Sexual Development

Commentary

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Concerning an Article by Ehl et al.: False Premise Leads to False Conclusions

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Error · PCR test · Pogona · Sex-linked marker · Sex reversal

In their recent paper, entitled "With or without W? Molecular and cytogenetic markers are not sufficient for identification of environmentally-induced sex reversal in the bearded dragon", Ehl et al. [2021] demonstrate that a published PCR sex test developed for the bearded dragon (F4-F1 marker) [Quinn et al., 2010] is only partially diagnostic for sex because the sex-linked sequence upon which the test is based is subject to recombination with sex and therefore inappropriate for studies of sex reversal. The authors use the deficiencies of this now superseded PCR F1-F4 sex test developed by Quinn et al. [2010] to call into question the more recent and robust sex reversal work of Holleley et al. [2015] and subsequent work on this system in *Pogona vitticeps* [Castelli et al., 2021].

This criticism is completely misplaced, because none of the sex reversal work criticised by Ehl et al. [2021] used the original Quinn F4-F1 PCR sex test.

Indeed, the limitation of the Quinn F4-F1 test was recognised early by our group, because some phenotypic males were genotyped as ZW individuals using the Quinn F4-F1 sex test. To address this limitation, different sex-linked sequences (using primers H2 and F of Quinn et al. [2010]) were characterised and developed as an improved PCR sex test (H2-F) [Holleley et al., 2015], which correctly assigned the individuals misassigned by the Quinn F4-F1 test. The robust Holleley H2-F PCR sex test has been used in all subsequent studies of sex reversal in the dragon lizard [e.g., Castelli et al., 2021; Whiteley et al., 2021] in which no phenotypic males scoring as ZW genotypes were reported.

We therefore completely reject the claims of Ehl et al. [2021] that the sex tests used in our sex reversal work on *P. vitticeps* were in any way inadequate.

The factual error by Ehl et al. [2021] evidently arose because of the failure of the authors to recognise the difference between these 2 molecular sex tests. The 2 tests use different PCR primers to amplify sequences from different members of a family of highly repetitive elements [Quinn et al., 2010], so it was unreasonable to assume that the 2 tests targeted the same region of the dragon W chromosome (as subsequently confirmed). The authors were evidently confused by the derivation of the 2 sets of sex-linked sequence upon which the tests were based from the same family of repetitive sequences that lie on the Z and W chromosome in high copy numbers relative to autosomes [Quinn et al., 2010]. The Quinn F4-F1 test is based on a sequence within this repetitive series with 4 W-specific SNPs detectable by PCR. The Holleley H2-F test is based on a different copy of the repetitive sequence at a different location on the W chromosome and is distinguished by the presence of 2 considerable W-specific deletions (150 bp and 14 bp). The 2 sequences are amplified by PCR using different primers. The sex specificity of the Quinn F4-F1 test is derived from one sex-specific SNP in

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the sequence to which one of the primers anneals; the sex specificity of the Holleley H2-F test uses amplicon length variation arising from the 2 W-specific indels in the amplified sequence. Thus, the 2 PCR tests have a fundamentally different basis.

The Quinn F4-F1 sequence is likely to be distant from the sex-determining locus on the W chromosome. Collating the results of our studies, we find that approximately 15% of phenotypic males score as having the Quinn W-specific sequence (n = 36 "ZW" males of a total of 229 phenotypic males; total number of tests = 517 individuals), validating the recombination inferred by Ehl et al. [2021] to have occurred in their familial lineage. In contrast, the Holleley H2-F sex-specific sequence must be very close to the sex-determining locus, since no recombinants have been detected among more than 900 individuals collated across our multiple studies, including those of the Castelli et al. [2021] study. Directly comparing the 2 tests, 339 individuals whose phenotypic sex was reliably known were tested with both PCR tests. The Quinn F1-F4 test yielded 11 individuals that scored as ZW but presented a male phenotype; in contrast, all 11 were correctly identified as ZZ using the Holleley H2-F test.

For these reasons, it is incorrect for Ehl et al. [2021] to equate the 2 loci and extend their criticism of the Quinn F4-F1 sex test to studies using the Holleley H2-F test. The 2 underlying tests are not equivalent; they target *different* sequences at *different* loci on the W chromosome. They reside at *different* distances from the sex-determining locus and indeed are subject to very *different* rates of recombination with sex, resulting in a detectable misidentification rate for the Quinn F1-F4 test and zero for the Holleley H2-F test. It is false to claim that the 2 tests differ only in the primer pairs used to isolate them.

Ehl et al. [2021] did not have access to the detailed data we have accumulated to demonstrate the different behaviours of the 2 PCR tests with respect to recombination. However, we believe sufficient information was readily available to Ehl et al. [2021] at the time of their experiments to indicate the 2 PCR tests could not be claimed to be equivalent and should not have been used as a premise in the absence of direct supporting evidence. The 2 tests had already been described in detail in published papers [Quinn et al., 2007, 2010; Holleley et al., 2015]. Details of the method for the Holleley H2-F PCR test were described in by Holleley et al. [2015], including the PCR conditions necessary to amplify the W-specific sequence and the internal positive control. Moreover, the distinction between the 2 tests would have been immediately evident had Ehl et al. [2021] compared the amplicons they generated with those reported from the Holleley H2-F test, which were published at the time (GenBank accession numbers EU938138.1 and KM508988) or that could have been generated by Ehl et al. [2021] with a simple PCR and Sanger sequencing.

Thus, Ehl et al. [2021] claimed equivalency of the 2 PCR tests without demonstrating equivalency when, in our opinion, there were ample indications at the time of publishing that this is not a reasonable premise, and it is the omission of this essential step that has led to false conclusions. Had Ehl et al. [2021] applied both PCR tests, the difference in misassignment rates would have become evident. Had Ehl et al. [2021] compared the sequence they amplified with the publicly available data, the difference in sequence identity would have become evident. But, from our understanding, they did neither.

We therefore completely reject the claims of Ehl et al. [2021] and Erratum that the validity of the Holleley sex test was unknown at the time of their experiments.

