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

Running title: Sex chromosomes in *Argiope bruennichi*

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], 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

*Correspondence address: Monica M. Sheffer, Zoological Institute and Museum, University of Greifswald, Loitzer Strasse 26, 17489 Greifswald, Germany

Correspondence E-Mail: monicasheffer.research@gmail.com

[‡]Current affiliation: Department of Biology, University of Washington, 98195 Seattle, WA, USA

[#]Current affiliation: Department of Molecular Biology and Genetics, Aarhus University, 8000 Aarhus, Denmark

Abstract:

Differences between sexes in growth, ecology and behavior 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, behavioral experiments, and ecological studies on e.g. 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 RT-qPCR sex markers. We found that genome size and GC content of this spider falls into the range 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 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 similarly sized X chromosomes in *A. bruennichi*. They are smaller chromosomes of the complement. Our findings open the door to new directions in spider evolutionary and ecological research.

Keywords: *Argiope bruennichi* – Araneae – Araneidae – genome size – karyotype – sex chromosome

Introduction

In most animals, sex is genetically determined. 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, Meisel, & Scott, 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 on the karyotype and the specifics of the sex chromosomes provides the basis for studying 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 easily be identified in adults and even less so in juveniles. The sex of early stages is often only determinable based on their internal anatomy (presence of testis 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). While they play an important role in terrestrial ecosystems, it is impossible to sex juvenile spiders in the field. Spiders possess complex sex chromosome systems. To date, karyotypes of more than 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 by 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).

Due to the different number 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 PCR. 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, required sample sizes, and on the availability of a reference genome (Al-Dous *et al.*, 2011; Picq *et al.*, 2014; Gautier, 2014; Hou *et al.*, 2015; Muyle *et al.*, 2016). In species with a chromosome-level genome assembly, comparing the sequencing coverage depth across chromosomes between individuals of each sex is a reliable method for

84 identifying sex chromosomes. Females, as the homogametic sex, would show twice as much
85 coverage as males on X chromosomes (i.e. (Vicoso & Bachtrog, 2011; Fraïsse, Picard, & Vicoso,
86 2017).
87 In 2021, Sheffer *et al.* published an annotated genome assembled to haploid chromosome level
88 of an entelegyne orb-weaving spider, *Argiope bruennichi* (Scopoli, 1772). With this
89 chromosome-level genome, we can employ the coverage depth approach to identify sex
90 chromosome sequences in this species. *A. bruennichi* is a sexually dimorphic species with a
91 Palearctic distribution, which has rapidly expanded its range in Europe over the course of the
92 last century (Krehenwinkel & Tautz, 2013; Krehenwinkel, Rödder, & Tautz, 2015; Wawer *et al.*,
93 2017). Sex differences in early life stages of these spiders are particularly interesting since
94 juveniles perform aerial dispersal with silk (ballooning), a behavior which is restricted to the
95 first few instars (Krüger, 2014) and might be sex biased (Krehenwinkel *et al.*, 2016).
96 The *A. bruennichi* genome assembly was based upon female specimens, and is comprised of 13
97 scaffolds, which suggests 2n = 26. This chromosome number is found in females of most orb-
98 weaving spiders, and it is consistent with the diploid chromosome number of the species
99 published by Zhang & Tong (1990). This study, however, contains no information on the
100 number or sex of the individual(s) examined. With additional karyological and sequence data
101 for males, it would be possible to ascertain the karyotype/sex determination system of the
102 species and identify the sex chromosomes in the published assembly.
103 We set out to (i) determine the karyotype and genome size of *A. bruennichi*, (ii) identify which
104 superscaffolds represent sex chromosomes in the genome assembly, and (iii) develop molecular
105 markers for sexing spiders using RT-qPCR.

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 found in subadult or young adult males of other araneids (Kotz and 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 (FCM) 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

125 polyvinylpyrrolidone (Sigma-Aldrich). After filtration through a nylon sifter, we added
126 fluorescent dye. We performed two concurrent FCM gauges, using: (i) Propidium iodide (PI, 50
127 µg/ml) – a nonspecific dye to establish genome size of the sample – and (ii) 4′,6-Diamidine-2′-
128 phenylindole dihydrochloride (DAPI, 4 µg/ml), a fluorochrome with higher specificity to AT
129 pairs. The resulting histograms were evaluated using FloMax software (Partec). We calculated
130 the 2C value as the ratio of PI fluorescence of sample and standard. We compared PI and DAPI
131 ratios to establish GC content as described in Šmarda *et al.* (2008). We measured each
132 individual twice for PI and twice for DAPI to account for machine fluctuations. We used fresh
133 leaves of *Pisum sativum* cultivar 'Ctirad' as an internal standard. We used the following genomic
134 values of this plant for calculations: 2C=7841.27 Mbp, GC=41.77% (Veselý *et al.*, 2012). Primary
135 data used for calculation of 2C and base content are available in Supplementary Table S1.

