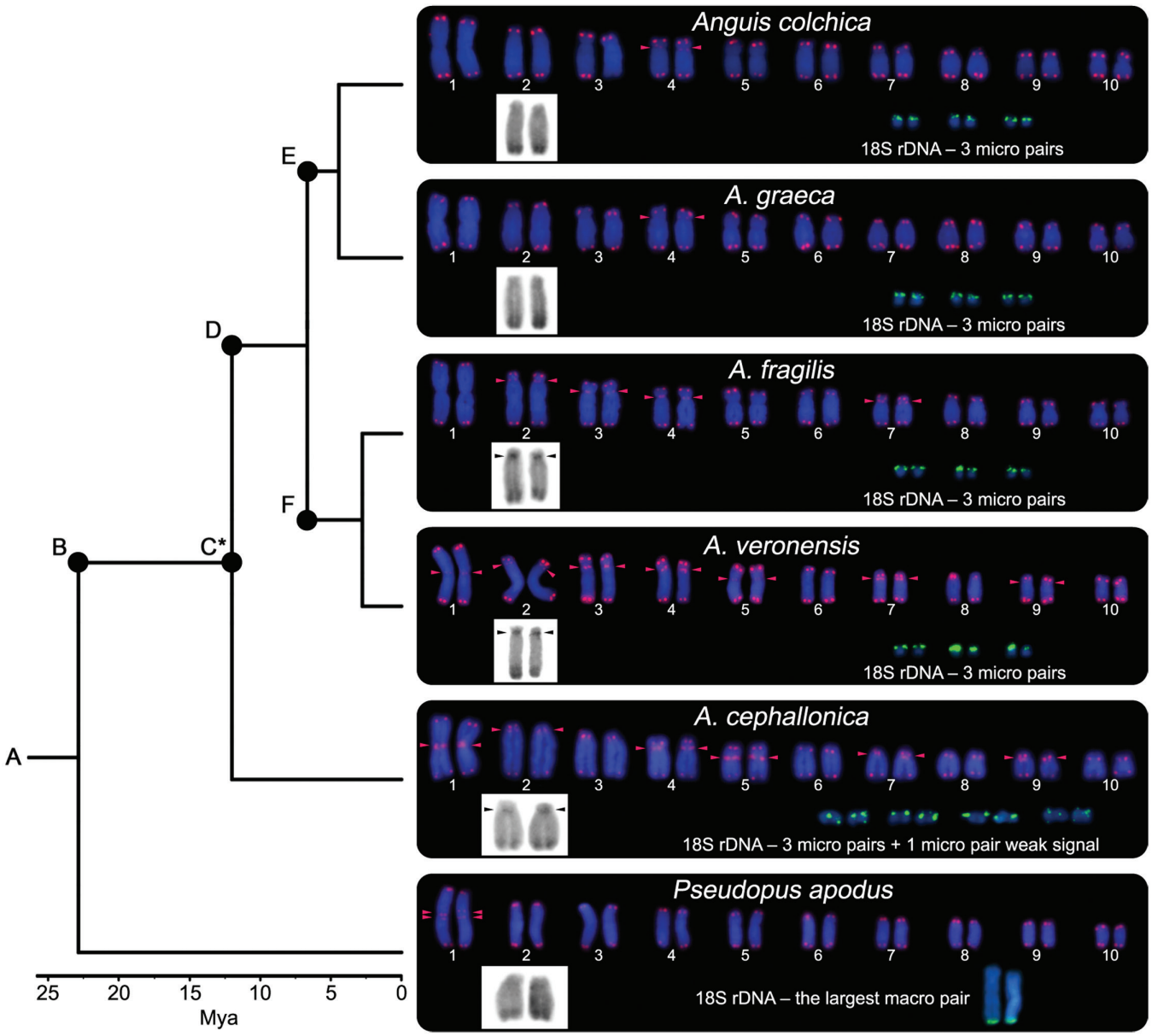

Figure 5. Evolutionary trends and patterns of repeat distribution in the karyotype of *Anguis* and *Pseudopus*. The summary presents: the distribution pattern of interstitial telomeric repeats (ITRs) in macrochromosomes (red arrowheads), the presence of constitutive heterochromatin in the centromeric region of chromosome No. 2 (black arrowheads), and the number and topology of 18S rDNA sites (green signals). Phylogenetic relationships follow Gvoždík *et al.* (2023). Although all six species share $2n = 44$ and macrochromosome morphology differs only subtly, several species-specific repeat accumulation patterns have been observed. A, The common ancestor of *Anguis* and *Pseudopus* had 10 macro- and 12 microchromosome pairs, and its metacentric chromosome No. 1 likely possessed ITRs in the centromeric region. B, Accumulation of ITRs on chromosomes Nos 2, 4, and 7, and heterochromatin on chromosome No. 2; translocation and accumulation of rDNA sites on microchromosomes in the *Anguis* ancestor. C, Accumulation (in *A. cephallonica*) or elimination (in the *A. fragilis* species complex ancestor) of ITRs on pair Nos 5 and 9 and of rDNA sites on one of the microchromosome pairs. An asterisk indicates two possible directions of chromosomal changes. D, Elimination of ITRs on pair No. 1 in the *A. fragilis* species complex ancestor. E, Elimination of ITRs on chromosomes Nos 2 and 7; elimination of heterochromatin on chromosome No. 2 in the common ancestor of *A. colchica* and *A. graeca*. F, Accumulation of ITRs on chromosome No. 3 in the ancestor of *A. fragilis* and *A. veronensis*. *Anguis veronensis* represents a composite ITR pattern of *A. cephallonica* and *A. fragilis*, providing support for the hypothesis of past contact between Italian and Peloponnese slow worms (Gvoždík *et al.* 2023), with remnants of these interactions likely persisting. An alternative hypothesis proposes a shared repeat pattern among all *Anguis* species, wherein the detection of ITRs depends on the repeat abundance and reveals accumulations only above the detection limit.

metaphase plates of *A. veronensis* which might have resulted in a different morphology comparing the same chromosome pair of *A. fragilis*. Based on the methodology used in our study, we could not assess the presence of the fragile site in *A. fragilis* and *A. veronensis* (or in other species). Nevertheless, we observed submetacentric chromosomes of pair No. 10 in all 12 sampled *A. fragilis* individuals, suggesting that either Dalcq (1921) and Margot (1946) studied populations with a fixed rearrangement,