In summary, although the general call for caution in Ehl et al. [2021] in the use of unvetted sex-linked sequences to infer sex reversal is legitimate, the authors were quite mistaken in using inadequacies of one PCR test (F1-F4 of Quinn et al. [2010]) to call into question other studies that did not use that test [e.g., Holleley et al., 2015; Castelli et al., 2021]. Their unfounded criticism of the application of the PCR sex test used by Holleley et al. [2015] and Castelli et al. [2021], and their uncalled-for suggestion that Castelli et al. [2021] were swayed by the allure of a good story in the face of opposing facts, are without foundation.

Conflict of Interest Statement

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Author Contributions

All authors evaluated the paper by Ehl et al. [2021] and contributed to writing this commentary.

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Sexual Development

Erratum

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Concerning the article "With or Without W? Molecular and Cytogenetic Markers Are Not Sufficient for Identification of Environmentally Induced Sex Reversal in the Bearded Dragon" [*Sexual Development*, this issue, DOI: 10.1159/000514195] by Ehl et al., the following additions should be observed.

1. The article describes the sexing protocol used in Quinn et al. [2010] as equivalent in the region amplified to that used in Holleley et al. [2015] and Castelli et al. [2020]. The protocol by Quinn et al. [2010] is based on the amplification of a fragment by the primer pair F4/F1, while the one used by Holleley et al. [2015] and Castelli et al. [2020] is by the primer pair H2/F, both published in Quinn et al. [2010]. Our claim of the equivalence of these tests was based on the information that both fragments are a part of the same short sequence assigned as "contig C" as depicted by Quinn et al. [2010] and also reported like this by Holleley et al. [2015]. However, after the online publication of our paper, Prof. Arthur Georges and members of his team informed us that the fragments amplified by these two primer pairs are not a part of the same short continuous sequence (contig), which was not possible to determine from published resources. Instead, according to their information, the amplified fragments are parts of different paralogs of a repetitive sequence previously assigned as "contig C". Therefore, these two protocols are not equivalent.

Sentence 3 of the Materials and Methods section should then read:

During a genetic screening of captive-bred central bearded dragons and the previous experiment with sex-reversed individuals [Ehl et al., 2017], we uncovered a male with a mismatch between the phenotypic sex and the genotype: it possessed a female-specific allele in the locus assigned as "contig C" by Quinn et al. [2010], serving as a sex-specific PCR marker previously used for detection of sex-reversed individuals in this species [Quinn et al., 2010; Ehl et al., 2017].

Consequently, the second part of the paragraph 3 of the Discussion (since the fifth sentence) should read:

In the recent study [Castelli et al., 2020], the mismatch between phenotypic and genotypic sex was assigned by the molecular marker used by Holleley et al. [2015] and was found in 5% out of 534 examined individuals of *P. vitticeps* covering the whole species range. Notably, all 28 animals with the mismatch were phenotypic females and they were recorded only in the south-western part of the species range. While this clustered distribution is consistent with non-random occurrence of sex reversals in certain environmental conditions at the edge of the species distribution as interpreted by Castelli et al. [2020], the spatial clustering can also reflect the geographic spread of a mutation, recombination, or other rearrangement concerning the region of the W chromosome containing the otherwise female-specific marker used for identification of the individuals with the mismatch. The current study discloses that the causes of the mismatch between the phenotypic and genotypic sex should be investigated more rigorously. Holleley et al. [2015] tested the reliability of their molecular marker by a congruence with cytogenetics, but our current work demonstrates that the presence of the accumulation of AAGG repeats is not a fully reliable marker as well.

2. The Editors also pointed out that the labelling of the metaphases in Figure 1 and supplementary Figure S2 could be misleading for readers and the figures should be relabelled. We state in the figure legends that "W^m in the caption in males reflects the presence of the W-specific marker in PCR", not the presence of the W chromosome. We hoped that it would prevent a misunderstanding that we claim that the male with the W-specific molecular fragment possesses the W chromosome (our interpretation of all the data is just the opposite). However, we agree that readers could be confused by our labelling. Therefore, we change the figures and their legends to the version below (Fig. 1, suppl. Fig. S2).

3. Figure 2 in the article presents the results for the W-specific fragment only, for clarity. The original gel produced as described in the methods including positive controls, and used for analysis, is included as supplementary Figure S3 below.

