Table S1 *Anguis* and *Pseudopus* sampling, origin of individuals and methods used. F – female, M – male, UN – juvenile with unknown sex; GR – Greece, CZ – Czech Republic, BG – Bulgaria, AL – Albania; CMA3 – Chromomycin A3 GC+ staining, FISH – fluorescence *in situ* hybridization, CGH – intersexual comparative genomic hybridization. Chromosome mapping of *Varanus* macrochromosome probes were applied to *A. fragilis* (AFR F4) and *P. apodus* (PAP UN).

Species	Individual and sex	Locality	Karyotyping, C-banding, CMA3 (GC+), 18S rDNA FISH, telomeric FISH	CGH
A. cephallonica	ACE F1	GR Kefalonia, 38.129N 20.795E	✓	✓
	ACE F2	GR Peloponnese, 38.136N 21.644E	✓	–
	ACE M1	GR Kefalonia, 38.129N 20.795E	✓	✓
	ACE M2	GR Kefalonia, 38.129N 20.795E	✓	✓
A. colchica	ACO F1	CZ Křišťanovice, 49.868N 17.508E	✓	✓
	ACO F2	CZ Odry-Dobešov, 49.673N 17.773E	✓	✓
	ACO F3	CZ Odry-Veselí, 49.647N 17.817E	✓	✓
	ACO F4	BG Gara Lakatnik, 43.095N 23.370E	✓	–
	ACO M1	CZ Potštát-Boškov, 49.623N 17.600E	✓	✓
	ACO M2	CZ Odry-Dobešov, 49.681N 17.768E	✓	✓
	ACO M3	CZ Rajnochovice-Kotáry, 49.370N 17.835E	✓	✓
	ACO M4	CZ Vítkov-Hadinka, 49.737N 17.693E	✓	✓
	ACO M5	BG Karlukovo, 43.180N 24.060E	✓	–
	ACO M6	BG Chibaovtsi, 42.916N 23.265E	✓	–
A. fragilis	AFR F1	CZ Želízy, 50.424N 14.463E	✓	✓
	AFR F2	CZ Želízy, 50.428N 14.468E	✓	✓
	AFR F3	CZ Bransouze, 49.302N 15.754E	✓	✓
	AFR F4	CZ Nová Knížecí Huť, 49.734N 12.456E	✓	✓
	AFR F5	CZ Aš-Mokřiny, 50.211N 12.213E	✓	✓
	AFR F6	BG Krushovitsa, 42.555N 23.610E	✓	–
	AFR F7	BG Krushovitsa, 42.555N 23.610E	✓	–
	AFR M1	CZ Hazlov-Podílná, 50.146N 12.238E	✓	✓
	AFR M2	CZ Praha-Modřany, 49.999N 14.438E	✓	✓
	AFR M3	CZ Praha-Modřany, 49.999N 14.438E	✓	✓
	AFR M4	CZ Želízy, 50.424N 14.471E	✓	✓
	AFR M5	BG Krushovitsa, 42.555N 23.610E	✓	–
A. graeca	AGR F1	AL Qazim Pali, 40.050N 19.841E	✓	✓
	AGR F2	GR Igoumenitsa, 39.519N 20.240E	✓	✓
	AGR F3	GR Ioannina, 39.684N 20.875E	✓	✓
	AGR M1	AL Muzinë, 39.938N 20.220E	✓	✓
	AGR M2	AL Qazim Pali, 40.032N 19.887E	✓	–
	AGR M3	GR Igoumenitsa, 39.519N 20.240E	✓	✓
	AGR M4	GR Igoumenitsa, 39.519N 20.240E	✓	✓
P. apodus	PAP UN	AL Baks i Ri, 41.910N 19.431E	✓	–