136 Whole-genome sequencing of male and female *Argiope bruennichi*

137 *DNA extraction and library preparation*

138 We will refer to chromosome-level scaffolds in the genome assembly as "superscaffolds" and
139 number all of them by length. For karyotype, we use the traditional approach and number
140 autosomes by length and refer to X chromosomes separately.
141 In order to identify the X chromosomes in the *A. bruennichi* genome assembly, we sequenced
142 one adult male and one adult female collected near Klausdorf, Germany at the end of July 2020
143 (54.424N, 13.029E). We extracted DNA from eight legs of the male and four legs of the female,
144 as follows: we disrupted the leg tissue with a mortar, pestle, and liquid nitrogen. The powdered

145 tissue was transferred into tubes containing Proteinase K and cell lysis buffer (10mM Tris pH 8,
146 100mM NaCl, 10mM EDTA pH 8, 0.5% SDS, double-distilled water). We incubated the tubes
147 overnight at 55°C, cooled them to room temperature and added RNAse A. We used 5M NaCl to
148 precipitate proteins and Isopropanol to precipitate DNA. We cleaned the DNA extract using 70%
149 Ethanol and eluted it in TE buffer. We prepared the libraries with the NEBNext Ultra II FS DNA
150 Library Prep Kit (New England Biolabs, Ipswich, Massachusetts, USA) according to the
151 manufacturer's protocol. We indexed and amplified the libraries with six PCR cycles, then
152 pooled them and sequenced the 2x75 bp paired-end libraries on one Flowcell of an Illumina
153 NextSeq 550 with a High Output v2 kit (150 cycles) (Illumina Inc., San Diego, California, USA).

154 *Bioinformatic processing and coverage comparison*

155 The reads were demultiplexed using the Illumina bcl2fastq tool in BaseSpace. We removed
156 adapter sequences using AdapterRemoval (v.2.2.2) (Schubert, Lindgreen, & Orlando, 2016) with
157 the --identify-adapters flag. Read quality was assessed using FastQC (v0.11.8) (Andrews, 2010);
158 all reads were high quality, and no further quality trimming was necessary. Adapter-trimmed
159 reads are available under BioProject PRJNA629526. We mapped the reads of each specimen
160 onto the *A. bruennichi* genome assembly using BWA-MEM (v.0.7.12-r1039) with default settings
161 (Li, 2013), sorted and indexed the mapped reads using SAMtools sort and SAMtools index
162 (SAMtools v.1.3.1) (Li *et al.*, 2009), then calculated the coverage of each of the 13
163 superscaffolds using QualiMap (v.2.2.1) (Okonechnikov, Conesa, & García-Alcalde, 2016).

164 # Development of sex markers using RT-qPCR

165 ## *DNA extraction*

166 In total, we extracted genomic DNA from six males collected in Greifswald (54.093N, 13.366E)
167 and six females collected near Klausdorf (54.424N, 13.029E), Germany at the beginning of
168 August 2020, following either the procedure outlined above, or a protocol based on the
169 Promega ReliaPrep™ gDNA Tissue Miniprep System (see Table S2). Prior to using the Promega
170 kit, we ground two legs each from females or four legs each from males with a Dounce
171 homogenizer; we added 200 µl of Tail Lysis Buffer and 30 µl of Proteinase K Solution to the
172 sample, and extracted the DNA following the manufacturer's instructions. We carried out the
173 final elution step twice to a total elution volume of 100 µl.
174 Further, we extracted DNA from ten first instar spiderlings that came from one egg sac, laid by a
175 female collected in Pärnu, Estonia in 2018 (58.297N, 24.597E). Genomic DNA was extracted as
176 described in the preceding section. Whole spiderling bodies were used, rather than legs, due to
177 their small size. First instar spiderlings from this population have an average mass of
178 approximately 0.3 mg (Sheffer, personal observation). We determined the DNA concentration
179 for each sample using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) and
180 a Qubit 4 Fluorometer with the Quant-iT™ Qubit™ dsDNA BR Assay Kit (Thermo Scientific,
181 Wilmington, DE). DNA concentrations can be found in Table S2.

182 *Primer design, PCR and RT-qPCR*

183 We designed primers for loci on the putative X chromosomes (superscaffolds 9 and 10 in the *A.*
184 *bruennichi* assembly; see results section) and 2 putative autosomes, superscaffolds 5 and 7,
185 using Primer3 (version 4.1.0; (Untergasser *et al.*, 2012) with the following parameters: length
186 between 20 and 25 nucleotides, melting temperature between 57 and 62°C, region amplified
187 between 150 and 200 bp in length, with one GC clamp (Table S3). We used additional data to
188 select the loci, such as the location of homologous sequences in spiders *Trichonephila*
189 *senegalensis* (Araneidae) (Walckenaer, 1841) (Grewoldt *et al.,* unpublished data) and
190 *Stegodyphus mimosarum* (Eresidae) (Pavesi, 1883) (Bechsgaard *et al.*, 2019). We tested the
191 amplification with conventional PCR, checked on a 1.5% agarose gel with ethidium bromide
192 staining and UV transillumination. We then conducted RT-qPCR using the EvaGreen® qPCR-Mix
193 II, on a ThermoFisher StepOne™ Real-Time PCR System (Thermo Fisher Scientific, Waltham,
194 USA). Primer efficiencies were measured by diluting 20 ng/µl of template DNA from a female in
195 a stepwise manner, 1:10 down to the lowest concentration of 0.02 ng/µl, to obtain a standard
196 curve. After ensuring comparable efficiencies for all primer pairs, we used DNA from the six
197 females and males as templates and simultaneously measured amplification in real time for
198 each primer pair in triplicate. One non template control was used for each primer combination.
199 All amplification protocols were conducted with the following reaction mix: 2 µL EvaGreen®
200 qPCR-Mix II 5x, 0.2 µL forward primer, 0.2 µL reverse primer, 1µL template DNA, and 6.6 µL
201 nuclease free water. We used the following program: initial denaturation for 15 minutes at 95°C,
202 40 cycles of denaturation (15 seconds at 95°C), annealing (20 seconds at 60°C), and extension

