Biological Journal of the Linnean Society, 2022, **136**, 405–416. With 2 figures.

Identification of sex chromosomes using genomic and cytogenetic methods in a range-expanding spider, *Argiope bruennichi* (Araneae: Araneidae)

MONICA M. SHEFFER[1,*,†], MATHILDE CORDELLIER[2], MARTIN FORMAN[3], MALTE GREWOLDT[2,‡], KATHARINA HOFFMANN[2], CORINNA JENSEN[4,5], MATĚJ KOTZ[3], JIŘÍ KRÁL[3], ANDREAS W. KUSS[4,5], EVA LÍZNAROVÁ[3,6] and GABRIELE UHL[1]

[1]*Zoological Institute and Museum, University of Greifswald, 17489 Greifswald, Germany*
[2]*Institute of Animal Cell and Systems Biology, University of Hamburg, 20146 Hamburg, Germany*
[3]*Department of Genetics and Microbiology, Charles University, Viničná 5, 128 44 Prague 2, Czech Republic*
[4]*Center for Functional Genomics of Microbes, University of Greifswald, 17489 Greifswald, Germany*
[5]*Interfaculty Institute for Genetics and Functional Genomics, University of Greifswald, 17489 Greifswald, Germany*
[6]*Department of Botany and Zoology, Masaryk University, 611 37 Brno, Czech Republic*

Received 6 October 2021; revised 18 March 2022; accepted for publication 24 March 2022

Differences between sexes in growth, ecology and behaviour strongly shape species biology. In some animal groups, such as spiders, it is difficult or impossible to identify the sex of juveniles based on external morphology. This information would be useful for field surveys, behavioural experiments and ecological studies, such as those on sex ratios and dispersal. In species with sex chromosomes, sex can be determined based on the specific sex chromosome complement. Additionally, information on the sequence of sex chromosomes provides the basis for studying sex chromosome evolution. We combined cytogenetic and genomic data to identify the sex chromosomes in the sexually dimorphic spider *Argiope bruennichi* and designed quantitative real-time polymerase chain reaction sex markers. We found that the genome size and GC content of this spider fall into the ranges reported for the majority of araneids. The male karyotype is formed by 24 acrocentric chromosomes with an X_1X_20 sex chromosome system, with little similarity between X chromosomes, suggesting an origin of these chromosomes by X chromosome fission or early duplication of an X chromosome and subsequent independent differentiation of the copies. Our data suggest X chromosomes of similar sizes in *A. bruennichi*. They are smaller chromosomes of the complement. Our findings open the door to new directions in spider evolutionary and ecological research.

ADDITIONAL KEYWORDS: Araneae – Araneidae – *Argiope bruennichi* – genome size – karyotype – sex chromosome.

INTRODUCTION

In most animals, sex is determined genetically. Genotypic mechanisms of sex determination are very diverse, depending, for example, on the ratio of X chromosomes to autosomes, on sex-determining factor(s) localized on autosomes and/or sex chromosomes, on the presence or absence of a sex chromosome specific for the heterogametic sex (Y or W), or on ploidy level, as in many Hymenoptera (Bachtrog *et al.*, 2014; Hamm *et al.*, 2015; Vicoso, 2019).

In animal species with genotypic sex determination, females and males often differ in the type and/or number of sex chromosomes, with many variations. Knowledge about the karyotype and the specifics of the sex chromosomes provides the basis for studying

*Corresponding author. E-mail: monicasheffer.research@gmail.com
†Current address: Department of Biology, University of Washington, Seattle, WA 98195, USA.
‡Current address: Department of Molecular Biology and Genetics, Aarhus University, 8000 Aarhus, Denmark.

a plethora of ecological and evolutionary questions. For ecological studies, it is desirable to know the sex of collected individuals. Offspring sex allocation, sex-specific growth and dispersal strategies, and sex differences in early developmental pathways and physiological plasticity can then be investigated (Cordellier *et al.*, 2020). However, in many animal taxa the sexes cannot be identified easily in adults and even less so in juveniles. The sex of early stages is often only determinable based on their internal anatomy (presence of testes or ovaries) or on their sex chromosome complement (e.g. Avilés & Maddison, 1991).

Spiders belong to one of the most diverse animal orders (Coddington & Levi, 1991) and play an important role in terrestrial ecosystems; however, it is impossible to sex juvenile spiders in the field. Spiders possess complex sex chromosome systems. To date, karyotypes of > 800 spider species have been characterized (Araujo *et al.*, 2021). In 67% of karyotyped spiders (calculated at the time of publication), and particularly in the entelegyne spiders, which are the most studied, there are two X chromosomes, X_1 and X_2 (X_1X_20 system), where the male exhibits one copy and the female two copies ($♂X_1X_2/♀X_1X_1X_2X_2$) (Araujo *et al.*, 2012). This pattern is probably ancestral for entelegynes (Král *et al.*, 2006). Systems with three X chromosomes or more also exist. These X chromosomes are supposedly non-homologous based on their achiasmatic pairing during male meiosis (Kořínková & Král, 2013). Some lineages of entelegyne spiders exhibit an X0 system, which has originated via X chromosome fusions, or neo-sex chromosomes, which arose by rearrangements between the X chromosome(s) and autosome(s). Neo-sex chromosome systems contain both X and Y chromosome(s). Systems formed by two or more X chromosomes are termed multiple X chromosome systems (White, 1973).

Owing to the different numbers of X chromosome copies in males and females, sexing could be achieved in spiders once targeted molecular markers for X chromosomes are available. The relative copy number of sequences on the X chromosomes, with males having half the number of copies relative to females, could be assessed through quantitative real-time polymerase chain reaction (qPCR). Sexing performed in this manner can be done at a high throughput, making it reasonable for ecological studies with relatively large sample sizes.

In recent years, several approaches to identify X chromosomal sequences have been introduced that rely on high-throughput sequencing methods. These methods were reviewed by Palmer *et al.* (2019) and vary in their costs, in required sample sizes and on the availability of a reference genome (Al-Dous *et al.*, 2011; Gautier, 2014; Picq *et al.*, 2014; Hou *et al.*, 2015; Muyle *et al.*, 2016). In species with a chromosome-level genome assembly, comparison of the sequencing coverage depth across chromosomes between individuals of each sex is a reliable method for identifying sex chromosomes. Females, as the homogametic sex, would show twice as much coverage as males on X chromosomes (i.e. Vicoso & Bachtrog, 2011; Fraïsse *et al.*, 2017).

An annotated genome assembled to the haploid chromosome level was published by Sheffer *et al.* (2021) for an entelegyne orb-weaving spider, *Argiope bruennichi* (Scopoli, 1772). With this chromosome-level genome, we can use the coverage depth approach to identify sex chromosome sequences in this species. *Argiope bruennichi* is a sexually dimorphic species with a Palaearctic distribution, which has rapidly expanded its range in Europe over the course of the last century (Krehenwinkel & Tautz, 2013; Krehenwinkel *et al.*, 2015; Wawer *et al.*, 2017). Sex differences in the early life stages of these spiders are particularly interesting, given that juveniles perform aerial dispersal with silk (ballooning), a behaviour which is restricted to the first few instars (Krüger, 2014) and might be sex biased (Krehenwinkel *et al.*, 2016).