or the morphology of the smallest macrochromosome was misidentified at that time.

Microchromosomes of *Anguis* display higher GC levels compared to macrochromosomes, which is consistent with other cytogenetic and genomic studies on reptiles (e.g. Ezaz *et al.* 2006, Kuraku *et al.* 2006, Kasai *et al.* 2019, Schield *et al.* 2019, Suryamohan *et al.* 2020, Oliveira *et al.* 2021, Koochekian *et al.* 2022; but see Alföldi *et al.* (2011) for a rather homogenous GC/AT content in *Anolis carolinensis* chromosomes). We observed no remarkable GC+ blocks on the macrochromosomes, except for the region bearing the rDNA clusters in *Pseudopus*, therefore the GC/AT pattern obtained does not indicate any apparent past fusion of macro- and microchromosomes (contrary to birds, where the GC/AT pattern revealed fusions of chromosomes in several lineages; O'Connor *et al.* 2019) and the 24 microchromosomes may represent the conserved, putatively ancestral state of squamates (Gorman 1973).

Contact zones and hybridization

Conserved karyotypes may facilitate hybridization between species in their secondary contact zones (e.g. Sember *et al.* 2020, Bedoya and Leaché 2021, Poignet *et al.* 2021). It may apply even in relatively deeply divergent, non-sister species such as *A. fragilis*–*A. colchica* (Szabó and Vörös 2014, Gvoždík *et al.* 2015, Benkovský *et al.* 2021) or *A. fragilis*–*A. graeca* (Mikulíček *et al.* 2018), both of which have the MRCA dated to 6.7 Mya (Gvoždík *et al.* 2023). In contrast, the sister-species pair *A. colchica*–*A. graeca* (MRCA 4.4 Mya) with very similar karyotypes do not hybridize due to lack of geographic contact, as their distribution ranges are separated by the range of *A. fragilis* (Jablonski *et al.* 2021). Their very similar karyotypes are likely a persistent ancestral condition from their MRCA, although past gene flow between *A. colchica* and *A. graeca* cannot be ruled out. Population size dynamics models show that the South Balkan lineage of *A. fragilis* has expanded since the last glacial maximum (LGM), whereas *A. graeca* has rather reduced its population size and possibly also its range (Jablonski *et al.* 2016). Thus, *A. graeca* and *A. colchica* are probably geographically separated at least since the LGM. The youngest sister-species pair *A. fragilis*–*A. veronensis* with a MRCA dated to about 2.7 Mya then forms a rather narrow hybrid zone (Dufresnes *et al.* 2023) despite similar karyotypes.