203 (20 seconds at 72°C), with a final extension at 72°C for 10 minutes, and a melting curve
204 (ramping from 65°C to 95°C). Ct values were collected for further analysis (Table S2).
205 After validation of the method and determination of effective markers (see results) using the
206 adult specimens, we used a subset of markers (see Tables S2 and S3) to determine the sex of
207 the spiderlings.

208 *RT-qPCR data analysis*

209 We applied the $2^{-\Delta\Delta Ct}$ method according to (Rao *et al.*, 2013) to analyze the RT-qPCR data. To
210 account for different primer efficiencies (E_{real}), the Ct values were normalized to correspond to
211 an optimal efficiency of 100% (E_{opt}). We averaged the Ct values of the three technical replicates;
212 if any of the three replicates showed a standard deviation >1, it was excluded from the average.
213 The ΔCt value was calculated from the difference between reference sequences (autosomal
214 loci) and a target sequence of interest (X-linked locus). Further, the female data points were
215 used as the reference, as females possess two sex-linked and two autosomal gene copies.
216 Consequently, if the number of gene copies between females and males is the same, a value of
217 1 is expected, whereas if the number of gene copies in males is half that in females, a value of
218 0.5 is expected.

219 **Results**

220 Chromosomes

221 The male karyotype of *A. bruennichi* was formed by 24 acrocentric chromosomes; the size
222 difference between chromosomes was gradual from the longest to the shortest (Figure 1A).

223 Analysis of male meiotic division revealed an X_1X_20 sex chromosome system (Figure 1B, E-J).
224 Chromosomes X_1 and X_2 were similar in length; X_2 length corresponded to 91.3% (±5.3) of X_1.
225 We observed nearly the complete course of male meiotic division except anaphase I, telophase
226 I, and telophase II. X chromosomes were positively heteropycnotic (i.e., they stained more
227 intensively) from the beginning of meiosis until diakinesis (Figure 1B-E). The intensity of
228 heteropycnosis decreased during metaphase I; X chromosomes were isopycnotic or slightly
229 positively heteropycnotic during this period (Figure 1F). Positive heteropycnosis reappeared
230 during prophase II (Figure 1G). During metaphase II (Fig. 1H), X chromosomes exhibited a slight
231 positive heteropycnosis only. This pattern was also displayed at late anaphase II (Figure 1J).
232 Tight pairing of X_1 and X_2 chromosomes was initiated at zygotene (Figure 1C). During zygotene
233 and pachytene, sex chromosomes formed a body on the periphery of the plate (Figure 1C, D).
234 During late prophase I (diplotene-diakinesis), X chromosome pairing was restricted to
235 centromeric regions (Figure 1E). From metaphase I onwards, X chromosomes were arranged in
236 parallel (Figure 1F, G, J).

237 Genome size and GC content

238 Female 2C was 4,079.809 Mbp (standard error (SE) ±256.265) and male 2C 3,921.083 Mbp (SE
239 ±207.799). The average chromosome size (2C/2n) was 160.147 Mbp (156.916 Mbp based on
240 female and 163.378 Mbp based on male data, respectively). X chromosomes contained 158.727
241 Mbp of DNA, based on the difference in female and male 2C. Taking in account the length ratio
242 of X chromosomes, size of X_1 chromosome can be estimated as 82.973 Mbp and size of X_2 as
243 75.754 Mbp. The size of the X chromosomes was below the average chromosome size.

244 Therefore, X chromosomes belonged to small chromosomes of the karyotype. The genome
245 contained a low GC proportion, 35.500 % of GC (SE ±1.289) in females and 36.730% (SE ±1.189)
246 in males, respectively.

247 Coverage of male and female

248 The mean coverage (± standard deviation) across the 13 superscaffolds was 15.34 ±2.57 fold for
249 the male and 16.50 ±0.76 fold for the female. We divided the coverage of the male for each
250 superscaffold by the coverage of the female, to get the male's relative coverage. 11/13
251 superscaffolds have nearly equal coverage of male relative to female, while two superscaffolds
252 (9 and 10) have half coverage in the male (Figure 2A).