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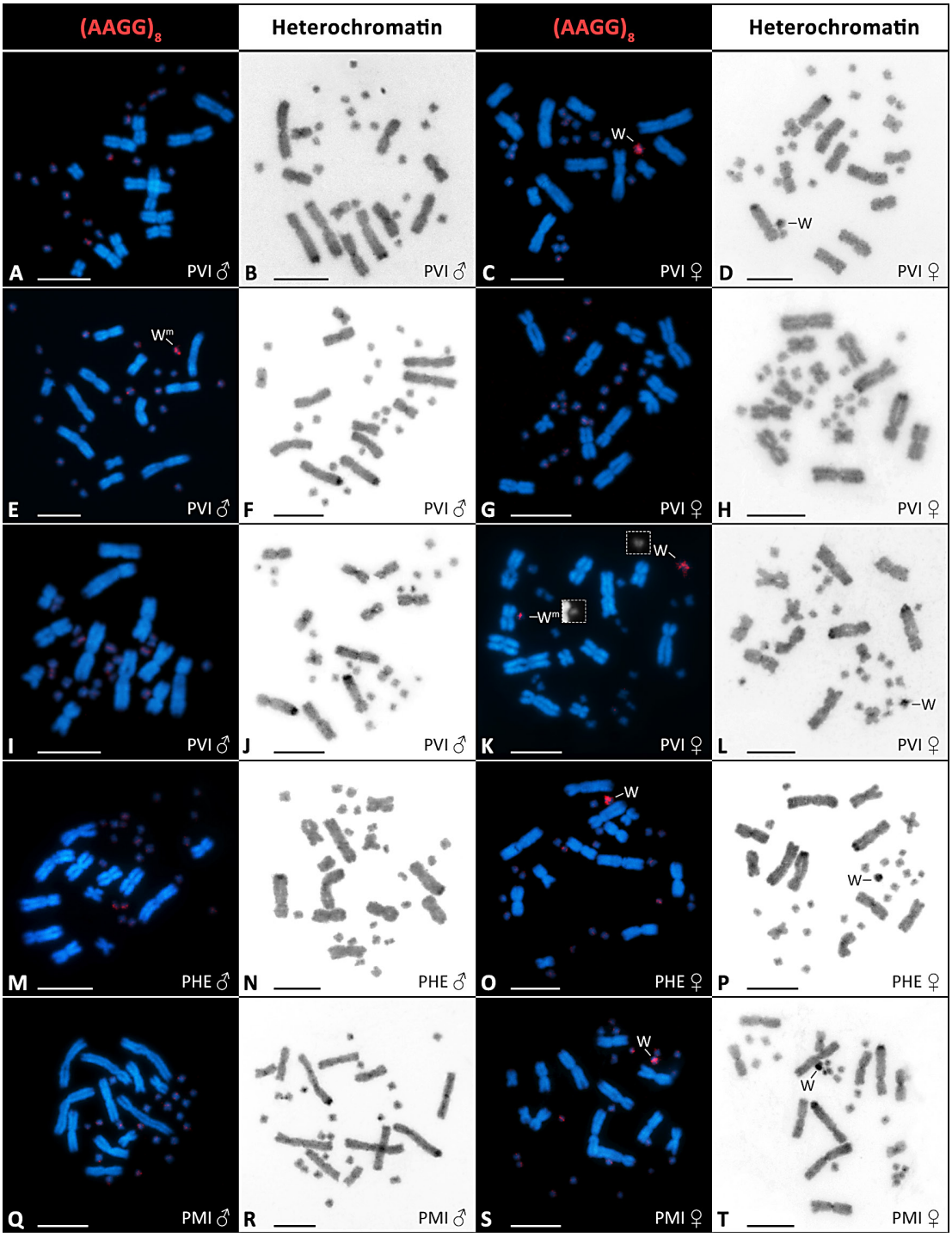
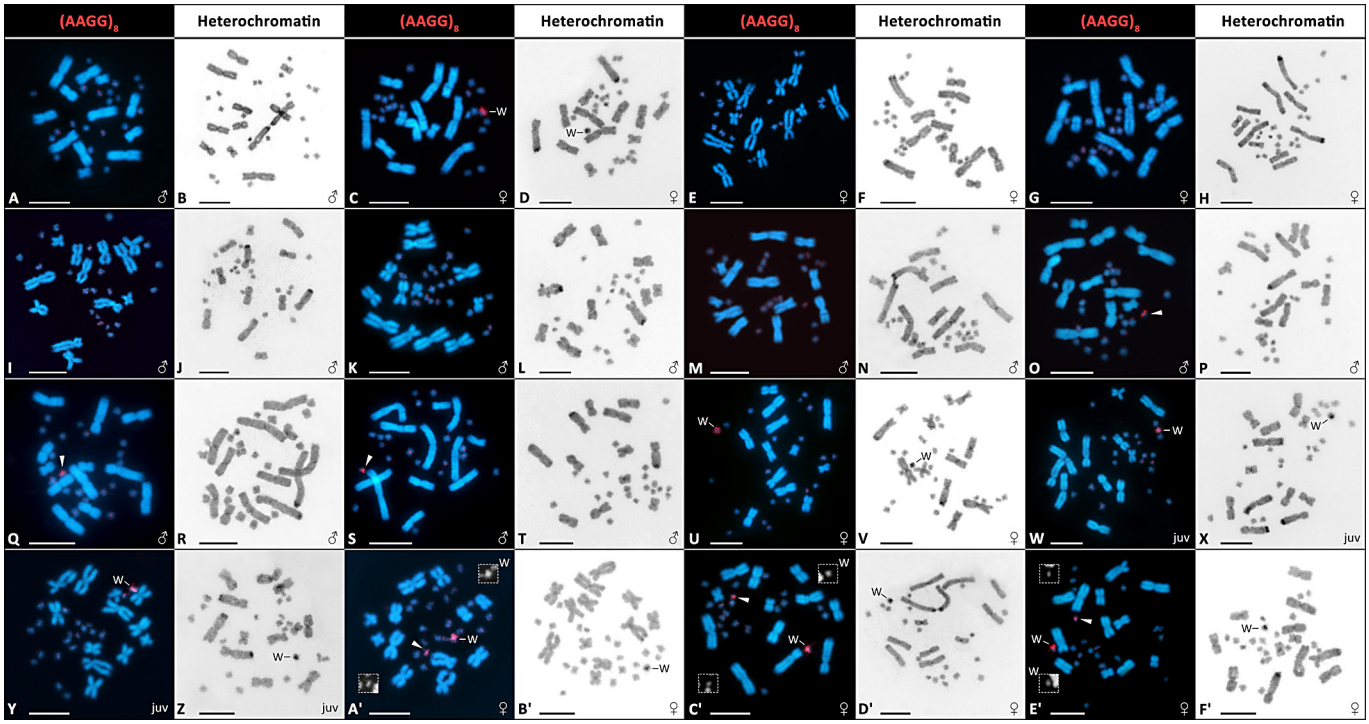
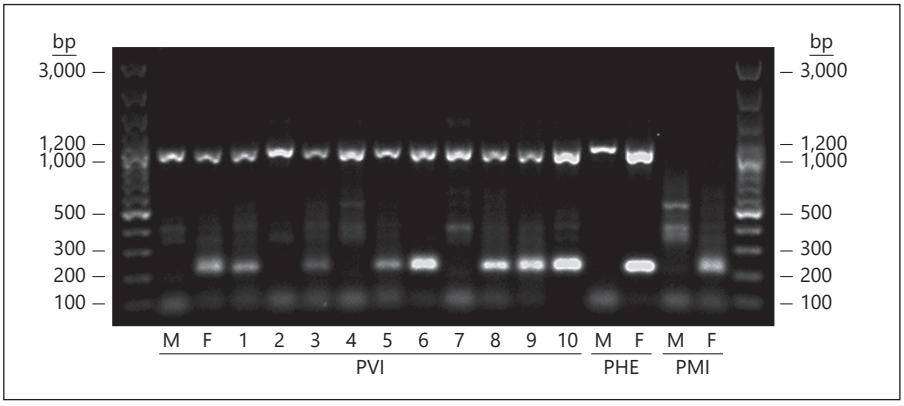