It is also worth noting that the phylogenetically and chromosomally (rDNA and ITR pattern, morphology of chromosome 8) most divergent *A. cephallonica* occurs in partial sympatry with *A. graeca* (Jablonski *et al.* 2016, 2021), but the two species also probably do not hybridize (Thanou *et al.* 2021). Differentiated karyotypes in sympatric species may indicate the presence of a reproductive isolation barrier. For example, Hooper and Price (2017) found in passerine birds that differences in chromosomal inversions correlate with overlapping ranges. However, how karyotypes change across contact zones of individual species of slow worms remains to be investigated.

Chromosome homeology with *Varanus* and karyotype evolution in Anguiformes

The *Anguis* and *Pseudopus* karyotypes differ in the number and shape of chromosomes from other anguiform lizards, which implies they likely underwent complex chromosomal rearrangements. Comparison of the available data on reptile chromosome synteny (Srikulnath *et al.* 2009, 2013, Lind *et al.* 2019, Koochekian *et al.* 2022; and reviewed in Deakin *et al.* 2016, Deakin and Ezaz 2019) with our results from mapping whole-macrochromosome probes of *V. komodoensis* onto the chromosomes of *A. fragilis* and *P. apodus* revealed four main evolutionary features. For simplicity, hereafter, we refer only to *Anguis* as the results for both anguinine genera were the same. First, chromosomes Nos 1 and 2 of *Varanus* share genetic content with chromosome Nos 2 and 1 of iguanians and snakes (i.e. the other two clades within toxicoferans besides anguiforms), but in *Anguis* these biarmed chromosomes split into AFR 2 and 8, and AFR 3 and 4, respectively. These results are also indirectly supported by the synteny mapping and genomic data from earlier studies (Pokorná *et al.* 2011, 2012, Lind *et al.* 2019). Second, chromosome 3 in *Varanus* is conserved with iguanians, but in snakes it forms two chromosomes (Deakin *et al.* 2016) and in *Anguis* it represents chromosome pair Nos 9 and 10. Third, chromosome 1 in *Anguis*, the largest metacentric chromosome in the karyotype, is formed by VKO 6 + 8. Considering these syntenies among Toxicofera, the macrochromosomes of *Varanus* and *Anguis* underwent one and three fissions with subsequent pericentromeric inversions, respectively, therefore we suggest that none of those two lineages represents an ancestral situation in Anguiformes (Fig. 6). The difficulties in detecting clear signals from VKO 4, 5 and overlap of VKO 6 + 8 probes might be attributed to turnover in repetitive DNA content (cf. Yoshido *et al.* 2013, Barby *et al.* 2019) between *Varanus* and *Anguis*. In this regard, future painting using avian whole-chromosome probes might be helpful (Pokorná *et al.* 2011, 2012), as avian genomes consist of a smaller proportion of non-coding repeats compared to non-avian reptiles (Primmer *et al.* 1997, Zhang *et al.* 2014).

Reconstruction of the ancestral karyotype of Anguiformes based solely on 2*n* and karyotype morphology is hampered by the extensive variation in both features and limited and unequal cytogenetic exploration across this group of lizards (Augstenová *et al.* 2021). In reptiles, major ribosomal RNAs (rDNAs) and telomeric repeats are the most used cytogenetic markers for estimating chromosome rearrangement rates and studying karyotype evolution. Both markers are conserved across vertebrates, making their detection via fluorescence *in situ* hybridization easy and reliable. In squamate reptiles, tandemly arrayed 18S and 28S rDNA sequences usually accumulate on only one chromosome pair (and more often on macrochromosomes, which is considered as a derived state; Sochorová *et al.* 2018, 2021), and only rarely have multiple rDNA clusters been found [e.g. in some iguanians (Altmanová *et al.* 2016, Rovatsos *et al.* 2019b)]. Anguiform lizards, including *P. apodus*, exhibit hybridization signals on one pair of micro- or macrochromosomes (*Heloderma* is an exception with two macrochromosome pairs; Augstenová *et al.* 2021). Therefore, it seems that the multiple rDNA clusters in *Anguis* are an evolutionary novelty. The rDNA loci are frequent recombination spots and their multiplication may originate from ectopic recombination and/or the activity of mobile elements or transposons (Eickbush and Eickbus 2007, Cazaux *et al.* 2011). The location in subtelomeric regions and co-localization with heterochromatin suggest that the rDNA loci in *Anguis* were amplified by some of the above mechanisms rather than by chromosomal rearrangements. Hence, the combination of missing data for most species of Anguioidea (so far