253 RT-qPCR

254 *Sex marker validation in adults*

255 To compare sex-linked with autosomal loci, the efficiency of the primers for each locus were
256 evaluated. Primer efficiencies ranged from 83.3- 147%, for the chosen loci (Table S3). Likewise,
257 a melting curve was generated for each locus, confirming specific amplification during RT-qPCR.
258 X-linkage was validated using RT-qPCR on DNA extracted from six females and six males (Figure
259 2B, C). The respective loci, sex-linked as well as autosomal, were normalized against each
260 autosomal locus, as well as the average of all autosomal loci used. After comparing these
261 normalization methods, we chose to normalize all loci against one autosomal locus, ABA-7, as
262 this provided consistent results during validation. To validate the markers on individuals with
263 known sex, the relative DNA abundance, or fold-change, in adult males and females, in

264 comparison to females, was determined using the $2^{-(\Delta\Delta Ct)}$ method (Figure 2B, C). For females,
265 autosomal loci showed an average fold-change of 1.09, and sex-linked loci showed an average
266 fold-change of 1.00. One male individual was excluded from our dataset due to inexplicable
267 fold-change values. With this male excluded, for a total of five males, the sex-linked loci on
268 superscaffold 9 exhibited on average a 0.42-0.61 fold-change in DNA abundance in males, while
269 the locus on superscaffold 10 had an average fold-change of 0.99 in males. The presumably
270 autosomal locus on superscaffold 5 had an average fold-change of 0.84 in males (Figure 2C).
271 Sex-linked loci on superscaffold 9 thus show about half the DNA abundance in males with an
272 average of 0.48 compared to females, and an average of 1.01 for females compared to females.
273 These markers on superscaffold 9 are therefore reliable for determining the sex of juveniles.
274 Due to their inconsistent results, ABA-5 and ABS-10C were not used for sexing juveniles.

275 *Application of sex markers in juveniles*

276 We used one autosomal locus (ABA-7) and three sex-linked loci (ABS-9.3, ABS-9.4, and ABS-9.5)
277 to determine the probable sex of ten individual spiderlings from a single clutch (Table S4). As
278 shown in Figure 2D, two clear groups of spiderlings are visible: five individuals have an average
279 fold change of 0.49 for the sex-linked loci, indicating that they are males, while the other 5 had
280 an average fold change of 0.98 for these loci, indicating females. In this clutch, our newly-
281 developed markers thus indicate an unbiased sex-ratio of 1:1.

282 Discussion

283 Our study determined the male karyotype of *A. bruennichi*. It is composed of 24 chromosomes
284 with an X_1X_20 system. The same karyotype was found in two other *Argiope* species, namely *A.*

285 *minuta* (Datta & Chatterjee, 1988), and *A. pulchella* (Bole-Gowda, 1958). This karyotype is the
286 most common in analyzed species of the superfamily Araneoidea, and is probably an ancestral
287 feature of this clade (Araujo *et al.*, 2015). As in most other entelegyne spiders, the karyotype of
288 *A. bruennichi* is formed by acrocentric chromosomes (Kořínková & Král, 2013); this karyotype
289 structure can be considered ancestral for entelegynes (Král *et al.*, 2006). Data on three other
290 *Argiope* species are incomplete or different from *A. bruennichi*: the male karyotype of *A.*
291 *amoena* is also formed by 24 chromosomes, including the X_1X_20 system, but the chromosome
292 morphology was not determined (Suzuki, 1951). The same diploid number was found in *A.*
293 *catenulata* (Amalin, Barrion, & Rueda, 1992) and *A. luzona* (Carandang & Barrion, 1994), but the
294 chromosome complement of *A. luzona* is reported to consist of both monoarmed (acrocentric
295 and subtelocentric) and biarmed (metacentric and submetacentric) chromosomes (Carandang
296 & Barrion, 1994). This karyotype structure is exceptional in entelegynes, and should be revisited
297 by analysis of metaphase II, when centromeres are unequivocally identifiable. Biarmed
298 chromosomes of *A. luzona* could arise from acrocentric chromosomes, such as those found in *A.*
299 *bruennichi*, by pericentric inversions. The karyotype of *A. catenulata* is reported to be
300 composed of holocentric chromosomes with an XY system (Amalin *et al.*, 1992). In this case, the
301 authors have most likely mistaken acrocentric chromosomes for holocentric ones, as they do
302 not report any features which support holocentric structure of chromosomes. Moreover, other
303 studied *Argiope* species have monocentric chromosomes (Kořínková & Král, 2013).
304 Furthermore, the determination of the XY system by Amalin *et al.* (1992) is probably also
305 incorrect, as they do not provide any supporting information on the mode of sex chromosome
306 pairing or sex chromosome segregation during male meiosis.