The *A. bruennichi* genome assembly was based upon female specimens and is composed of 13 scaffolds, which suggests $2n = 26$. This number of chromosomes is found in females of most orb-weaving spiders, and it is consistent with the diploid chromosome number of the species published by Zhang & Tong (1990). Their study, however, contains no information on the number or sex of the individual(s) examined. With additional karyological and sequence data for males, it would be possible to ascertain the karyotype/sex determination system of the species and identify the sex chromosomes in the published assembly.

We set out to determine the karyotype and genome size of *A. bruennichi*, identify which superscaffolds represent sex chromosomes in the genome assembly and develop molecular markers for sexing spiders using qPCR.

MATERIAL AND METHODS

Chromosome preparations and their evaluation

We collected eight subadult *A. bruennichi* males for chromosomal analysis at village Březová, Czech Republic (49.901N, 13.887E) in late June 2021. The number of meiotic cells in the testes of these males was lower than that found in subadult or young adult males of other araneids (M. Kotz and M. Forman, unpublished observation). We followed the protocol of Dolejš *et al.* (2011) for preparation of chromosome slides and Giemsa staining. We inspected the slides under an Olympus BX 50 microscope and photographed selected plates using an Olympus DP 71

CCD camera. We measured chromosome lengths using ImageJ (https://imagej.nih.gov/ij/index.html). The length ratio of sex chromosomes was established using 20 randomly selected diakinesis and metaphase I plates. Chromosome morphology was based on centromere position, which was apparent during early metaphase II.

Determination of genome size and GC content

We determined the diploid genome size (2C value) and guanosine cytosine (GC) content of *A. bruennichi* by flow cytometry using five adult males and five females (collected in July 2020, Albrechtice, Czech Republic, 49.924N, 16.658E, stored at –80 °C). Sample preparation was based on the protocol of Král *et al.* (2019), with a different buffer. We chopped the legs together with an internal plant standard in GBP buffer (Loureiro *et al.*, 2007), with the pH adjusted to 9.5 and 1.5% (v/v) of Triton X-100 (Sigma-Aldrich) and 1.5% (v/v) of polyvinylpyrrolidone (Sigma-Aldrich). After filtration through a nylon sifter, we added fluorescent dye. We performed two concurrent flow cytometry gauges, using: (1) propidium iodide (PI; 50 µg/mL), a non-specific dye to establish the genome size of the sample; and (2) 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; 4 µg/mL), a fluorochrome with higher specificity for AT pairs. The resulting histograms were evaluated using FloMax software (Partec). We calculated the 2C value as the ratio of PI fluorescence of the sample and the standard. We compared PI and DAPI ratios to establish the GC content as described by Šmarda *et al.* (2008). We measured each individual twice with PI and twice with DAPI to account for machine fluctuations. We used fresh leaves of *Pisum sativum* cultivar 'Ctirad' as an internal standard. We used the following genomic values of this plant for calculations: 2C = 7841.27 Mbp, GC = 41.77% (Veselý *et al.*, 2012). Primary data used for calculation of 2C and base content are available in the Supporting Information (Table S1).

Whole-genome sequencing of male and female *A. bruennichi*

DNA extraction and library preparation

We refer to chromosome-level scaffolds in the genome assembly as 'superscaffolds' and number all of them by length. For karyotype, we take the traditional approach and number autosomes by length and refer to X chromosomes separately.

In order to identify the X chromosomes in the *A. bruennichi* genome assembly, we sequenced one adult male and one adult female collected near Klausdorf, Germany at the end of July 2020 (54.424N, 13.029E). We extracted DNA from eight legs of the male and four legs of the female, as follows. We disrupted the leg tissue with a mortar, pestle and liquid nitrogen. The powdered tissue was transferred into tubes containing proteinase K and cell lysis buffer (10 mM Tris pH 8, 100 mM NaCl, 10 mM EDTA pH 8, 0.5% sodium dodecyl sulfate and double-distilled water). We incubated the tubes overnight at 55 °C, cooled them to room temperature and added RNAse A. We used 5 M NaCl to precipitate proteins and isopropanol to precipitate DNA. We cleaned the DNA extract using 70% ethanol and eluted it in TE buffer. We prepared the libraries with the NEBNext Ultra II FS DNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol. We indexed and amplified the libraries with six PCR cycles, then pooled them and sequenced the 2 × 75 bp paired-end libraries on one Flowcell of an Illumina NextSeq 550 with a High Output v.2 kit (150 cycles) (Illumina, San Diego, CA, USA).

Bioinformatic processing and coverage comparison

The reads were demultiplexed using the Illumina bcl2fastq tool in BaseSpace. We removed adapter sequences using AdapterRemoval (v.2.2.2; Schubert *et al.*, 2016) with the --identify-adapters flag. Read quality was assessed using FastQC (v.0.11.8; Andrews, 2010); all reads were high quality, and no further quality trimming was necessary. Adapter-trimmed reads are available under BioProject PRJNA629526. We mapped the reads of each specimen onto the *A. bruennichi* genome assembly using BWA-MEM (v.0.7.12-r1039) with default settings (Li, 2013), sorted and indexed the mapped reads using SAMtools sort and SAMtools index (SAMtools v.1.3.1; Li *et al.*, 2009), then calculated the coverage of each of the 13 superscaffolds using QualiMap (v.2.2.1; Okonechnikov *et al.*, 2016).

Development of sex markers using qPCR

DNA extraction

In total, we extracted genomic DNA from six males collected in Greifswald (54.093N, 13.366E) and six females collected near Klausdorf (54.424N, 13.029E), Germany at the beginning of August 2020, following either the procedure outlined above or a protocol based on the Promega ReliaPrep gDNA Tissue Miniprep System (see Supporting Information, Table S2). Before using the Promega kit, we ground two legs each from females or four legs each from males with a Dounce homogenizer; we added 200 µL of tail lysis buffer and 30 µL of proteinase K solution to the sample

and extracted the DNA following the manufacturer's instructions. We carried out the final elution step twice, to a total elution volume of 100 µL.

Furthermore, we extracted DNA from ten first instar spiderlings that came from one egg sac, laid by a female collected in Pärnu, Estonia in 2018 (58.297N, 24.597E). Genomic DNA was extracted as described in the previous section. Whole spiderling bodies were used, rather than legs, owing to their small size. First instar spiderlings from this population have an average mass of ~0.3 mg (M.M. Sheffer, personal observation). We determined the DNA concentration for each sample using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and a Qubit 4 Fluorometer with the Quant-iT Qubit dsDNA BR Assay Kit (Thermo Scientific). The DNA concentrations are given in the Supporting Information (Table S2).

Primer design, PCR and qPCR

We designed primers for loci on the putative X chromosomes (superscaffolds 9 and 10 in the *A. bruennichi* assembly; see Results section) and two putative autosomes, superscaffolds 5 and 7, using PRIMER3 (v.4.1.0; Untergasser *et al.*, 2012) with the following parameters: length between 20 and 25 nucleotides, melting temperature between 57 and 62 °C, region amplified between 150 and 200 bp in length, with one GC clamp (Supporting Information, Table S3). We used additional data to select the loci, such as the location of homologous sequences in the spiders *Trichonephila senegalensis* (Araneidae) (Walckenaer, 1841) (M. Grewoldt, J. Schneider, and M. Cordellier, unpublished data) and *Stegodyphus mimosarum* (Eresidae) Pavesi, 1883 (Bechsgaard *et al.*, 2019). We tested the amplification with conventional PCR, checked on a 1.5% agarose gel with ethidium bromide staining and ultraviolet transillumination. We then conducted qPCR using the EvaGreen qPCR-Mix II on a ThermoFisher StepOne Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Primer efficiencies were measured by diluting 20 ng/µL of template DNA from a female in a stepwise manner, 1:10 down to the lowest concentration of 0.02 ng/µL, to obtain a standard curve. After ensuring comparable efficiencies for all primer pairs, we used DNA from the six females and males as templates and measured amplification simultaneously in real time for each primer pair in triplicate. One non-template control was used for each primer combination. All amplification protocols were conducted with the following reaction mix: 2 µL EvaGreen qPCR-Mix II 5×, 0.2 µL forward primer, 0.2 µL reverse primer, 1 µL template DNA and 6.6 µL nuclease-free water. We used the following programme: initial denaturation for 15 min at 95 °C, 40 cycles of denaturation (15 s at 95° C), annealing (20 s at 60 °C) and extension (20 s at 72 °C), with a final extension at 72 °C for 10 min and a melting curve (ramping from 65 to 95 °C). The cycle threshold (*Ct*) values were collected for further analysis (Supporting Information, Table S2).