Fig. 1. Visualization of the accumulation of AAGG repeats and heterochromatin in selected individuals of *P. vitticeps* (PVI; **A–L**), *P. henrylawsoni* (PHE; **M–P**), and *P. minor* (PMI; **Q–T**). In *P. vitticeps*, the figure represents successively a standard male ZZ karyotype with no accumulations (**A**, **B**), standard female ZW karyotype with AAGG (**C**) and heterochromatin accumulation (**D**), karyotype of a male with the W-specific molecular marker amplified by primers F4/F1 and with AAGG accumulation (**E**) but without heterochromatinization (**F**), karyotype of a sex-reversed ZZ female with no accumulations (**G**, **H**), karyotype of a male positive for the molecular marker and no accumulations (**I**, **J**), and karyotype of a female with 2 AAGG accumulations (**K**) and unpaired heterochromatic block (**L**). In *P. henrylawsoni* and *P. minor,* male karyotypes with no accumulations (**M**, **N**, **Q**, **R**) and female karyotypes with AAGG (**O**, **S**) and heterochromatin accumulations (**P**, **T**) are shown. Boxes in **K** show the W chromosome and the chromosome with the AAGG accumulation inherited from the father in separated blue channel mode to present their size difference. Scale bars, 10 μm.



Suppl. Fig. S2. Visualization of accumulation of AAGG repeats and heterochromatin in 16 individuals of *Pogona vitticeps*. The figure represents male offspring of sex-reversed ZZ female and male positive for the W-specific molecular marker amplified by primers F4/F1 and with AAGG accumulation – the karyotype with no accumulations (**A**, **B**); standard female with AAGG (**C**) and heterochromatin accumulation (**D**) used in the cross of the ZW female with the marker-positive male; karyotypes of two sex-reversed ZZ females with no AAGG accumulations (**E**, **G**) and no heterochromatinization (**F**, **H**). In the following part of the Figure the offspring of a standard ZW female and marker-positive male as it follows: karyotypes of 3 males positive for the marker with no accumulation (**I**–**N**); karyotypes of 3 males positive for the marker with one microchromosome bearing AAGG accumulation (**O**, **Q**, **S**; arrowheads) but without heterochromatinization (**P**, **R**, **T**); karyotypes of 1 ZW female and 2 juveniles with undetermined sex showing AAGG (**U**, **W**, **Y**) and heterochromatin accumulation on one microchromosome (**V**, **X**, **Z**); karyotypes of 3 females with 2 AAGG accumulations (**A′**, **C′**, **E′**) and unpaired heterochromatic blocks (**B′**, **D′**, **F′**). Boxes (**A′**, **C′**, **E′**) show W and the chromosome with the AAGG accumulation in separated blue channel mode to present their size difference. Scale bars, 10 μm.



Suppl. Fig. S3. Results of duplex PCR with E/C primers used for positive control and F4/F1 (W-specific fragment) primers in *Pogona vitticeps* (PVI), *P. henrylawsoni* (PHE), and *P. minor* (PMI) following the protocol of Quinn et al. (2010). All animals show the specific control product (around 1,000 bp) except for both PMI individuals. Standard females (F) of PVI, PHE and PMI display the W-specific fragment (224 bp), whereas in standard males (M) this fragment is not amplified. PVI individuals 1–10 include: (1) male with the W-specific PCR fragment and AAGG accumulation, used for the crosses, (2) sex-reversed ZZ female, (3, 4) male offspring of sex-reversed females and the male positive for the W-specific PCR fragment with AAGG accumulation, (5) offspring of unknown sex from the same cross, (6) standard ZW female used in the cross with this male, i.e. the mother of the offspring 7–10 depicted here (7, 8) male offspring of standard female and the male positive for the W-specific PCR fragment with AAGG accumulation, and (9, 10) female offspring of standard ZW female and the male positive for the W-specific PCR fragment with AAGG accumulation.

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With or Without W? Molecular and Cytogenetic Markers are Not Sufficient for Identification of Environmentally-Induced Sex Reversal in the Bearded Dragon

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Keywords
Molecular markers · Reversal · Sex chromosomes · Sex linkage · Vertebrates

Abstract
Transitions from environmental sex determination (ESD) to genotypic sex determination (GSD) require an intermediate step of sex reversal, i.e., the production of individuals with a mismatch between the ancestral genotypic and the phenotypic sex. Among amniotes, the sole well-documented transition in this direction was shown in the laboratory in the central bearded dragon, *Pogona vitticeps*, where very high incubation temperatures led to the production of females with the male-typical (ZZ) genotype. These sex-reversed females then produced offspring whose sex depended on the incubation temperature. Sex-reversed animals identified by molecular and cytogenetic markers were also reported in the field, and their increasing incidence was speculated as a climate warming-driven transition in sex determination. We show that the molecular and cytogenetic markers normally sex-linked in *P. vitticeps* are also sex-linked in *P. henrylawsoni* and *P. minor*, which points to quite ancient sex chromosomes in this lineage. Nevertheless, we demonstrate, based on a crossing experiment with a male bearded dragon who possesses a mismatch between phenotypic sex and genotype, that the used cytogenetic and molecular markers might not be reliable for the identification of sex reversal. Sex reversal should not be considered as the only mechanism causing a mismatch between genetic sex-linked markers and phenotypic sex, which can emerge also by other processes, here most likely by a rare recombination between regions of sex chromosomes which are normally sex-linked. We warn that sex-linked, even apparently for a long evolutionary time, and sex-specific molecular and cytogenetic markers are not a reliable tool for the identification of sex-reversed individuals in a population and that sex reversal has to be verified by other approaches, particularly by observation of the sex ratio of the progeny.