307 As mentioned above, our karyotype analysis revealed 2n=24, X_1X_20 in male *A. bruennichi*. Given
308 the X_1X_20 system, where males have one copy each of the two X chromosomes, this result fits
309 with the 13 superscaffolds in the haploid genome assembly of a female *A. bruennichi* published
310 by Sheffer *et al.* (2021): 11 autosomes + X_1 + X_2. The result showing half coverage for
311 superscaffolds 9 and 10 in the male indicates that these represent the X chromosomes in the *A.*
312 *bruennichi* genome assembly. These superscaffolds are very similar in length (123.24 Mbp and
313 122.82 Mbp), which does not match the lengths of X_1 and X_2 (82.973 Mbp and 75.754 Mbp)
314 derived from the comparison of male and female genome sizes, with the X_1:X_2 ratio from
315 image-based measurements. However, the genome size measurements had a relatively high
316 standard error, and the superscaffold lengths fall within this error margin. The fact that we
317 identified two superscaffolds as putative X chromosomes supports the hypothesis that X
318 chromosomes are highly divergent in spiders (Kořínková & Král, 2013)..
319 In addition to the multiple X chromosomes, the karyotype of two distantly related entelegyne
320 families, Agelenidae and Lycosidae (Král, 2007; Král *et al.*, 2011; Sember *et al.*, 2020) contains a
321 specific XY sex chromosome pair that associates at the centromeres with the multiple X
322 chromosomes during male meiosis (Král, 2007). This pair has been termed "cryptic sex
323 chromosome pair" (CSCP) (Sember *et al.*, 2020) since the chromosomes are homomorphic (i.e.,
324 exhibit no morphological differentiation). Available data suggest that the chromosomes forming
325 the pair are mostly homologous (Sember et al. 2020). The CSCP can only be detected under
326 optimal conditions, as their meiotic association with the X chromosomes is fragile. Due to its
327 discovery in some entelegyne lineages, as well as in more basally branching spider groups, it has
328 been hypothesized that the CSCP represents ancestral spider sex chromosomes (Král, 2007; Král

329 *et al.*, 2011, 2013; Sember *et al.*, 2020). Whether or not *A. bruennichi* possesses the CSCP is
330 beyond the scope of this study. However, if it does exist in *A. bruennichi*, the male Y and X
331 sequences of the CSCP are very similar and the Y likely maps to the X in the genome assembly.
332 The X_1X_20 system of entelegyne spiders is thought to have arisen by X chromosome fission (e.g.,
333 (Pätau, 1948), nondisjunction of the X chromosome of an X0 system (e.g., (Postiglioni & Brum-
334 Zorrilla, 1981), or nondisjunction of the X chromosome of the CSCP (Král, 2007). The lack of
335 sequence similarity of X_1 and X_2 chromosomes found in *A. bruennichi* could reflect their origin
336 by X chromosome fission. Alternatively, lack of sequence similarity could reflect a homologous
337 origin via early duplication of the X chromosome in an X0 system (or early duplications of the X
338 chromosome of CSCP) followed by a long period of differentiation of X_1 and X_2 chromosomes by
339 gene mutations and chromosomal rearrangements. Recent analyses of karyotype evolution of
340 araneomorph spiders suggest a considerable age of X_1 and X_2 chromosomes of the entelegyne
341 X_1X_20 system (Král *et al.*, 2006; Ávila Herrera *et al.*, 2021). Differentiation of X chromosome
342 copies could be accelerated by the inactivation of X chromosome bivalents during meiosis in
343 spider females (Král, 2007).
344 Interestingly, superscaffold 9 (i.e., chromosome X_1) contains Hox cluster "A" in the *A. bruennichi*
345 genome assembly (Sheffer *et al.*, 2021). Hox genes are a set of highly conserved genes that
346 regulate the body plan organization of animals (Pearson, Lemons, & McGinnis, 2005). This
347 finding should be tested with fluorescence *in situ* hybridization, and investigated in other
348 spiders, when chromosome-level assemblies and sequences for X chromosomes become
349 available for more species. The localization of the cluster has potential implications of dosage

350 differences in Hox genes for males and females, and the suspected compensation mechanisms
351 should be investigated further.
352 Our measurements of genome size and GC content roughly matched data from the published
353 genome assembly (Sheffer *et al.*, 2021). The female measurement of 2C=4,079.809 Mbp
354 ±256.265 corresponds to a haploid (1C) genome size of 2,039.904 Mbp ±128.132 (this study).
355 The genome assembly has a size of 1,670.286 Mbp (Sheffer *et al.*, 2021). Likewise, the average
356 chromosome size for females was 156.916 Mbp based on flow cytometry (this study), and
357 126.475 Mbp in the genome assembly (Sheffer *et al.*, 2021). The process of genome assembly
358 may collapse repetitive regions, and usually results in a smaller assembly size than measured
359 via flow cytometry (see Pflug *et al.*, 2020). The GC content in flow cytometry measurements
360 was 35.5% (this study), while the genome assembly has a GC content of 29.3%. Reduced GC
361 content is a common trait in spiders, and the estimates reported here fall within the range
362 reported for entelegyne spiders (27.2-35%, see Krehenwinkel *et al.*, 2019; Sheffer *et al.*, 2021),
363 and indeed all other investigated spider species (Král *et al.*, 2019). The GC content of females
364 ($X_1X_1X_2X_2$) and males (X_1X_20) was nearly identical, which indicates no substantial difference in
365 base ratio between the X chromosomes and the autosomal set. *A. bruennichi* exhibits a larger
366 genome than the two other studied *Argiope* species established using Feulgen densitometry, *A.*
367 *aurantia* (1,584 Mbp) and *A. trifasciata* (1,653 Mbp) (Gregory & Shorthouse, 2003), but falls
368 into the range of 1C reported for the majority of araneids. Excluding the large genome of
369 *Hyposinga* sp. (4,000 Mbp), araneids exhibit a low diversity of genome sizes (1,438 – 2,680
370 Mbp) (Gregory & Shorthouse, 2003).