Supplementary Methods

Chromosome preparations

Briefly, 100–150 μL of fresh anticoagulated full blood was cultivated in 4 mL of cultivation medium consisting of Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO, USA), enriched with 10% (v/v) foetal bovine serum (Biowest, Nuaillé, France), 3% (v/v) phytohaemagglutinin (GIBCO, Carlsbad, CA, USA), 1% (v/v) L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 1% (v/v) penicillin/streptomycin solution (GIBCO, Carlsbad, CA, USA) and 1% (v/v) lipopolysaccharide (Sigma-Aldrich, St. Louis, MO, USA) for one week at 28 °C. Prior to harvesting, the cell division was arrested in metaphase by adding 35 μL of colcemid (Roche, Basel, Switzerland) and incubating for 3.5 h at 28 °C. The cells were hypotonized in 5 mL of 0.075 M KCl for 40 min at 37 °C. Released nuclei and chromosomes were fixed by adding 100 μL of cold Carnoy's fixative solution (methanol:acetic acid in 3:1) and by following three rounds of fixation, i.e. replacing the supernatant by fresh cold Carnoy's solution. The chromosomal suspensions were stored at -20 °C.

C-banding

The chromosome spreads were aged for 1 h at 60 °C, then soaked in 0.2 N HCl for 30 min at RT, rinsed in dH_2O, soaked in 5% $Ba(OH)_2$ solution for 5 min at 49 °C, rinsed in 0.2 N HCl and in dH_2O and finally soaked in 2× saline-sodium citrate (SSC) buffer for 1 h at 60 °C. The slides with the chromosome spreads were stained and mounted in Vectashield Antifade Medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA).

18S rDNA probe preparation

The 18S rDNA sequence fragment was amplified from genomic DNA of *Anguis fragilis* (AFR F2) using the primers 18SF and 18SR (Cioffi et al. 2009). The 50 μL PCR reaction mix consisted of 20 μmol of each primer, 100 ng DNA, 25 μL PPP master mix (Top-Bio, Vestec, CZ), and PCR H_2O. Primer sequences and PCR conditions are specified in Table S2.

Table S2 Primer sequences and PCR conditions for amplification of 18S rDNA

Primer (5'–3')	**PCR conditions**			
	Step	**Temperature (°C)**	**Time (min)**	**Cycles**
Forward	Initial denaturation	95	5:00	1×
18SF: CCGAGGACCTCACTAAACCA	Denaturation	95	1:00	35×
Backward	Annealing	60	1:00	
18SR: CCGCTTTGGTGACTCTTGAT	Extension	72	1:30	
	Final extension	72	10:00	1×

PCR products were purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel GmbH, Düren, Germany), cloned to pDrive Cloning Vector (Qiagen, Hilden, Germany), and transformed into QIAGEN EZ Competent Cells (Qiagen). Candidate recombinant plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen) and sequenced bi-directionally in Macrogen. The homology of resulting sequences and target 18S rDNA gene were compared with existing libraries using BLAST search (Altschul et al. 1990) and selected clones were used for following probe preparation. Before the nick-translation labeling, the resulting plasmid DNA was amplified via PCR under the conditions described above. The sequence of the 18S rDNA fragment is available in Poignet at al. (2021; Supplementary data).

The probe was labeled by biotin-dUTP (Roche, Mannheim, Germany) using Nick Translation kit (Abbott Laboratories, Chicago, IL, USA) following the manufacturer's instructions and precipitated overnight at -20 °C with 2.5 µL of sonicated salmon sperm DNA (Sigma-Aldrich, St. Louis, MO, USA) and enriched by 0.1% (v/v) 3 M sodium acetate (ThermoFisher Scientific) and 2.5% (v/v) cold 96% ethanol. The probe was washed in 70% ethanol, air-dried, and resuspended in hybridization buffer (pH = 7) containing 50% formamide, 2× SSC, 10% dextran sulphate, 10% sodium dodecyl sulphate, and 1× Denhard's buffer, with the final concentration 20 ng/µL of labeled DNA. The probe was denatured for 6 min at 75 °C and chilled on ice prior to hybridization.