After validation of the method and determination of effective markers (see Results) using the adult specimens, we used a subset of markers (see Supporting Information, Tables S2 and S3) to determine the sex of the spiderlings.

qPCR data analysis

We applied the $2^{-\Delta\Delta Ct}$ method according to Rao *et al.* (2013) to analyse the qPCR data. To account for different primer efficiencies (E_{real}), the *Ct* values were normalized to correspond to an optimal efficiency of 100% (E_{opt}). We averaged the *Ct* values of the three technical replicates; if any of the three replicates showed a standard deviation greater than one, it was excluded from the average. The ΔCt value was calculated from the difference between reference sequences (autosomal loci) and a target sequence of interest (X-linked locus). Furthermore, the female data points were used as the reference, because females possess two sex-linked and two autosomal gene copies. Consequently, if the number of gene copies between females and males is the same, a value of one is expected, whereas if the number of gene copies in males is half that in females, a value of 0.5 is expected.

RESULTS

CHROMOSOMES

The male karyotype of *A. bruennichi* was formed by 24 acrocentric chromosomes; the size difference between chromosomes was gradual from the longest to the shortest (Fig. 1A). Analysis of male meiotic division revealed an X_1X_20 sex chromosome system (Fig. 1B, E–J). Chromosomes X_1 and X_2 were similar in length; the length of X_2 amounted to 91.3 ± 5.3% of X_1. We observed almost the complete course of male meiotic division, except for anaphase I, telophase I, and telophase II. The X chromosomes were positively heteropycnotic (i.e. they stained more intensively) from the beginning of meiosis until diakinesis (Fig. 1B–E). The intensity of heteropycnosis decreased during metaphase I; X chromosomes exhibited isopycnosis or a slight positive heteropycnosis during this period (Fig. 1F). Positive heteropycnosis reappeared during prophase II (Fig. 1G). During metaphase II (Fig. 1H), X chromosomes exhibited a slight positive heteropycnosis only. This pattern was also displayed at late anaphase II (Fig. 1J). Tight pairing of X_1 and X_2 chromosomes was initiated at

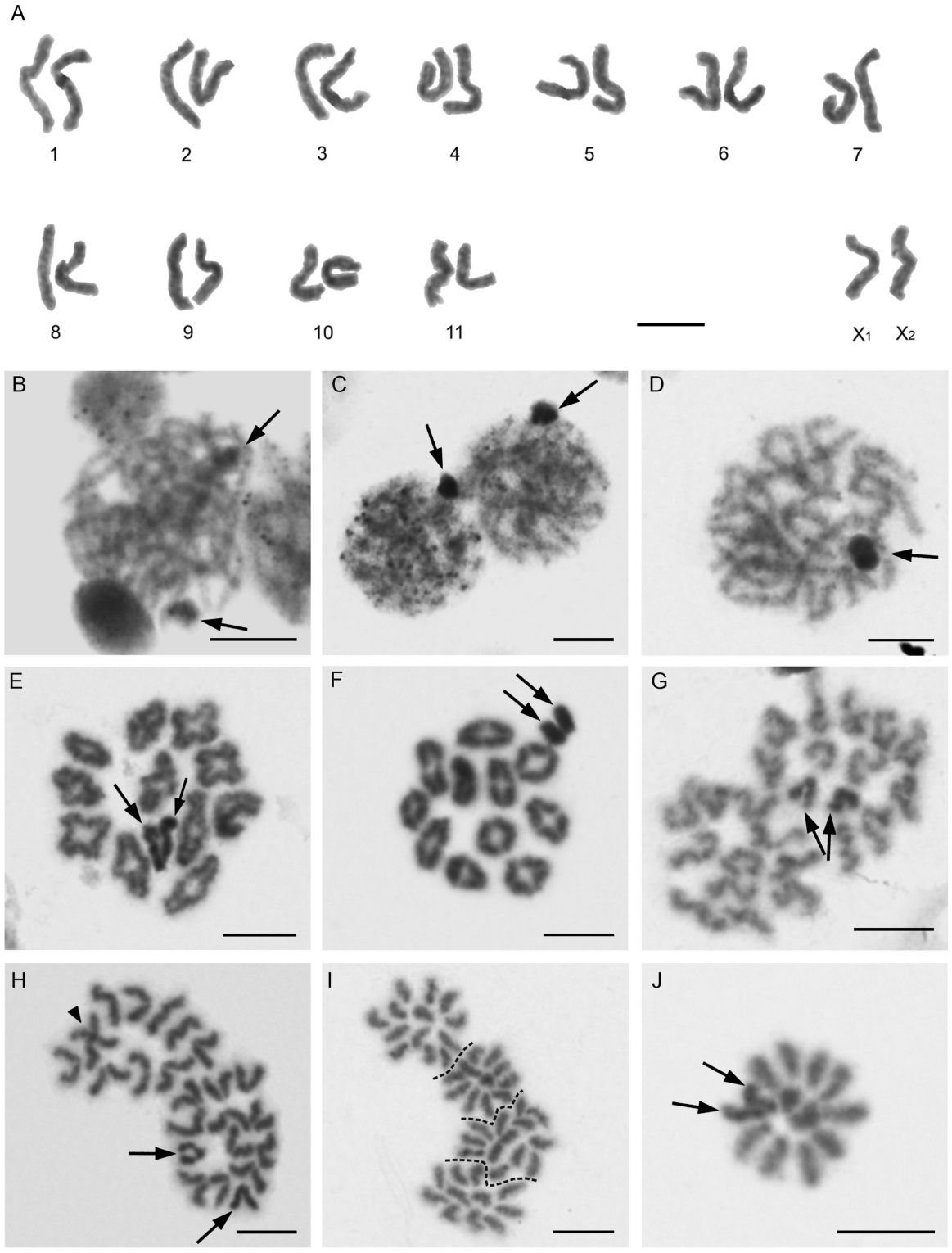

Figure 1. *Argiope bruennichi* male, $2n = 24$, X_1X_20, Giemsa stained mitotic (A) or meiotic (B–J) chromosomes. Arrows point to sex chromosomes. A, karyotype, based on spermatogonial prometaphase. B, leptotene, showing that chromosomes X1 and X2 are not associated. C, two zygotene plates, sex chromosomes form the body on the periphery of the nucleus. D, pachytene, showing the sex chromosome body persisting on the periphery of the nucleus. E, diplotene, showing that the heteropycnotic X1 and X2 chromosomes pair by their centromeric regions. F, metaphase I, note a slight positive heteropycnosis of X

zygotene (Fig. 1C). During zygotene and pachytene, sex chromosomes formed a body on the periphery of the plate (Fig. 1C, D). During late prophase I (diplotene–diakinesis), X chromosome pairing was restricted to centromeric regions (Fig. 1E). From metaphase I onwards, X chromosomes were arranged in parallel (Fig. 1F, G, J).