371 The DNA abundance values obtained by RT-qPCR in males for loci on superscaffold 9 show the
372 expected pattern of approximately half abundance relative to autosomal markers and can thus
373 be reliably used for sexing spiders. The values obtained for ABS-10C, a locus on superscaffold
374 10, and thus putatively sex-linked, on the other hand, resemble values obtained for autosomal
375 loci. Given the compelling evidence from the coverage comparison that supports superscaffold
376 10 as being one of the two X chromosomes, we are currently unsure what might explain this
377 phenomenon. We verified the designed primers *in silico* and found them to be specific to the
378 region we intended to amplify. Further, the coverage for this region was neither suspiciously
379 low nor high. However, an interpretation of the RT-qPCR results without *a priori* expectation
380 would let us think the amplified region is placed on an autosome. While the results obtained
381 with superscaffold 9 are robust, new primers should be designed for other fragments of
382 superscaffold 10. The method presented here is applicable to very small DNA quantities and
383 allowed us to identify the sex of early instar spiderlings, which are otherwise nearly impossible
384 to sex.
385 Having identified the sex chromosomes in the genome assembly, future work can investigate
386 sex chromosome evolution in *A. bruennichi*. In species in which males have one copy of each
387 sex chromosome, as in *A. bruennichi*, the sex chromosomes are expected to evolve faster at the
388 molecular level, relative to autosomes. This is due to several factors, such as fewer
389 recombination events, weaker purifying selection or higher positive selection on recessive
390 alleles relative to autosomes. These processes may lead to the acquisition of specific gene
391 content on X chromosomes and to an X chromosome to autosome divergence (Charlesworth,
392 Coyne, & Barton, 1987; Ellegren, 2009). A comparison of gene content between X

393 chromosomes within and among species from diverse clades of spiders (entelegynes,
394 haplogynes but also basally branching groups), would deliver exciting and novel insights into sex
395 chromosome evolution and eventually of the evolution of sexual differentiation in these
396 arachnids (Rice, 1984).

397 Conclusions

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

409 Data Availability

410 Sequencing reads from the male and female specimens are available on NCBI's Short Read
411 Archive, under BioProject PRJNA629526. Raw data for the flow cytometry data are available in

412 the supplementary material, as is the information on RT-qPCR primers and the Ct values from
413 the RT-qPCR experiments.

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427 Author contributions

428 Other than the first author position, the authors are listed in alphabetical order by last name,
429 not relative to contributions or institutional hierarchy. MMS, MC, GU, JK and MF conceived of
430 the study. MMS, MF, and GU collected spiders. MMS and CJ isolated DNA, prepared libraries for
431 the whole-genome sequencing and performed the sequencing, with input and infrastructure

432 from GU and AWK. MMS, KH, MG, and MC selected loci, designed primers, and performed the
433 RT-qPCR analysis. MK and MF performed the karyological analysis of chromosomes, with input
434 and infrastructure from JK. EL performed the flow cytometry measurements of genome size
435 and GC content. All authors contributed to manuscript writing and revision.

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584

585 # Figure legends

586 **Figure 1**. *A. bruennichi male*, 2n=24, X_1X_20, Giemsa stained mitotic (A) or meiotic (B-J)

587 chromosomes. Arrows points to sex chromosomes. (A) Karyotype, based on spermatogonial

588 prometaphase, (B) leptotene. Chromosomes X_1 and X_2 are not associated, (C) two zygotene

589 plates. Sex chromosomes form the body on the periphery of the nucleus, (D) pachytene. The

590 sex chromosome body persists on the periphery of the nucleus, (E) diplotene. Heteropycnotic

591 X_1 and X_2 pair by their centromeric regions, (F) metaphase I. Note a slight positive

592 heteropycnosis of X chromosomes, which are arranged in parallel on the periphery of the plate,

593 (G) two sister prophases II. The left plate contains 11 chromosomes and the right plate 13

594 chromosomes, (H) two sister metaphases II. Sex chromosomes are slightly positively

595 heteropycnotic (arrowhead - two overlapping chromosomes), (I) two sister anaphases II. Two

596 half plates with 11 chromosomes and two half plates with 13 chromosomes (half plates