Fluorescence* in situ *hybridization with 18S rDNA probe

Slides with chromosomal spreads were aged for 1 h at 60 °C and went through RNase A (150 µL with concentration 133 ng/µL in 2× SSC on each slide, 60 min at 37 °C; Sigma-Aldrich, St. Louis, MO, USA) and pepsin (150 µL with concentration 170 ng/µL in 0.001 N HCl on each slide, 10 min at 37 °C; Sigma-Aldrich, St. Louis, MO, USA) treatment, followed by treatment in 1% formaldehyde in 1× PBS (10 min) with intermediate washes in 1× PBS and final dehydration in ethanol series (70%, 85%, 96% ethanol). Chromosomes were denatured in 70% formamide/ 2× SSC for 3 min at 75 °C, dehydrated through an ethanol series and air-dried. On each side, 15 µL of probe (i.e., 300 ng of labeled DNA) was hybridized on chromosome spreads overnight at 37 °C.

Post-hybridization washes were performed in 50% formamide/ 2× SSC for 3×5 min at 40 °C, 2×5 min in 2× SSC at room temperature (RT), 1×5 min in 4× SSC/ 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) at RT. To minimize the unspecific fluorochrome binding, the blocking reagent (Roche, Basel, Switzerland) was applied for 30 min at 37 °C. The probe signal was amplified using Fluorescein Avidin D (30 min at 37 °C; Vector Laboratories, Burlingame, CA, USA) and Biotinylated Anti-Avidin D (20 min at 37 °C; Vector Laboratories, Burlingame, CA, USA) antibodies complex and one final application of Fluorescein Avidin D, with intermediate washes in 4× SSC/ 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) for 3×5 min at RT. After the last application of antibodies, the slides were washed in 4× SSC/ 0.1% Tween 20 for 2×5 min and in 1× phosphate-buffered saline (PBS)

solution for 5 min at RT and finally mounted and stained in Vectashield Antifade Medium containing DAPI.

Intersexual comparative genomic hybridization (CGH)

For each test, 1 μg of male DNA and 1 μg of female DNA were used for probe preparation. Whole genomic DNA was directly labeled during nick-translation using a commercial kit, male DNA was labeled with Fluorescein NT Labeling Kit (Jena Bioscience, Jena, Germany) and female DNA was labeled with Cy3 NT Labeling Kit (Jena Bioscience, Jena, Germany), following the manufacturer's protocols. After nick-translation, male and female products were co-precipitated together overnight at -20 °C with adding 5 μL of sonicated salmon sperm DNA (Sigma-Aldrich, St. Louis, MO, USA), 0.1% (v/v) 3M sodium acetate and 2.5% (v/v) cold 96% ethanol. The probe was then washed in 70% ethanol and resuspended in a 30 μL hybridization buffer (containing the same mixture as for rDNA probe preparation), 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, 15 μL of probe (i.e., 500 ng of male and 500 ng of female labeled DNA) was hybridized on chromosome spreads for 3 days at 37 °C. Post-hybridization washes were performed in 1× SSC for 2×5 min at 65 °C, in 4× SSC/ 0.1% Tween 20 for 5 min at 44 °C, soaked in 1× PBS for 1 min at RT and rinsed in dH_20. Slides were mounted and counterstained by Vectashield Antifade Mounting Medium with DAPI.