Genome size and GC content

Female 2C was 4079.809 Mbp (SE ± 256.265 Mbp) and male 2C 3921.083 ± 207.799 Mbp. The average chromosome size (2C/2*n*) was 160.147 Mbp (156.916 Mbp based on female data and 163.378 Mbp based on male data). The X chromosomes contained 158.727 Mbp of DNA, based on the difference in female and male 2C. Taking into account the length ratio of X chromosomes, the size of the X_1 chromosome can be estimated as 82.973 Mbp and the size of X_2 as 75.754 Mbp. The size of the X chromosomes was below the average chromosome size. Therefore, X chromosomes belonged to small chromosomes of the karyotype. The genome contained a low GC proportion, 35.500% of GC (SE ±1.289%) in females and 36.730 ± 1.189% in males.

Coverage of male and female

The mean ± SD coverage across the 13 superscaffolds was 15.34 ± 2.57-fold for the male and 16.50 ± 0.76-fold for the female. We divided the coverage of the male for each superscaffold by the coverage of the female to obtain the relative coverage for the male. Eleven of 13 superscaffolds had nearly equal coverage of male relative to female, and two superscaffolds (9 and 10) had half coverage in the male (Fig. 2A).

qPCR

Sex marker validation in adults

To compare sex-linked loci with autosomal loci, the efficiency of the primers for each locus was evaluated. Primer efficiencies ranged from 83.3 to 147% for the chosen loci (Supporting Information, Table S3). Likewise, a melting curve was generated for each locus, confirming specific amplification during qPCR.

We validated X linkage using qPCR on DNA extracted from six females and six males (Fig. 2B, C). The respective loci, both sex linked and autosomal, were normalized against each autosomal locus and against the average of all autosomal loci used. After comparing these normalization methods, we chose to normalize all loci against one autosomal locus, ABA-7, because this provided consistent results during validation.

To validate the markers on individuals with known sex, the relative DNA abundance, or fold change, in adult males and females, in comparison to females, was determined using the $2^{-\Delta\Delta Ct}$ method (Fig. 2B, C). For females, autosomal loci showed an average fold change of 1.09, and sex-linked loci showed an average fold change of 1.00. One male individual was excluded from our dataset owing to inexplicable fold-change values. With this male excluded, for a total of five males, the sex-linked loci on superscaffold 9 exhibited on average a 0.42- to 0.61-fold change in DNA abundance in males, and the locus on superscaffold 10 had an average fold change of 0.99 in males. The presumably autosomal locus on superscaffold 5 had an average fold change of 0.84 in males (Fig. 2C). Sex-linked loci on superscaffold 9 thus showed about half the DNA abundance in males, with an average of 0.48 compared with females, and an average of 1.01 for females compared with females. These markers on superscaffold 9 were therefore reliable for determining the sex of juveniles. Owing to their inconsistent results, ABA-5 and ABS-10C were not used for sexing juveniles.

Application of sex markers in juveniles

We used one autosomal locus (ABA-7) and three sex-linked loci (ABS-9.3, ABS-9.4 and ABS-9.5) to determine the probable sex of ten individual spiderlings from a single clutch (Supporting Information, Table S4). As shown in Figure 2D, two clear groups of spiderlings are visible: five individuals had an average fold change of 0.49 for the sex-linked loci, indicating that they were males, whereas the other five had an average fold change of 0.98 for these loci, indicating females. In this clutch, our newly developed markers thus indicated an unbiased sex ratio of 1:1.

chromosomes, which are arranged in parallel on the periphery of the plate. G, two sister prophases II, note a slight positive heteropycnosis of X chromosomes, which are arranged in parallel. H, two sister metaphases II, the left plate contains 11 chromosomes and the right plate 13 chromosomes (arrowhead points to two overlapping chromosomes). I, two sister anaphases II, note two half plates with 11 chromosomes and two half plates with 13 chromosomes (half plates separated by dashed lines). The X chromosomes are indistinguishable from the other chromosomes. J, late anaphase II, half plate. Note a slight positive heteropycnosis of X chromosomes. Scale bars: 5 µm.