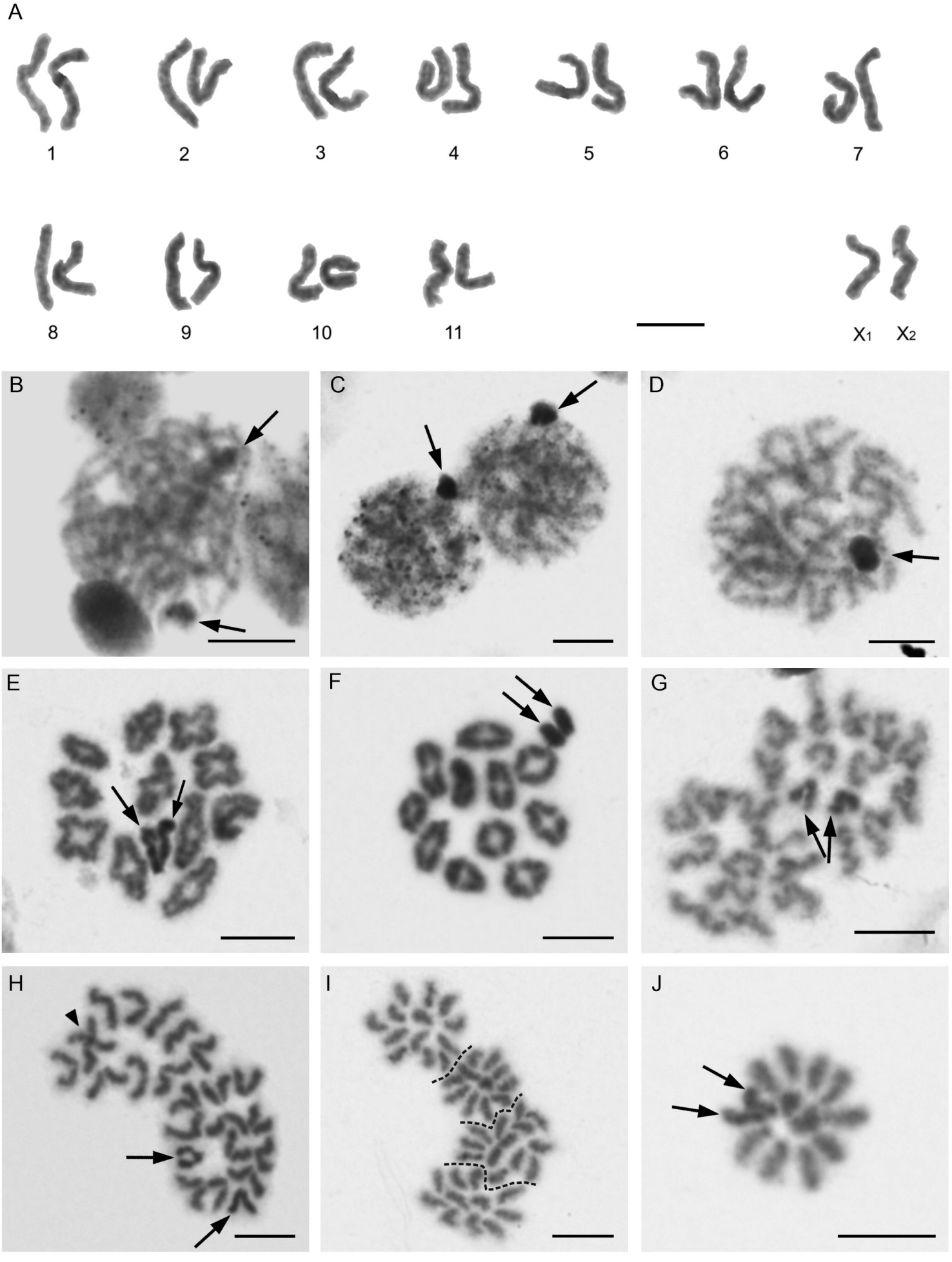
597 separated by dashed line). X chromosomes indistinguishable from the other chromosomes, (J)

598 late anaphase II, half plate. Note a slight positive heteropycnosis of X chromosomes. Scale bar =

599 5 µm.

600

601 **Figure 2.** Identification of the sex chromosomes in the *Argiope bruennichi* genome assembly.
602 The dashed horizontal lines represent (A) the expected coverage and (B-D) expected DNA
603 abundance for sex chromosomes in females (1) and males (0.5) relative to females. (A) Relative
604 sequencing coverage of males and females across the 13 superscaffolds (Scaff) in the genome
605 assembly. (B-D) Boxplots show the results of the $2^{-(\Delta\Delta Ct)}$ method, calculated from average
606 adjusted Ct values for each locus and individual. Symbols represent individual datapoints, boxes
607 represent the interquartile range, thick lines represent medians, and whiskers represent 1.5
608 times the interquartile range. (B) RT-qPCR results for females. (C) RT-qPCR results for males. (D)
609 RT-qPCR results for spiderlings for the subset of markers delivering reliable results (ABS-9.3,
610 ABS-9.4, ABS 9.5, ABA-7).
611