Introduction

Amniotes possess a large variability in sex determination. According to comparative studies, genotypic sex determination (GSD) and thus sex chromosomes evolved within amniotes independently around 40

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times [Johnson Pokorná and Kratochvíl, 2016; Rovatsos et al., 2016, 2019a]. Some species – among amniotes forming a minority – rely on environmental sex determination (ESD), where sexes do not differ in their genotypes and the sex of an individual is set by environmental conditions during a sensitive developmental period. ESD represents a special case of polyphenism. Once emerged, GSD seems to be very evolutionarily stable at least in some lineages of amniotes, as demonstrated by the stability of sex chromosomes in mammals [Waters et al., 2007; Cortez et al., 2014], birds [Shetty et al., 1999; Zhou et al., 2014], softshell turtles [Rovatsos et al., 2017], iguanas [Gamble et al., 2014; Rovatsos et al., 2014a,b; Altmanová et al., 2018], caenophidian snakes [Rovatsos et al., 2015a], lacertids [Rovatsos et al., 2016, 2019b], varanid lizards [Iannucci et al., 2019; Rovatsos et al., 2019c], and skinks [Kostmann et al., 2021]. While independent evolution of GSD within amniotes is well supported by its phylogenetic distribution and genomic evidence for nonhomology of sex chromosomes among particular amniote lineages (although some lineages co-opted the same genomic regions for the function of sex chromosomes) [Rovatsos et al., 2019a; Straková et al., 2020], independent origins of ESD from the ancestral GSD are still controversial. Some authors propose that transitions in this direction might be rapid and common [Janzen and Paukstis, 1991; Barske and Capel, 2008; Marshall Graves, 2008; Sarre et al., 2011; Ezaz et al., 2017; Pennel et al., 2018]. However, many of the reported GSD to ESD transitions suggested within lacertids, skinks, varanids, and chameleons appeared to be based on the erroneous assignment of GSD species as ESD [Rovatsos et al., 2015b; Hill et al., 2018; Nielsen et al., 2018; Iannucci et al., 2019; Kostmann et al., 2021]. Other authors question the frequent transitions from GSD to ESD based on the phylogenetic distribution of ESD clades and general evolutionary stability of GSD [Pokorná and Kratochvíl, 2009; Gamble et al., 2015; Johnson Pokorná and Kratochvíl, 2016; Straková et al., 2020].

A transition from GSD to ESD requires an intermediate step of sex reversal, i.e., the production of individuals with a mismatch between the ancestral genotypic and the actual phenotypic (gonadal) sex. Such a transition from the ancestral GSD to ESD was unequivocally demonstrated only in the laboratory in the central bearded dragon, *Pogona vitticeps* [Ahl, 1926] [Holleley et al., 2015]. Sex in this species is determined by female heterogamety (ZZ/ZW sex chromosomes), where the W sex chromosome is cytogenetically easily recognizable due to a large heterochromatic block containing an accumulation of repeats with the AAGG motif [Ezaz et al., 2005; Matsubara et al., 2016]. However, high constant incubation temperatures starting above 32°C feminized genotypic males, i.e., led to the production of females with the male-typical ZZ genotype [Quinn et al., 2007; Holleley et al., 2015]. In the next generation, such sex-reversed females mated with normal ZZ males produced offspring exclusively with the ZZ genotype whose sex depended on the incubation temperature. These laboratory studies demonstrated that the cytogenetically recognizable sex chromosome (here the W) can be lost in a single generation, and therefore, a short time is potentially needed for GSD to ESD transitions [Holleley et al., 2015]. At a population level, the derived ESD could then potentially sweep out GSD and become fixed in a population. Sex-reversed animals identified by molecular and cytogenetic markers were reported in the central bearded dragon also in the field [Holleley et al., 2015; Castelli et al., 2020], and their higher incidence in recent years is thought to be in connection with global warming [Holleley et al., 2015]. While the scenario of a climate change-driven transition in sex determination in the central bearded dragon sounds theoretically appealing, here we show that both cytogenetic and molecular genetic markers used for the demonstration of the occurrence of sex reversal in the field in this species are not sufficient for detection of sex reversals, hence evidence for sex-reversed animals in wild populations is not very strong. More generally, we investigated what evidence is needed for the identification of sex reversal, which has a profound influence on our understanding of the stability of sex determination in nature and points to limitations of the commonly used approaches based on genetic markers.

Materials and Methods

The central bearded dragon is an agamid lizard native to arid central Australia. It reaches approximately 40–60 cm in total length and has a body mass of around 200–500 g. Females typically lay 15–40 eggs per clutch. This species became a popular pet worldwide [Cogger, 1992; De Vosjoli et al., 2016]. During a genetic screening of captive bred central bearded dragons and the previous experiment with sex-reversed individuals [Ehl et al., 2017], we uncovered a male with a mismatch between the phenotypic sex and the genotype: it possessed a female-specific allele in the locus assigned as 'contig C' by Quinn et al. [2010], serving as a sex-specific PCR marker previously used for detection of sex-reversed individuals in this species [Quinn et al., 2010; Holleley et al., 2015; Ehl et al., 2017; Castelli et al., 2020]. The W-specific fragment represents an anonymous sequence which differs in 4 single nucleotide polymorphisms (SNPs) from the homologous Z-linked locus [Quinn et al., 2010]. We hypothesized that this individual could

represent female-to-male sex reversal and tested this possibility by experimental crosses to uncover inheritance of this putative sex-specific locus. Furthermore, as the W chromosome is well defined in this species and cytogenetics was also used for determination of sex-reversed individuals [Ezaz et al., 2005; Holleley et al., 2015], we performed cytogenetic analyses and determined how the molecular and cytogenetic markers correspond to the phenotypic sex. Additionally, 2 pairs of *P. henrylawsoni* (PHE) and 1 pair of *P. minor* (PMI) were analysed as an outgroup.

Experimental Crosses

We mated the male with the PCR W-linked fragment (the genotype assigned further as ZW^m, W^m indicates male W-like chromosome) with 3 unrelated females. One of the females possessed the typical ZW genotype, the other 2 females were sex reversed (ZZ sex chromosomes) by the high incubation temperature of 35.5°C [Ehl et al., 2017]. Eggs from these crosses were incubated at the constant temperature of 28°C, the standard incubation temperature which does not lead to thermally-induced sex reversal [Quinn et al., 2010; Holleley et al., 2015; Ehl et al., 2017]. These experimental crosses were done to elucidate the potential effect of the W^m in sex determination and viability of the progeny. Predictions on the sex ratio in the cross between the ZW^m male and the ZW female depend on the viability of WW^m individuals. In lineages with a degenerated W chromosome, the WW genotype is lethal [Harada and Buss 1981; Watts et al., 2006], while poorly differentiated sex chromosomes allow viable WW offspring [Roco et al., 2015]. Although sex chromosomes in *P. vitticeps* are cytogenetically easily distinguishable, no Z-specific gene (i.e., a gene missing on the W chromosome) was identified yet suggesting that its Z and W sex chromosomes do not differ substantially in gene content [Georges et al., 2015; Deakin et al., 2016]. Gonadal phenotype in ZW^m offspring should differentiate between sex reversal (ZW^m offspring incubated at the standard, non-reverting temperature should be females) versus its alternatives (the W^m chromosome does not participate in sex determination as the W and offspring should be ZW^m males).