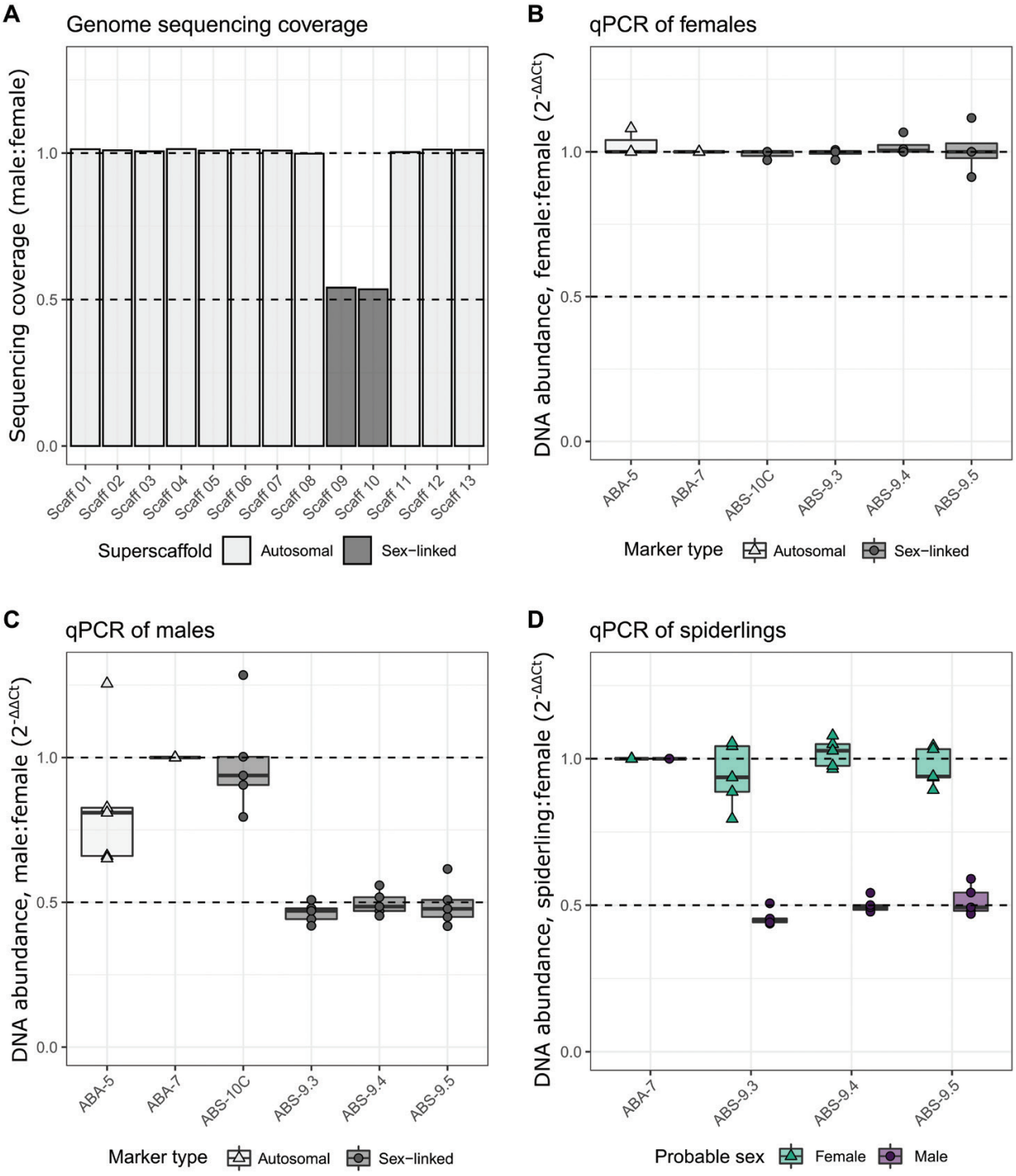

Figure 2. Identification of the sex chromosomes in the *Argiope bruennichi* genome assembly. The dashed horizontal lines represent the expected coverage (A) and the expected DNA abundance (B–D) for sex chromosomes in females (1) and males (0.5) relative to females. A, relative sequencing coverage of males and females across the 13 superscaffolds (Scaff) in the genome assembly. B–D, boxplots showing the results of the $2^{-\Delta\Delta Ct}$ method, calculated from average adjusted *Ct* values for each locus and individual. Symbols represent individual data points, boxes represent the interquartile range, thick lines represent medians, and whiskers represent 1.5 times the interquartile range. B, qPCR results for females. C, qPCR results for males. D, qPCR results for spiderlings for the subset of markers delivering reliable results (ABS-9.3, ABS-9.4, ABS 9.5 and ABA-7).

DISCUSSION

Our study determined the male karyotype of *A. bruennichi*. It was composed of 24 chromosomes, with an X_1X_20 system. The same karyotype was found in two other *Argiope* species, namely *Argiope minuta* Karsch, 1879 (Datta & Chatterjee, 1988), and *Argiope pulchella* Thorell, 1881 (Bole-Gowda, 1958). This karyotype is the most common in analysed species of the superfamily Araneoidea, and it is probably an ancestral feature of this clade (Araujo *et al.*, 2015). As in most other entelegyne spiders, the karyotype of *A. bruennichi* is formed by acrocentric chromosomes (Kořínková & Král, 2013); this karyotype structure can be considered ancestral for entelegynes (Král *et al.*, 2006). Data on three other *Argiope* species are incomplete or different from *A. bruennichi*: the male karyotype of *Argiope amoena* L. Koch, 1878 is also formed by 24 chromosomes, including the X_1X_20 system, but the chromosome morphology was not determined (Suzuki, 1951). The same diploid number was found in *Argiope catenulata* (Doleschall, 1859) (Amalin *et al.*, 1992) and *Argiope luzona* (Walckenaer, 1841) (Carandang & Barrion, 1994), but the chromosome complement of *A. luzona* is reported to consist of both monoarmed (acrocentric and subtelocentric) and biarmed (metacentric and submetacentric) chromosomes (Carandang & Barrion, 1994). This karyotype structure is exceptional in entelegynes and should be revisited by analysis of metaphase II, when centromeres are identifiable unequivocally. Biarmed chromosomes of *A. luzona* could arise from acrocentric chromosomes, such as those found in *A. bruennichi*, by pericentric inversions. The karyotype of *A. catenulata* is reported to be composed of holocentric chromosomes with an XY system (Amalin *et al.*, 1992). In this case, the authors have most probably mistaken acrocentric chromosomes for holocentric ones, because they do not report any features that support a holocentric structure of chromosomes. Moreover, other studied *Argiope* species have monocentric chromosomes (Kořínková & Král, 2013). Furthermore, the determination of the XY system by Amalin *et al.* (1992) is probably also incorrect, because they do not provide any supporting information on the mode of sex chromosome pairing or sex chromosome segregation during male meiosis.

As mentioned above, our karyotype analysis revealed $2n = 24$, X_1X_20 in male *A. bruennichi*. Given the X_1X_20 system, in which males have one copy each of the two X chromosomes, this result fits with the 13 superscaffolds in the haploid genome assembly of a female *A. bruennichi* published by Sheffer *et al.* (2021): 11 autosomes + X_1 + X_2. The result showing half coverage for superscaffolds 9 and 10 in the male indicates that these represent the X chromosomes in the *A. bruennichi* genome assembly. These superscaffolds are very similar in length (123.24 and 122.82 Mbp), which does not match the lengths of X_1 and X_2 (82.973 and 75.754 Mbp) derived from the comparison of male and female genome sizes, with the X_1:X_2 ratio from image-based measurements. However, the genome size measurements had a relatively high standard error, and the superscaffold lengths fall within this margin of error. The fact that we identified two superscaffolds as putative X chromosomes supports the hypothesis that X chromosomes are highly divergent in spiders (Kořínková & Král, 2013).

In addition to the multiple X chromosomes, the karyotype of two distantly related entelegyne families, Agelenidae and Lycosidae (Král, 2007; Král *et al.*, 2011; Sember *et al.*, 2020), contains a specific XY sex chromosome pair that associates at the centromeres with the multiple X chromosomes during male meiosis (Král, 2007). This pair has been termed a 'cryptic sex chromosome pair' (CSCP) (Sember *et al.*, 2020), because the chromosomes are homomorphic (i.e. they exhibit no morphological differentiation and their meiotic association with X chromosomes is fragile, detectable only in optimal conditions). Available data suggest that the chromosomes forming the pair are mostly homologous (Sember *et al.*, 2020). Owing to the discovery of the CSCP in some entelegyne lineages and in more basally branching spider groups, it has been hypothesized that this pair represents ancestral spider sex chromosomes (Král, 2007; Král *et al.*, 2011, 2013; Sember *et al.*, 2020). Whether or not *A. bruennichi* possesses the CSCP is beyond the scope of the present study. However, if the CSCP does exist in *A. bruennichi*, the male Y and X sequences of the CSCP are very similar, and the Y is likely to map to the X in the genome assembly.

The X_1X_20 system of entelegyne spiders is thought to have arisen by X chromosome fission (e.g. Pätau, 1948), non-disjunction of the X chromosome of an X0 system (e.g. Postiglioni & Brum-Zorrilla, 1981) or non-disjunction of the X chromosome of the CSCP (Král, 2007). The lack of sequence similarity of X_1 and X_2 chromosomes found in *A. bruennichi* could reflect their origin by X chromosome fission. Alternatively, lack of sequence similarity could reflect a homologous origin via early duplication of the X chromosome in an X0 system (or early duplication of the X chromosome of CSCP) followed by a long period of differentiation of X_1 and X_2 chromosomes by gene mutations and chromosomal rearrangements. Recent analyses of karyotype evolution of araneomorph spiders suggest a considerable age of X_1 and X_2 chromosomes of the entelegyne X_1X_20 system (Král *et al.*, 2006; Ávila Herrera *et al.*, 2021). Differentiation of X chromosome copies could be accelerated by the inactivation of X chromosome bivalents during meiosis in spider females (Král, 2007).

Interestingly, superscaffold 9 (i.e. chromosome X_1) contains Hox cluster 'A' in the *A. bruennichi* genome assembly (Sheffer *et al.*, 2021). Hox genes are a set of highly conserved genes that regulate organization of the body plan of animals (Pearson *et al.*, 2005). This finding should be tested with fluorescence *in situ* hybridization and investigated in other spiders when chromosome-level assemblies and sequences for X chromosomes become available for more species. The localization of the cluster has potential implications of dosage differences in Hox genes for males and females, and the suspected compensation mechanisms should be investigated further.