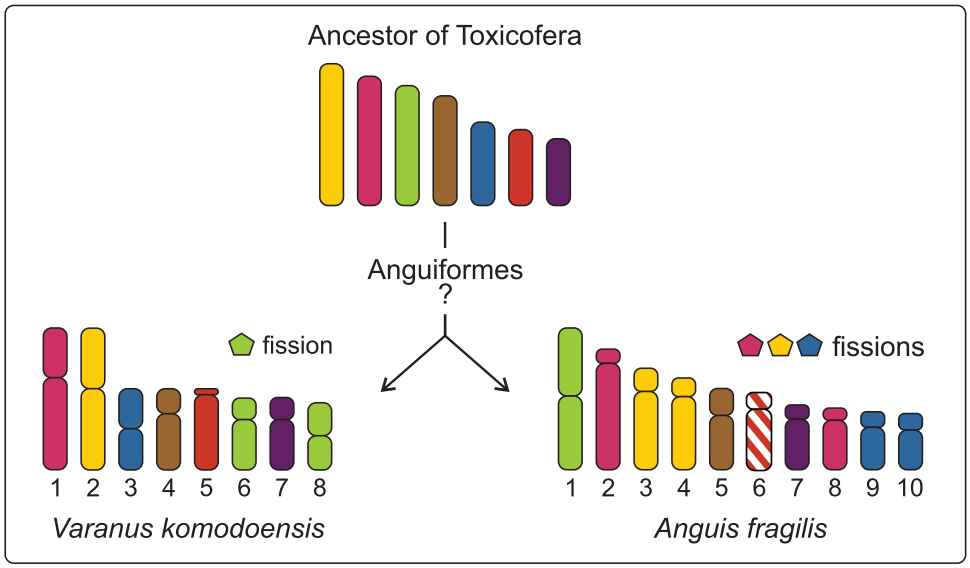

Figure 6. Schematic illustration of the homeology of the *Varanus* and *Anguis* + *Pseudopus* macrochromosomes with respect to the putative toxicoferan ancestor. The simplified arrangement of the macrochromosomes of the toxicoferan ancestor follows the hypothesis of Deakin and Ezaz (2019). Based on the fission(s) leading to *Varanus* and *Anguis*, we can assume that both lizards exhibit a derived stage of macrochromosome organization rather than variants of the putative ancestral arrangement of their common anguiform ancestor. The homeology of VKO 5 and AFR 6 (red and white hatched) is tentative and requires further evidence. The colour code depicts the chromosome homeology.

approximately 20% of species diversity has been explored cytogenetically, with the majority of them only by conventional karyotyping methods; reviewed in Augstenová *et al.* 2021), in general variable distribution of rDNA clusters (including different macrochromosomes and microchromosomes), and multiple rDNA loci in slow worms does not allow us to interpret the putative homeology of *Anguis* microchromosomes or to trace chromosome rearrangements and thus understand better the karyotype evolution in Anguiformes.