The schematic overview of the experimental crosses in *P. vitticeps* is presented in online supplementary Figure 1 (for all online suppl. material, see www.karger.com/doi/10.1159/514195).

PCR Genotyping

Approximately 200 μL of blood was collected from the ventral tail vein for cytogenetic approaches and DNA isolation. In individuals who died during/early after hatching the tail tip was collected instead. DNA was extracted using DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions. For PCR genotyping, we used the protocol by Quinn et al. [2010]. This duplex PCR was designed to simultaneously amplify the W-specific fragment present only in females (224 bp, primers F4-F1) and an additional fragment present in both sexes (963 bp, primers E-C) as a positive control.

The W-specific PCR products from 4 individuals were sequenced in order to check the amplification of specific sequences and the presence of female-specific SNPs. The PCR products were purified with QIAquick PCR Purification Kit (Qiagen). Sequencing reactions were performed with BigDye Terminator Cycle Sequencing Kit 1.1 (Applied Biosystems) according to the manufacturer's protocol. Sequence products were purified using DyeEx 2.0 Spin Kit (Qiagen) and sequenced bi-directionally on an Applied Biosystems 3130 Genetic Analyser using 30 cm long capillaries and POP-7 polymer. The obtained sequences were assembled and checked by eye using the SeqMan II version 5.05 module of the Lesergene software package (DNASTAR). Obtained sequences were checked by BLASTN search [Altschul et al., 1990] for similarity with sequences already existing in GenBank. Consensus sequences were aligned in Sequencher 4.1.4 (Gene Codes Corporation) together with reference sequences from the previous study of Quinn et al. [2010].

Cytogenetic Analyses

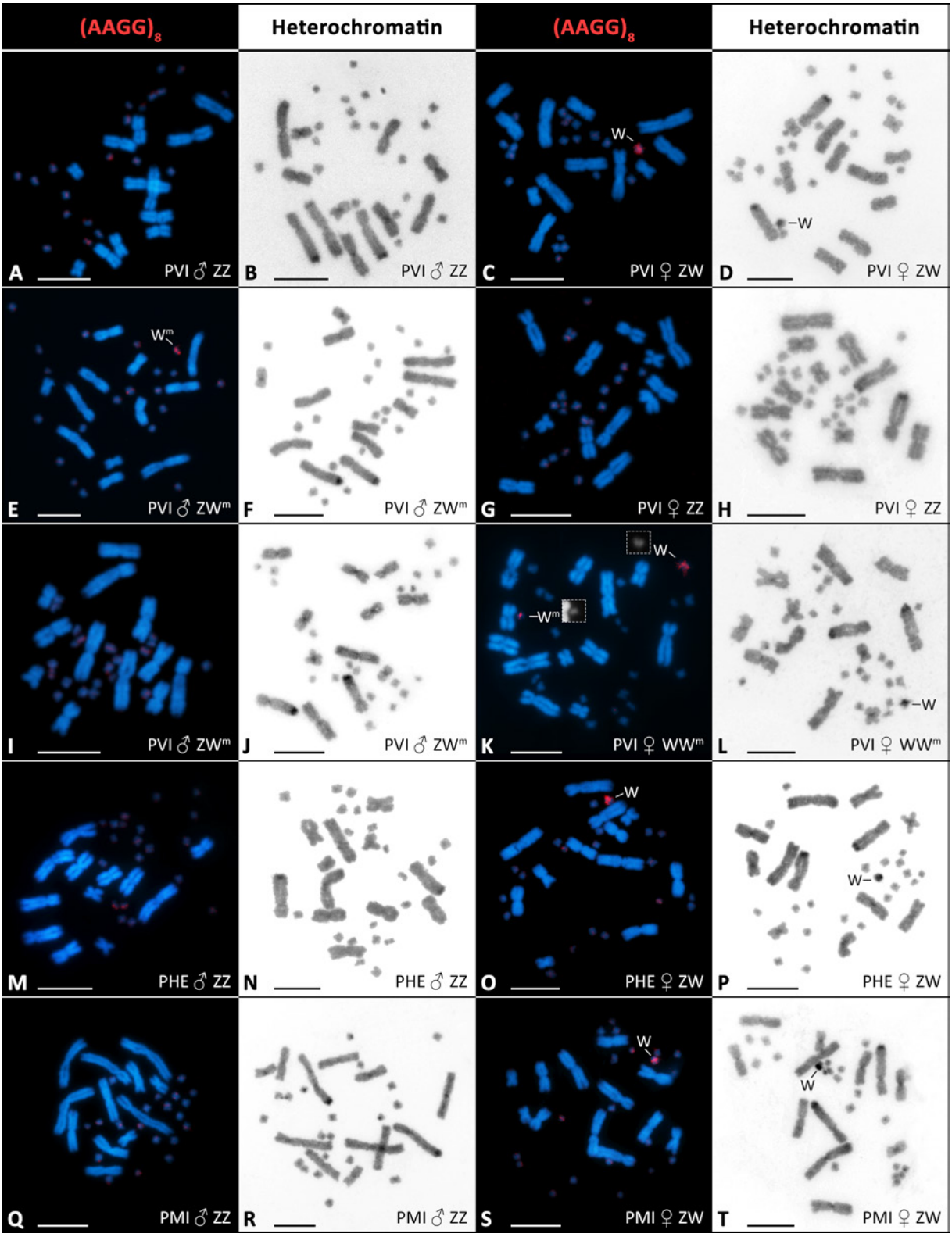
According to previous cytogenetic studies, Z and W are microchromosomes and the W chromosome is highly heterochromatic [Ezaz et al., 2005] and possesses notable accumulations of the AAGG repetitive motif [Matsubara et al., 2016]. Therefore, we applied C-banding visualizing heterochromatin [Sumner, 1972] and FISH with the $(AAGG)_8$ motif as a probe [for detailed methodology see Altmanová et al., 2016; Suwala et al., 2020] to chromosome spreads from 22 individuals representing all experimental groups: parents and offspring of all genotypic combinations (summarized in online suppl. Table 1). Chromosome spreads were obtained by cultivation of whole blood according to our standard protocol [Pokorná et al., 2014]. In all cytogenetically tested individuals (online suppl. Tables 1, 2), we scored at least 10 metaphases per each individual and method.