Our measurements of genome size and GC content roughly matched data from the published genome assembly (Sheffer *et al.*, 2021). Based on the measurement of female 2C = 4079.809 ± 256.265 Mbp it is possible to calculate a haploid (1C) genome size as 2039.904 ± 128.132 Mbp (this study). The genome assembly has a size of 1670.286 Mbp (Sheffer *et al.*, 2021). Likewise, the average chromosome size for females was 156.916 Mbp based on flow cytometry (this study) and 126.475 Mbp in the genome assembly (Sheffer *et al.*, 2021). The process of genome assembly can collapse repetitive regions and usually results in a smaller assembly size than measured via flow cytometry (see Pflug *et al.*, 2020). The GC content in flow cytometry measurements was 35.5% (this study), and the genome assembly has a GC content of 29.3%. Reduced GC content is a common trait in spiders, and the estimates reported here fall within the range reported for entelegyne spiders (27.2–35%; see Krehenwinkel *et al.*, 2019; Sheffer *et al.*, 2021) and for all other investigated spider species (Král *et al.*, 2019). The GC content of females ($X_1X_1X_2X_2$) and males (X_1X_20) was nearly identical, which indicates no substantial difference in base ratio between the X chromosomes and the autosomal set. *A. bruennichi* exhibits a larger genome than the two other studied *Argiope* species established using Feulgen densitometry, *Argiope aurantia* Lucas, 1833 (1584 Mbp) and *Argiope trifasciata* (Forsskål, 1775) (1653 Mbp) (Gregory & Shorthouse, 2003), but falls into the range of 1C reported for the majority of araneids. Excluding the large genome of *Hyposinga* sp. (4000 Mbp), araneids exhibit a low diversity of genome sizes (1438–2680 Mbp) (Gregory & Shorthouse, 2003).

The DNA abundance values obtained by qPCR in males for loci on superscaffold 9 show the expected pattern of approximately half abundance relative to autosomal markers and can thus be used reliably for sexing spiders. The values obtained for ABS-10C, a locus on superscaffold 10 and thus putatively sex-linked, in contrast, resemble values obtained for autosomal loci. Given the compelling evidence from the coverage comparison that supports superscaffold 10 as being one of the two X chromosomes, we are currently unsure what might explain this phenomenon. We verified the designed primers *in silico* and found them to be specific to the region we intended to amplify. Furthermore, the coverage for this region was not suspiciously low or high. However, an interpretation of the qPCR results without a priori expectation would let us think the amplified region is placed on an autosome. Although the results obtained with superscaffold 9 are robust, new primers should be designed for other fragments of superscaffold 10. The method presented here is applicable to very small quantities of DNA and allowed us to identify the sex of early instar spiderlings, which are otherwise nearly impossible to sex.

Having identified the sex chromosomes in the genome assembly, future work can investigate sex chromosome evolution in *A. bruennichi*. In species in which males have one copy of each sex chromosome, as in *A. bruennichi*, the sex chromosomes are expected to evolve faster at the molecular level, relative to autosomes. This is attributable to several factors, such as fewer recombination events, weaker purifying selection or higher positive selection on recessive alleles relative to autosomes. These processes might lead to the acquisition of specific gene content on X chromosomes and to an X chromosome to autosome divergence (Charlesworth *et al.*, 1987; Ellegren, 2009). A comparison of gene content between X chromosomes within and among species from diverse clades of spiders (entelegynes and haplogynes, but also basally branching groups) would deliver exciting new insights into sex chromosome evolution and, eventually, the evolution of sexual differentiation in these arachnids (Rice, 1984).

Conclusions

We determined the *A. bruennichi* karyotype, genome size and GC content, which are consistent with the existing genome assembly. By comparison of the sequencing coverage of males and females, we were able to identify which of the superscaffolds in the genome assembly represent the multiple X chromosomes. This result enabled us to develop the first qPCR markers allowing for a molecular approach to sexing spiders early during their development. Furthermore, having knowledge on the identity of the sex chromosomes in the genome assembly will allow studies on sex determination, sex allocation, sex-specific dispersal and growth, and sex-dependent physiological and developmental trajectories. Extending the taxonomic scope will allow comparisons of the gene content of X chromosomes within genomes and among species and will deliver invaluable information on the evolution of sex chromosome systems formed by X chromosomes.

ACKNOWLEDGEMENTS

We would like to thank Henrik Krehenwinkel (Trier, Germany) and Stefan Prost (Vienna, Austria) for discussions and brainstorming on this project early on. We also thank Lars Jensen (Greifswald, Germany) for his input on library preparation and sequencing and Douglas Araujo (Campo Grande, Brazil) for access to the paper on the karyotype of *A. luzona*. We are grateful to Shou-Wang Lin (Greifswald, Germany), who provided a translation of the original *A. bruennichi* karyotype paper, which started us on this path to generate new data. We thank the Deutsche Forschungsgemeinschaft (DFG) for the funding of this study as part of the Research Training Group 2010 RESPONSE (GRK 2010) awarded to G.U. The cytogenetic part of the study was supported by the Grant Agency of Charles University (project 1000119) and the Ministry of Education, Youth, and Sports of the Czech Republic (project LTAUSA 19142). Lastly, we sincerely thank three anonymous reviewers for comments that substantially improved our manuscript. Other than the first author position, the authors are listed in alphabetical order by last name, not relative to contributions or institutional hierarchy. M.M.S., M.C., G.U., J.K. and M.F. conceived the study. M.M.S., M.F. and G.U. collected spiders. M.M.S. and C.J. isolated DNA, prepared libraries for the whole-genome sequencing and performed the sequencing, with input and infrastructure from G.U. and A.W.K. M.M.S., K.H., M.G. and M.C. selected loci, designed primers and performed the qPCR analysis. M.K. and M.F. performed the karyological analysis of chromosomes, with input and infrastructure from J.K. E.L. performed the flow cytometry measurements of genome size and GC content. All authors contributed to manuscript writing and revision. We have no conflicts of interest to declare.

DATA AVAILABILITY

Sequencing reads from the male and female specimens are available on the NCBI Short Read Archive, under BioProject PRJNA629526. Raw data for the flow cytometry are available in the Supporting Information, as is the information on qPCR primers and the *Ct* values from the qPCR experiments.

REFERENCES

Al-Dous EK, George B, Al-Mahmoud ME, Al-Jaber MY, Wang H, Salameh YM, Al-Azwani EK, Chaluvadi S, Pontaroli AC, Debarry J, Arondel V, Ohlrogge J, Saie IJ, Suliman-Elmeer KM, Bennetzen JL, Kruegger RR, Malek JA. 2011. De novo genome sequencing and comparative genomics of date palm (*Phoenix dactylifera*). *Nature Biotechnology* **29:** 521–527.

Amalin DM, **Barrion AA**, **Rueda LM. 1992.** Morphology and cytology of *Argiope catenulata* (Doleschall) (Araneae: Araneidae). *Asia Life Sciences* **1:** 35–44.

Andrews S. 2010. FastQC: a quality control tool for high throughput sequence data. Available at: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Araujo D, **Paula-Neto E**, **Brescovit AD**, **Cella DM**, **Schneider MC. 2015.** Chromosomal similarities between Nephilidae and Tetragnathidae indicate unique evolutionary traits among Araneoidea. *Italian Journal of Zoology* **82:** 513–520.