A
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X1
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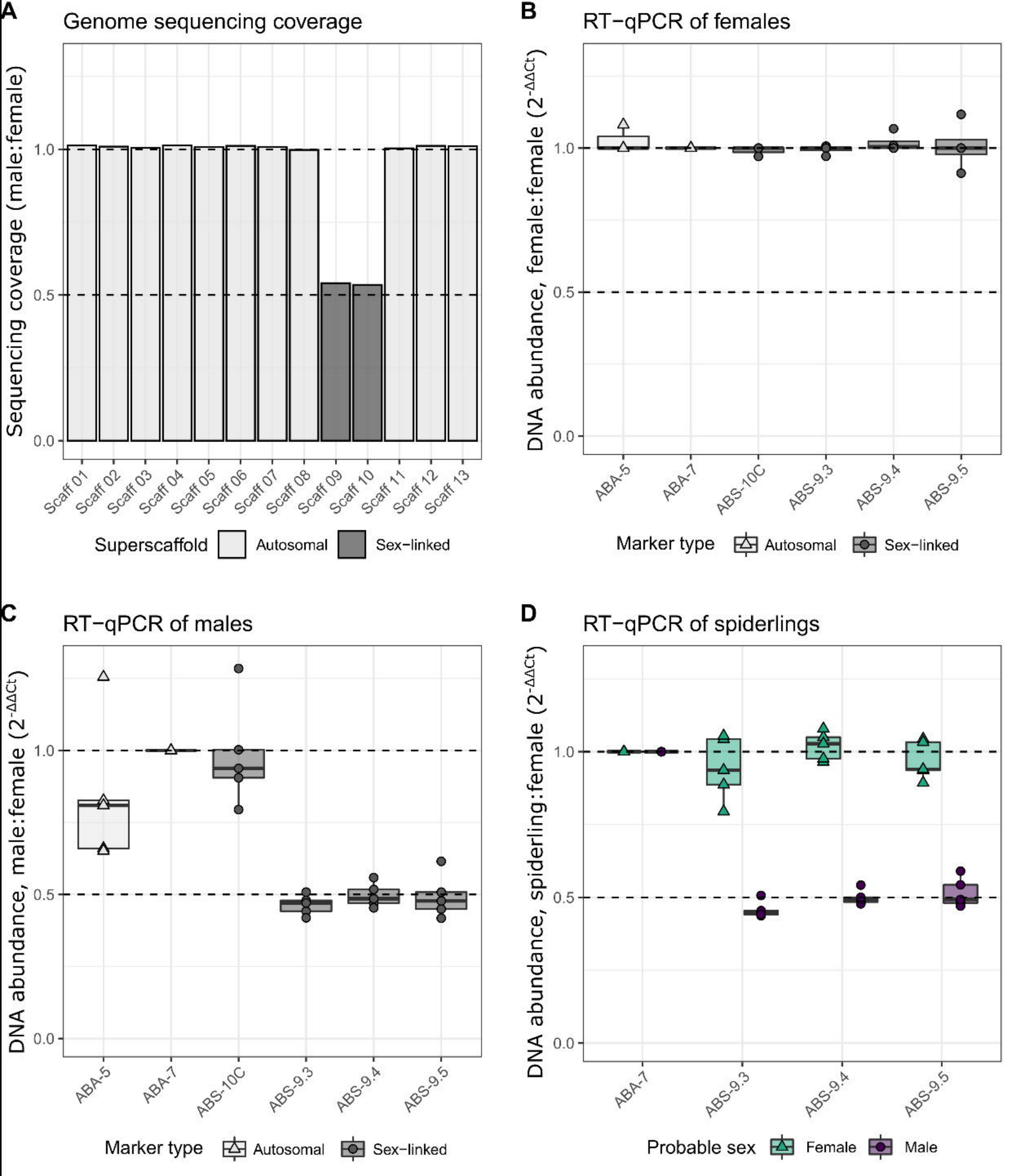
A
Genome sequencing coverage
Sequencing coverage (male:female)
1.0
0.5
0.0
Scaff 01
Scaff 02
Scaff 03
Scaff 04
Scaff 05
Scaff 06
Scaff 07
Scaff 08
Scaff 09
Scaff 10
Scaff 11
Scaff 12
Scaff 13
Superscaffold
Autosomal
Sex−linked
B
RT−qPCR of females
DNA abundance, female:female ($2^{-\Delta\Delta Ct}$)
1.0
0.5
0.0
ABA-5
ABA-7
ABS-10C
ABS-9.3
ABS-9.4
ABS-9.5
Marker type
Autosomal
Sex−linked
C
RT−qPCR of males
DNA abundance, male:female ($2^{-\Delta\Delta Ct}$)
1.0
0.5
0.0
ABA-5
ABA-7
ABS-10C
ABS-9.3
ABS-9.4
ABS-9.5
Marker type
Autosomal
Sex−linked
D
RT−qPCR of spiderlings
DNA abundance, spiderling:female ($2^{-\Delta\Delta Ct}$)
1.0
0.5
0.0
ABA-7
ABS-9.3
ABS-9.4
ABS-9.5
Probable sex
Female
Male