Phenotypic Sexing

We determined the sex of the experimental animals by gonadal inspection, breeding history, and/or external morphology (enlarged hemipenes). The phenotypic sex can be determined by the presence of an enlarged hemipenes in males. However, according to our experience, the determination of the phenotypic sex based on external morphology is reliable only in older animals with a larger body size. Therefore, we consider determination of sex based on external morphology as reliable only in subadult animals above 200 g. The phenotypic sex determined by external morphology was verified by dissection and examination of gonad morphology in 10 individuals from the ZW female × ZW^m male cross (5 males, 5 females).

Results

All 22 karyotyped individuals of *P. vitticeps* (listed in online suppl. Table 1) had 32 chromosomes, consisting of 12 macrochromosomes and 20 microchromosomes, which is congruent with previous studies [Witten, 1983; Ezaz et al., 2005]. We did not observe aneuploidy or chromosomal abnormalities in any tested individual. C-banding revealed heterochromatin in the centromeres of macrochromosomes and at a distal part of the second largest chromosome pair in all individuals, confirming that the method was applied successfully (Fig. 1; online suppl. Fig. 2). A notable heterochromatic accumulation lacking in a control, standard male (Fig. 1B) was observed in a control female on the W chromosome (Fig. 1D) as reported pre-



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(For legend see next page.)

viously by Ezaz et al. [2005]. FISH with the probe containing the AAGG repetitive motif detected a weak accumulation of this motif on several microchromosome pairs [similarly to Holleley et al., 2015] and a strong accumulation on the W chromosome (Fig. 1C). This accumulation was absent in a control male (Fig. 1A). As expected, the female-specific PCR marker was amplified only in the standard female and not in the male (Fig. 2). Similarly to *P. vitticeps*, *P. henrylawsoni* (2 males and 2 females examined; the results of only 1 pair are shown in Fig. 1M–P) and *P. minor* (1 male and 1 female; Fig. 1Q–T) possessed $2n = 32$ chromosomes. Also heterochromatin and accumulations of the AAGG repetitive motifs were distributed in the same way as in *P. vitticeps* with apparently strong accumulation on the W chromosomes of females (Fig. 1O, S). The female-specific PCR marker was also amplified only in females of these 2 species (Fig. 2). The results of all studied individuals of *P. henrylawsoni* and *P. minor* are summarized in online suppl. Table 2. None of the ZZ females of *P. vitticeps* which were sex-reversed by high temperature exhibited the female-specific cytogenetic markers (heterochromatic block and AAGG accumulation, Fig. 1G, H' ; online suppl. Fig. 2E–H and Table 1) or the female-specific PCR marker (Fig. 2). The male with the W-specific PCR fragment (Fig. 2) lacked the female-specific heterochromatic block (Fig. 1F) but possessed the otherwise female-specific AAGG accumulation, although under closer examination the signal was fainter than in females and the chromosome bearing it was notably smaller than the W chromosome (Fig. 1E).

Sex-Reversed ZZ Female × ZW^m Male

Among the progeny of the cross between the male carrying the W-specific marker (ZW^m) and the 2 ZZ sex-reversed females, exactly 50% out of 40 offspring inherited the W-specific marker obviously from their father, confirming Mendelian inheritance of this locus. As the offspring from this cross were used in another project, there was only 1 offspring carrying the W-specific marker and 1 lacking it from this cross available for reliable sexing and karyotyping (Fig. 2). Both these individuals were males, fertility was confirmed in the male with the W-specific marker (online suppl. Table 1). The male without the W-specific marker had the same cytogenetic

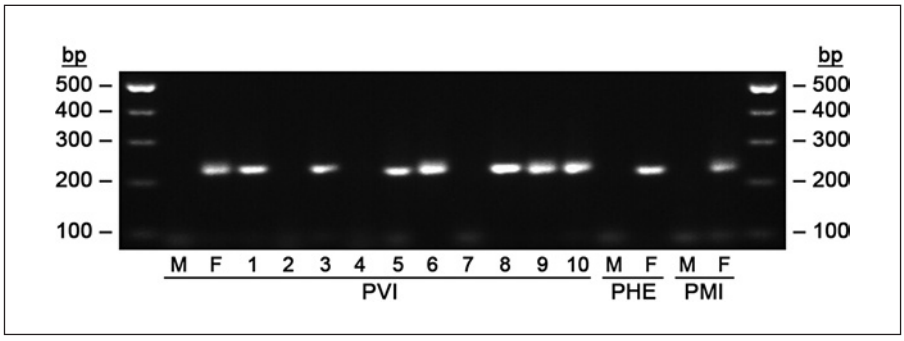

Fig. 2. Results of PCR test with primers for the W-specific fragment in *P. vitticeps* (PVI), *P. henrylawsoni* (PHE), and *P. minor* (PMI) visualized by agarose gel electrophoresis. Standard females (F) of PVI, PHE, and PMI display the W-specific fragment (224 bp), whereas in standard males (M) this fragment is not amplified. PVI individuals 1–10 include: 1) ZW^m male; 2) sex-reversed ZZ female; 3, 4) male offspring of sex-reversed females and ZW^m male; 5) offspring of unknown sex from the same cross; 6) standard female used in the ZW × ZW^m cross, i.e., the mother of the offspring 7–10 depicted here; 7, 8) male offspring of standard female and ZW^m male; and 9, 10) female offspring of standard female and ZW^m male.