Araujo D, **Schneider MC**, **Paula-Neto E**, **Cella DM. 2012.** Sex chromosomes and meiosis in spiders: a review. In: Swan A, ed. *Meiosis: molecular mechanisms and cytogenetic diversity*. Rijeka: InTechOpen, 87–108.

Araujo D, **Schneider MC**, **Paula-Neto E**, **Cella DM. 2021.** The spider cytogenetic database. Available at: www.arthropodacytogenetics.bio.br/spiderdatabase

Ávila Herrera IM, **Král J**, **Pastuchová M**, **Forman M**, **Musilová J**, **Kořínková T**, **Šťáhlavský F**, **Zrzavá M**, **Nguyen P**, **Just P**, **Haddad CR**, **Hiřman M**, **Koubová M**, **Sadílek D**, **Huber BA. 2021.** Evolutionary pattern of karyotypes and meiosis in pholcid spiders (Araneae: Pholcidae): implications for reconstructing chromosome evolution of araneomorph spiders. *BMC Ecology and Evolution* **21:** 75.

Avilés L, **Maddison W. 1991.** When is the sex ratio biased in social spiders?: Chromosome studies of embryos and male meiosis in *Anelosimus* species (Araneae, Theridiidae). *Journal of Arachnology* **19:** 126–135.

Bachtrog D, **Mank JE**, **Peichel CL**, **Kirkpatrick M**, **Otto SP**, **Ashman TL**, **Hahn MW**, **Kitano J**, **Mayrose I**, **Ming R**, **Perrin N**, **Ross L**, **Valenzuela N**, **Vamosi JC**, **Mank JE**, **Peichel CL**, **Ashman TL**, **Blackmon H**, **Goldberg EE**, **Hahn MW**, **Kirkpatrick M**, **Kitano J**, **Mayrose I**, **Ming R**, **Pennell MW**, **Perrin N**, **Valenzuela N**, **Vamosi JC. 2014.** Sex determination: why so many ways of doing it? *PLoS Biology* **12:** e1001899.

Bechsgaard J, **Schou MF**, **Vanthournout B**, **Hendrickx F**, **Knudsen B**, **Settepani V**, **Schierup MH**, **Bilde T. 2019.** Evidence for faster X chromosome evolution in spiders. *Molecular Biology and Evolution* **36:** 1281–1293.

Bole-Gowda BN. 1958. A study of the chromosomes during meiosis in twenty-two species of Indian spiders. *Proceedings of the Zoological Society of Bengal* **11:** 69–108.

Carandang RB, **Barrion AA. 1994.** Karyotype of the egg chromosomes of *Argiope luzona* (Walck), an orb-weaving spider (Araneae, Araneidae). *Philippine Entomologist* **9:** 443–447.

Charlesworth B, **Coyne JA**, **Barton NH. 1987.** The relative rates of evolution of sex chromosomes and autosomes. *The American Naturalist* **130:** 113–146.

Coddington JA, **Levi HW. 1991.** Systematics and evolution of spiders (Araneae). *Annual Review of Ecology and Systematics* **22:** 565–592.

Cordellier M, **Schneider JM**, **Uhl G**, **Posnien N. 2020.** Sex differences in spiders: from phenotype to genomics. *Development Genes and Evolution* **230:** 155–172.

Datta SN, **Chatterjee K. 1988.** Chromosomes and sex determination in 13 araneid spiders of North-Eastern India. *Genetica* **76:** 91–99.

Dolejš P, **Kořínková T**, **Musilová J**, **Opatová V**, **Kubcová L**, **Buchar J**, **Král J. 2011.** Karyotypes of central European spiders of the genera *Arctosa, Tricca*, and *Xerolycosa* (Araneae: Lycosidae). *European Journal of Entomology* **108:** 1–16.

Ellegren H. 2009. The different levels of genetic diversity in sex chromosomes and autosomes. *Trends in Genetics* **25:** 278–284.

Fraïsse C, **Picard MAL**, **Vicoso B. 2017.** The deep conservation of the Lepidoptera Z chromosome suggests a non-canonical origin of the W. *Nature Communications* **8:** 1486.

Gautier M. 2014. Using genotyping data to assign markers to their chromosome type and to infer the sex of individuals: a Bayesian model-based classifier. *Molecular Ecology Resources* **14:** 1141–1159.

Gregory TR, **Shorthouse DP. 2003.** Genome sizes of spiders. *Journal of Heredity* **94:** 285–290.

Hamm RL, **Meisel RP**, **Scott JG. 2015.** The evolving puzzle of autosomal *versus* Y-linked male determination in *Musca domestica*. *G3: Genes, Genomes, Genetics* **5:** 371–384.

Hou J, **Ye N**, **Zhang D**, **Chen Y**, **Fang L**, **Dai X**, **Yin T. 2015.** Different autosomes evolved into sex chromosomes in the sister genera of *Salix* and *Populus*. *Scientific Reports* **5:** 9076.

Kořínková T, **Král J. 2013.** Karyotypes, sex chromosomes, and meiotic division in spiders. In: Nentwig W, ed. *Spider ecophysiology*. Berlin: Springer, 159–171.

Král J. 2007. Evolution of multiple sex chromosomes in the spider genus *Malthonica* (Araneae: Agelenidae) indicates unique structure of the spider sex chromosome systems. *Chromosome Research* **15:** 863–879.

Král J, **Forman M**, **Kořínková T**, **Lerma ACR**, **Haddad CR**, **Musilová J**, **Řezáč M**, **Herrera IMA**, **Thakur S**, **Dippenaar-Schoeman AS**, **Marec F**, **Horová L**, **Bureš P. 2019.** Insights into the karyotype and genome evolution of haplogyne spiders indicate a polyploid origin of lineage with holokinetic chromosomes. *Scientific Reports* **9:** 3001.

Král J, **Kořínková T**, **Forman M**, **Krkavcová L. 2011.** Insights into the meiotic behavior and evolution of multiple sex chromosome systems in spiders. *Cytogenetic and Genome Research* **133:** 43–66.

Král J, **Kořínková T**, **Krkavcová L**, **Musilová J**, **Forman M**, **Herrera IMA**, **Haddad CR**, **Vítková M**, **Henriques S**, **Vargas JGP**, **Hedin M. 2013.** Evolution of karyotype, sex chromosomes, and meiosis in mygalomorph spiders (Araneae: Mygalomorphae). *Biological Journal of the Linnean Society* **109:** 377–408.

Král J, **Musilová J**, **Šťáhlavský F**, Řezáč **M**, **Akan Z**, **Edwards RL**, **Coyle FA**, **Almerje CR. 2006.** Evolution of the karyotype and sex chromosome systems in basal clades of araneomorph spiders (Araneae: Araneomorphae). *Chromosome Research* **14:** 859–880.

Krehenwinkel H, **Graze M**, **Rödder D**, **Tanaka K**, **Baba YG**, **Muster C**, **Uhl G. 2016.** A phylogeographical survey of a highly dispersive spider reveals eastern Asia as a major glacial refugium for Palaearctic fauna. *Journal of Biogeography* **43:** 1583–1594.

Krehenwinkel H, **Meese S**, **Mayer C**, **Ruch J**, **Schneider J**, **Bilde T**, **Künzel S**, **Henderson JB**, **Russack J**, **Simison WB**, **Gillespie R**, **Uhl G. 2019.** Cost effective microsatellite isolation and genotyping by high throughput sequencing. *Journal of Arachnology* **47:** 190–201.