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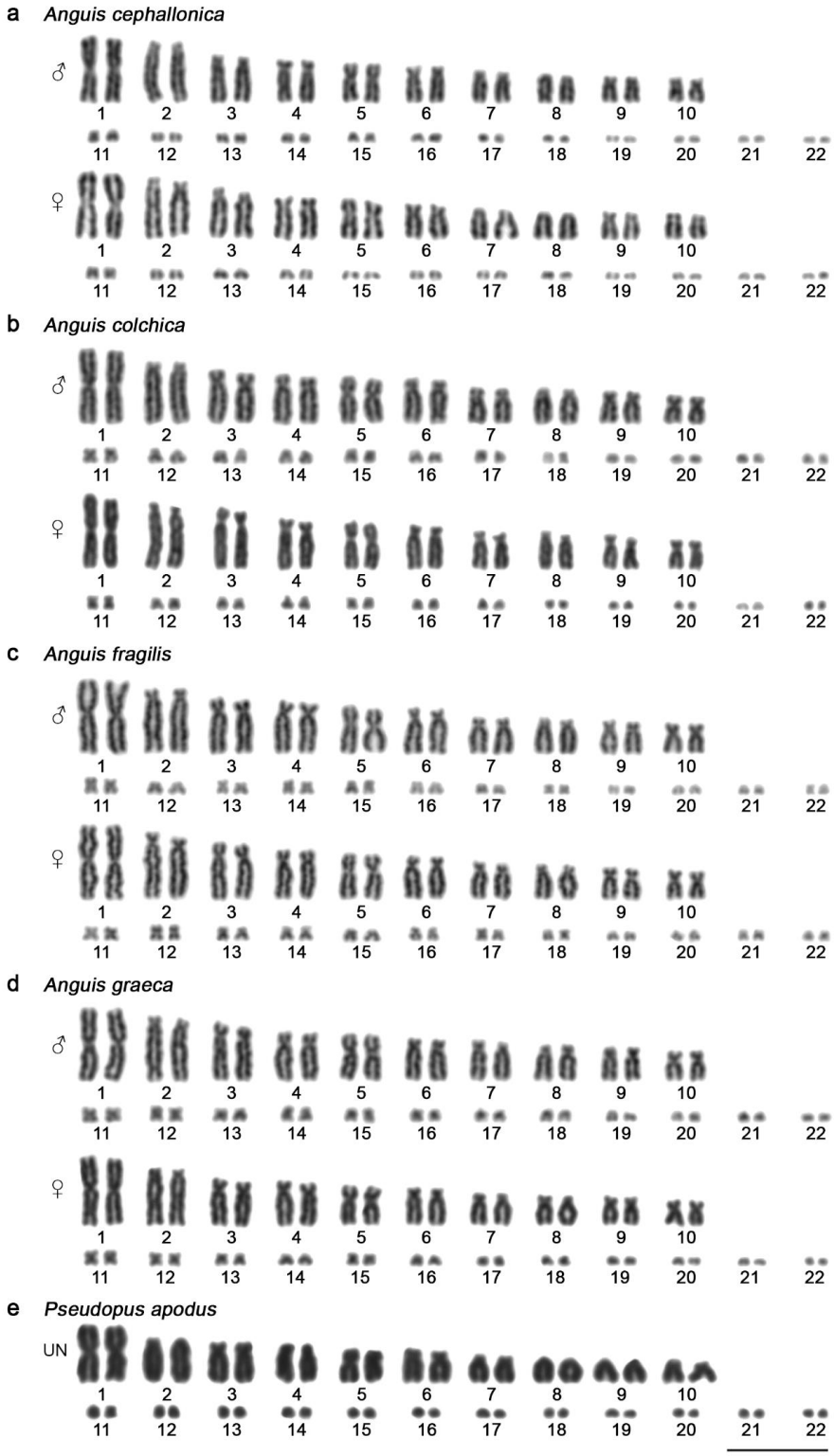

Fig. S1 Karyograms of *Anguis* and *Pseudopus*. Karyogram of *Pseudopus apodus* originates from juvenile of unknown sex. All tested individuals share the karyotype with $2n = 44$ consisting of 20 macrochromosomes and 24 microchromosomes. No sex differences are apparent. Scale bar = 10 μm