It is noteworthy that seven out of ten *Anguis* macrochromosome pairs possess (peri)centromeric ITRs (Fig. 5), particularly as four of these chromosomes have been proven by chromosome painting to be involved in chromosomal rearrangements (Fig. 6). However, without direct evidence of chromosome synteny, we can only speculate if ITRs are remnants of true telomeres resulting from pericentromeric inversions in the common *Anguis* + *Pseudopus* ancestor. For instance, ITRs are also found in the (peri)centromeric region of the largest chromosome of *A. cephallonica* and *P. apodus*, where intrachromosomal rearrangements cannot be ruled out, nevertheless, this chromosome appears to represent an ancestral syntenic group based on comparisons with the other two toxicoferan clades, iguanians and snakes (Deakin and Ezaz 2019; Fig. 6). Furthermore, ITRs were observed also on chromosomes 5 and 7 of *A. cephallonica* and *A. veronensis* (and also chromosome 7 in *A. fragilis*), but not in other species studied here, despite these chromosomes appearing to be conserved based on measured centromeric indices across *Anguis* + *Pseudopus*. In addition to chromosomal rearrangements, ITRs might be attributed to telomere-like sequences having originated as a common component of the satellite repetitive motif in most centromeres (as reported e.g. in Rovatsos *et al.* 2011) or as a result of retrotransposon activity (reviewed in Bolzán 2017, Vicari *et al.* 2022), and their detectability depends on amplification/erosion of repeats during genome evolution. ITRs have been found in many squamate reptiles (Rovatsos *et al.* 2015), and in some of them the repeat accumulations were abundant and could be linked with chromosomal rearrangements (e.g. iguanians or snakes; Altmanová *et al.* 2016, Viana *et al.* 2016, Augstenová *et al.* 2019, Rovatsos *et al.* 2019b). In anguiforms, however, ITRs are relatively rare despite extensive karyotype changes, and in the superfamily Anguioidea they have so far only been detected in the xenosaurid *Xenosaurus platyceps* King & Thompson, 1968 and the diploglossid *Caribicus warreni* (Augstenová *et al.* 2021). Therefore, we hypothesize that ITRs could emerge in the common *Anguis* + *Pseudopus* ancestor during the chromosome rearrangements and/or via a stochastic process of mobile element or transposon activity, followed by species-specific amplification or erosion of these repeats. The comparison of chromosome synteny and centromeric content within related lineages would address these questions.

Sex determination in *Anguis* in the context of Anguiformes

Sex chromosomes have been cytogenetically detected in several species of Anguiformes, but most notably in the genera *Heloderma* and *Varanus* (Johnson Pokorná *et al.* 2014, 2016, Matsubara *et al.* 2014, Iannucci *et al.* 2019b, Augstenová *et al.* 2021, Mezzasalma *et al.* 2021 and references therein). Putative sex chromosomes have also been revealed in one species in the genera *Abronia* and *Gerrhonotus* (Anguidae, Gerrhonotinae) and one species of *Caribicus* (Diploglossidae) (Augstenová *et al.* 2021). All of them were of the ZZ/ZW type with apparent heterochromatinization and/or accumulation of female-specific genetic content in the W chromosome. Chromosome painting with the VKO Z probe revealed sex chromosome homology across nine *Varanus* species (Iannucci *et al.* 2019b), later confirmed by molecular testing of sex-linked genes, and evidence of the common sex chromosomes

was further extended to other *Varanus* species and to the genera *Heloderma* and *Abronia*, suggesting that all Anguiformes could have conserved ZW sex chromosomes (Rovatsos *et al.* 2019a). Interestingly, *Abronia*, a New World anguid, also possesses these homologous sex chromosomes but, on the other hand, *Anguis fragilis* shows a (pseudo)autosomal pattern regarding potential sex chromosomes (Rovatsos *et al.* 2019a).

Neither previous cytogenetic research in *A. veronensis* (Mezzasalma *et al.* 2013), nor our experiments on the herein studied *Anguis* species, revealed evidence of differentiated sex chromosomes. The success of the CGH technique to detect sex chromosomes depends on the amount and level of differentiation of sex-specific genetic content (e.g. Altmanová *et al.* 2016, Deon *et al.* 2020, Augstenová *et al.* 2021, Kostmann *et al.* 2021, Štundlová *et al.* 2022). In the *Anguis* species tested here, CGH either detected no sex difference, as in *A. colchica* and *A. cephallonica*, or it revealed regions predominantly stained by either a female- or male-specific probe on a few microchromosomes in *A. fragilis* and *A. graeca*. However, we assume that the positive signals are more likely to reflect differences in rDNA gene copy number between individuals, because the signals were both male- and female-specific on chromosome spreads in both sexes (for a similar case, see Marajó *et al.* 2022). We are aware that the sex chromosomes of some reptile species harbour rDNA sites (e.g. Montiel *et al.* 2016, Kostmann *et al.* 2020, 2021, Mazzoleni *et al.* 2020). While this possibility for *Anguis* cannot be entirely excluded, our results do not provide any conclusion on this matter.