Krehenwinkel H, **Rödder D**, **Tautz D. 2015.** Eco-genomic analysis of the poleward range expansion of the wasp spider *Argiope bruennichi* shows rapid adaptation and genomic admixture. *Global Change Biology* **21:** 4320–4332.

Krehenwinkel H, **Tautz D. 2013.** Northern range expansion of European populations of the wasp spider *Argiope bruennichi* is associated with global warming-correlated genetic admixture and population-specific temperature adaptations. *Molecular Ecology* **22:** 2232–2248.

Krüger J. 2014. Präballooning-Verhalten bei der Wespenspinne *Argiope bruennichi*. Unpublished Diploma Thesis, University of Greifswald.

Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv*.

Li H, **Handsaker B**, **Wysoker A**, **Fennell T**, **Ruan J**, **Homer N**, **Marth G**, **Abecasis G**, **Durbin R. 2009.** The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25:** 2078–2079.

Loureiro J, **Rodriguez E**, **Doležel J**, **Santos C. 2007.** Two new nuclear isolation buffers for plant DNA flow cytometry: a test with 37 species. *Annals of Botany* **100:** 875–888.

Muyle A, **Käfer J**, **Zemp N**, **Mousset S**, **Picard F**, **Marais GA. 2016.** SEX-DETector: a probabilistic approach to study sex chromosomes in non-model organisms. *Genome Biology and Evolution* **8:** 2530–2543.

Okonechnikov K, **Conesa A**, **García-Alcalde F. 2016.** Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data. *Bioinformatics* **32:** 292–294.

Palmer DH, **Rogers TF**, **Dean R**, **Wright AE. 2019.** How to identify sex chromosomes and their turnover. *Molecular Ecology* **28:** 4709–4724.

Pätau K. 1948. X-segregation and heterochromasy in the spider *Aranea reaumuri*. *Heredity* **2:** 77–100.

Pearson JC, **Lemons D**, **McGinnis W. 2005.** Modulating Hox gene functions during animal body patterning. *Nature Reviews Genetics* **6:** 893–904.

Pflug JM, **Holmes VR**, **Burrus C**, **Johnston JS**, **Maddison DR. 2020.** Measuring genome sizes using read-depth, k-mers, and flow cytometry: methodological comparisons in beetles (Coleoptera). *G3: Genes, Genomes, Genetics* **10:** 3047–3060.

Picq S, **Santoni S**, **Lacombe T**, **Latreille M**, **Weber A**, **Ardisson M**, **Ivorra S**, **Maghradze D**, **Arroyo-Garcia R**, **Chatelet P**, **This P**, **Terral JF**, **Bacilieri R. 2014.** A small XY chromosomal region explains sex determination in wild dioecious *V. vinifera* and the reversal to hermaphroditism in domesticated grapevines. *BMC Plant Biology* **14:** 229.

Postiglioni A, **Brum-Zorrilla N. 1981.** Karyological studies on Uruguayan spiders II. Sex chromosomes in spiders of the genus *Lycosa* (Araneae-Lycosidae). *Genetica* **56:** 47–53.

Rao X, **Huang X**, **Zhou Z**, **Lin X. 2013.** An improvement of the 2^(−delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. *Biostatistics, Bioinformatics and Biomathematics* **3:** 71–85.

Rice WR. 1984. Sex chromosomes and the evolution of sexual dimorphism. *Evolution; international journal of organic evolution* **38:** 735–742.

Schubert M, **Lindgreen S**, **Orlando L. 2016.** AdapterRemoval v2: rapid adapter trimming, identification, and read merging. *BMC Research Notes* **9:** 88.

Sember A, **Pappová M**, **Forman M**, **Nguyen P**, **Marec F**, **Dalíková M**, **Divišová K**, **Doležálková-Kaštánková M**, **Zrzavá M**, **Sadílek D**, **Hrubá B**, **Král J. 2020.** Patterns of sex chromosome differentiation in spiders: insights from comparative genomic hybridisation. *Genes* **11:** 849.

Sheffer MM, **Hoppe A**, **Krehenwinkel H**, **Uhl G**, **Kuss AW**, **Jensen L**, **Jensen C**, **Gillespie RG**, **Hoff KJ**, **Prost S. 2021.** Chromosome-level reference genome of the European wasp spider *Argiope bruennichi*: a resource for studies on range expansion and evolutionary adaptation. *GigaScience* **10:** giaa148.

Šmarda P, **Bureš P**, **Horová L**, **Foggi B**, **Rossi G. 2008.** Genome size and GC content evolution of *Festuca*: ancestral expansion and subsequent reduction. *Annals of Botany* **101:** 421–433.

Suzuki S. 1951. A comparative study of the chromosomes in the family Argiopidae. *Journal of Science of the Hiroshima University, Series B, Division 1* **12:** 67–98.

Untergasser A, **Cutcutache I**, **Koressaar T**, **Ye J**, **Faircloth BC**, **Remm M**, **Rozen SG. 2012.** Primer3—new capabilities and interfaces. *Nucleic Acids Research* **40:** e115.

Veselý P, **Bureš P**, **Šmarda P**, **Pavlíček T. 2012.** Genome size and DNA base composition of geophytes: the mirror of phenology and ecology? *Annals of Botany* **109:** 65–75.

Vicoso B. 2019. Molecular and evolutionary dynamics of animal sex-chromosome turnover. *Nature Ecology & Evolution* **3:** 1632–1641.

Vicoso B, **Bachtrog D. 2011.** Lack of global dosage compensation in *Schistosoma mansoni*, a female-heterogametic parasite. *Genome Biology and Evolution* **3:** 230–235.

Wawer W, **Rutkowski R**, **Krehenwinkel H**, **Lutyk D**, **Pusz-Boscheńska K**, **Bogdanowicz W. 2017.** Population structure of the expansive wasp spider (*Argiope bruennichi*) at the edge of its range. *Journal of Arachnology* **45:** 361–369.

White MJD. 1973. *Animal cytology and evolution*. London: Cambridge University Press.

Zhang YJ, **Tong SJ. 1990.** The routine method for preparing the chromosomes in spiders. *Chinese Journal of Zoology* **25:** 30–31.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Primary data of flow cytometry measurements. The fluorescence intensity (FI) is the mean value of the peak for a standard (*Pisum sativum*) or a sample (*Argiope bruennichi*) for certain fluorochromes and their ratio. The coefficient of variation (CV; as a percentage) of each peak is given in the next column. The dye factor (DAPI) is estimated as the ratio of FI ratios (base-specific fluorochrome/non-selective fluorochrome). The dye factor is the parameter used for calculation of base content [for details, see Šmarda *et al.* (2008) and references therein].

Table S2. Quantities of DNA obtained before qPCR or conventional PCR ('conv. PCR'), and triplicate *Ct* values for each locus in each individual from qPCR. A minus sign (–) indicates no data or missing data (i.e. if a marker was not run or one replicate was excluded owing to high deviation). The extraction column lists the extraction method used [Promega kit ('promega') or basic principles extraction ('basic')].

Table S3. Primers used for quantification in the qPCR approach. The autosomal loci are ABA-5 and ABA-7, whereas ABS-10C, ABS-9.3, ABS-9.4 and ABS-9.5 should be sex linked. Primers marked with an asterisk (*) were used to sex spiderlings. The two efficiencies provided in the final column represent different efficiencies in different laboratories (Hamburg/Greifswald).

Table S4. Fold-change values of sex-linked loci in spiderlings. Values are rounded to two decimal places.