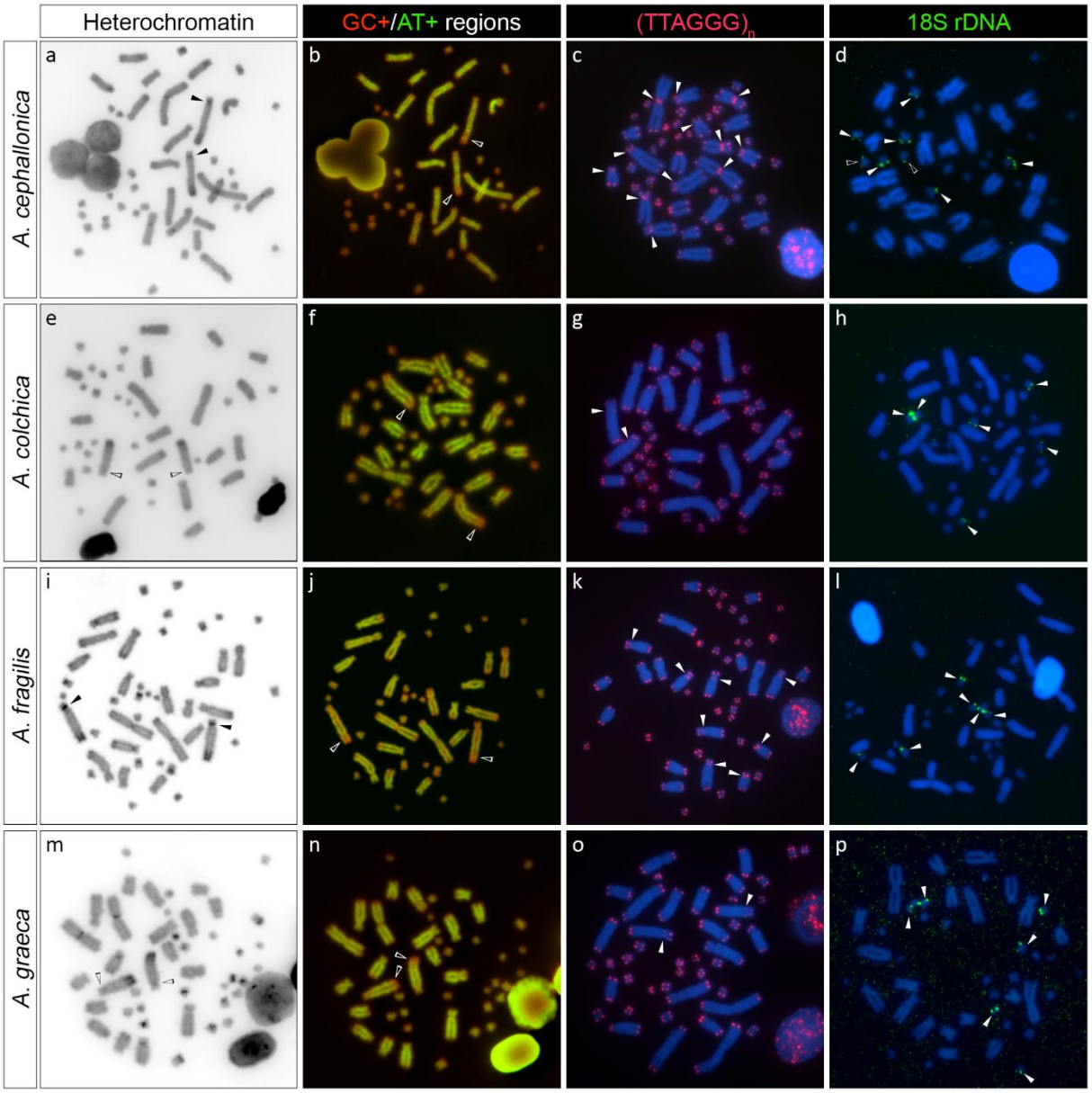

Fig. S2 Detection of constitutive heterochromatin (first column from the left side), GC/AT base-specific staining (second column, pseudocolored), distribution of telomeres and ITRs (third column), topology of 18S rDNA gene clusters (fourth column) in Anguis females. *First column (a, e, i, m):* Presence (full arrowhead) and absence (empty arrowhead) of centromeric heterochromatin in chromosome pair 2. *Second column (b, f, j, n):* Diffused GC+ pattern in distal part of pair 2 (empty arrowhead). *Third column (c, g, k, o):* ITRs (full arrowhead). *Fourth column (d, h, l, p):* Hybridization of 18S rDNA on three pairs of microchromosomes (full arrowheads) and additional weak signal on another microchromosome pair (empty arrowheads).