Although we cannot rule out environmental sex determination in *Anguis*, we incline to the possibility that slow worms have genetic sex determination with cryptic sex chromosomes. These may (i) be of the same origin as those found in some other anguiform lizards (e.g. *Abronia*, *Heloderma*, *Varanus*), but restored recombination between most of Z and W chromosomes may have caused an autosomal pattern; or (ii) evolved from a different linkage group, and remained at a stage of differentiation that does not allow them to be detected by the methods applied here. Recently, a cryptic ZW sex chromosome system evolved from a different linkage group has been described in *Shinisaurus crocodilurus* Ahl, 1930, which is closely related to *Varanus* (Pinto *et al.* 2023). Thus, *S. crocodilurus* brings the first direct evidence of sex chromosome turnover in Anguiformes, suggesting that besides the *Varanus*-like ZW sex chromosomes, other cryptic or poorly differentiated sex chromosome systems can exist in this group. For the spectrum of sex chromosome fates in vertebrates, see the review by Kratochvíl *et al.* (2021b). To conclude, the future search for subtle sex-specific differences in *Anguis* will require deeper and more sensitive methods such as genomic or transcriptomic approaches, as has been shown in similar case studies (Acosta *et al.* 2019, Nielsen *et al.* 2019, Pinto *et al.* 2023).

CONCLUSIONS

Our findings underscore the importance of comparative cytogenomic methods because conserved karyotypes as represented by slow worms may undergo different structural rearrangements that may remain hidden to conventional cytogenetic techniques. Although slow worms exhibit remarkably conserved karyotypes that may facilitate interspecific hybridization in secondary contact zones, species-specific patterns of distribution of repetitive sequences accompanied by heterochromatinization can be detected upon closer examination. These detected cytogenetic differences could be of interest for hybrid zone studies, as species-specific patterns appear to be conserved across different intraspecific populations, including geographically distant localities or distinct genetic lineages/haplogroups. In the context of deeper phylogeny, our results suggest that karyotype evolution in anguiform lizards is a complex process involving inter- and intrachromosomal rearrangements and deserves further investigation. The same applies for the sex determination mechanism of slow worms, which remains unknown.

SUPPLEMENTARY DATA

Supplementary data is available at *Zoological Journal of the Linnean Society* Journal online as Supporting information.

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AUTHOR CONTRIBUTIONS

Marie Altmanová (Conceptualization, Methodology, Resources, Investigation, Formal Analysis, Visualization, Writing—original draft, Writing—review & editing), Marie Doležálková–Kaštánková (Conceptualization, Methodology, Resources, Investigation, Formal Analysis, Writing—review & editing), Daniel Jablonski (Resources, Writing—review & editing), Ilias Strachinis (Resources, Writing—review & editing), Vladislav Vergilov (Resources, Writing—review & editing), Emiliya Vacheva (Resources, Writing—review & editing), Alessio Iannucci (Resources, Investigation, Writing—review & editing), Lukáš Choleva (Resources, Writing—review & editing), Petr Ráb (Methodology, Supervision, Resources, Writing—review & editing), Jiří Moravec (Conceptualization, Supervision, Funding acquisition, Resources, Investigation, Writing—review & editing), Václav Gvoždík (Conceptualization, Supervision, Funding acquisition, Resources, Investigation, Writing—original draft, Writing—review & editing).

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All procedures followed ethical statements, and the manipulation of animals was minimized and their fitness not harmed. The blood samples were collected by accredited persons (M. Altmanová: CZ01223, V. Gvoždík: CZ02519, J. Moravec: CZ01242) under approved experimental project no. MZP/2018/630/2449. The field research in Albania was conducted with the permission No. 480/2019, issued by the Ministry of Tourism and Environment, National Agency of Protected Areas. The sample of *Anguis veronensis* was collected in Tuscany (Italy) under the permit number 0115032 of 23 March 2020 granted to A.I. by Regione Toscana.

DATA AVAILABILITY STATEMENT

All the data obtained are contained in this article and its supplementary information. New DNA sequences are available in the GenBank Nucleotide Database under accession numbers OR352076–OR352110.

COMPETING INTERESTS

The authors declare no competing interests.

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