Fig. 1. Visualization of the accumulation of AAGG repeats and heterochromatin in selected individuals of *P. vitticeps* (PVI; **A–K**), *P. henrylawsoni* (PHE; **M–P**), and *P. minor* (PMI; **Q–T**). In *P. vitticeps*, the figure represents successively a standard male ZZ karyotype with no accumulations (**A**, **B**), standard female ZW karyotype with AAGG (**C**) and heterochromatin accumulation (**D**), karyotype of a ZW^m male with AAGG accumulation (**E**) but without heterochromatinization (**F**), karyotype of a sex-reversed ZZ female with no accumulations (**G**, **H**), karyotype of a ZW^m male with no accumulations (**I**, **J**), and karyotype of a WW^m female with 2 AAGG accumulations (**K**) and unpaired heterochromatic block (**L**). In *P. henrylawsoni* and *P. minor,* male karyotypes with no accumulations (**M**, **N**, **Q**, **R**) and female karyotypes with AAGG (**O**, **S**) and heterochromatin accumulations (**P**, **T**) are shown. W^m in the caption in males reflects the presence of the W-specific marker in PCR, the assignment in females is based on cytogenetics. Boxes in **K** show W and W^m chromosomes in separated blue channel mode to present their size difference. Scale bars, 10 μm.

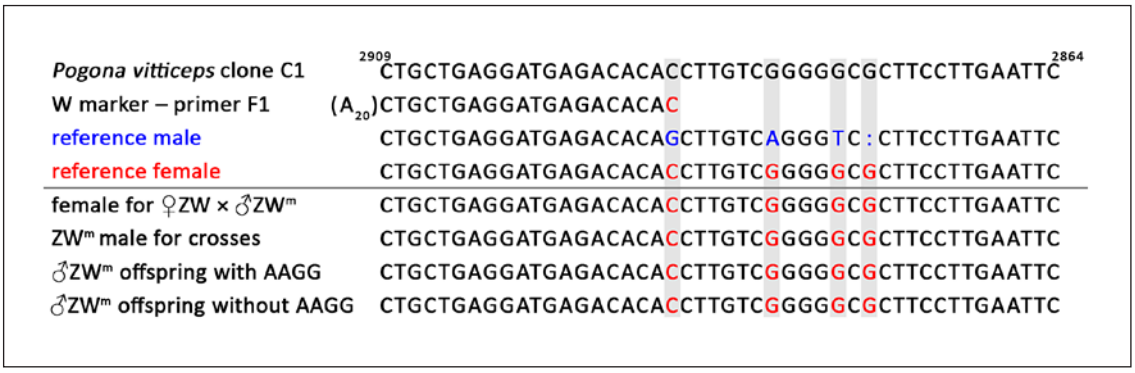

Fig. 3. Sequences of the W-specific fragment with the male and female reference. *P. vitticeps* clone C1 sex chromosome anonymous locus genomic sequence (GenBank sequence ID: EU938138.1, compared using BLASTN search), primer F1 used for the amplification of the W-specific fragment, male and female reference sequences differing in 4 SNPs from Quinn et al. [2010], and 4 individuals used in the study identical with the female reference sequence are shown. These individuals are a standard ZW female and a male possessing the W-specific marker and the AAGG accumulation involved in ♀ZW × ♂ZW^m cross and their offspring, the male possessing the W-specific marker and the AAGG accumulation. The last depicted sequence represents the male possessing the W-specific marker but without the AAGG accumulation originating from the cross between female sex reversal and the male with the W-specific marker (♀ZZ × ♂ZW^m).

characteristics as the standard male (online suppl. Fig. 2A,B), i.e., no microchromosome with heterochromatic and AAGG blocks. The cytogenetic profile of the male carrying the W-specific marker was not identical to his father: he lacked heterochromatin accumulation, but also a notable AAGG accumulation (Fig. 1I, J').

Standard ZW Female × ZW^m Male

Among offspring from the cross between the ZW^m male with the standard ZW female, 42 out of 57 juveniles possessed the W-specific marker. The ratio of individuals without and with the W-specific marker from this cross was 1:2.8, which is neither statistically different from 1:3 ($\chi^2 = 0.46$, $df = 1$, $p = 0.50$), which is expected if the WW^m genotype is viable, nor from 1:2 ($\chi^2 = 0.63$, $df = 1$, $p = 0.43$), which is expected for the lethality of the WW^m genotype. Nevertheless, the cytogenetic examinations revealed that the WW^m genotype is viable (see below). The offspring with the W-specific PCR marker were of both sexes. Determination of phenotypic sex by external morphology was successfully validated in all 10 examined individuals by dissection and gonad inspection. Gonads of 5 animals with the male-typical external morphology were undoubtedly testes, while ovaries were found in all 5 dissected animals with a female-typical external morphology. Three cytogenetically examined males from the cross with the W-specific PCR marker possessed male-typical cytogenetic characteristics, i.e., absence of AAGG and heterochromatin accumulation (online suppl. Fig. 2K–P). In other 3 cytogenetically examined males with the W-specific PCR marker, the examination revealed a prominent AAGG accumulation of the motif on a microchromosome notably smaller than the W chromosome (online suppl. Fig. 2O,Q,S), but no heterochromatic accumulation (online suppl. Fig. 2P,R,T). Among 7 cytogenetically examined individuals from the cross, 1 female and 2 juveniles with undetermined sex had typical female cytogenetic characteristics (online suppl. Fig. 2U–Z). Four females possessed the W chromosome with the strong AAGG accumulation and heterochromatic block together with a smaller chromosome without heterochromatin but with notable AAGG accumulation (Fig. 1K, L; online suppl. Fig. 2A′–F′, summarized in online suppl. Table 1).

Sequence of the W^m Fragment

BLASTN search of the obtained 201 bp long sequences after exclusion of the A_{20} F1 primer motif found 99–100% cover and 92–97% similarity with *P. vitticeps* clone C1 sex chromosome anonymous locus genomic sequence [GenBank sequence ID: EU938138.1, published in Quinn et al., 2010]. A partial 46 bp long sequence was determined by Quinn et al. [2010] to be informative for the identification of the W-specific fragment with the Z- and W-specific alleles differing in 4 SNPs. Fragments in all 4 individuals sequenced by us, i.e., standard ZW female and 3 males with the amplified W-specific marker, possess the 4 SNPs assigned as female-specific (Fig. 3).

Discussion

The initial result, stimulating further experiments described in the current study, was the discovery of the amplification of the molecular W-specific marker among the offspring of the thermally-reversed ZZ female with a standard, presumably ZZ, male. Further testing including cytogenetics confirmed that the mother was indeed a reversed ZZ female, whereas the father together with around a half of the offspring displayed markers for the W chromosome (ZW^m individuals; summarized in online suppl. Table 1 and Fig. 1–3). The same results were obtained from the cross of the same male with another sex-reversed ZZ female. The linkage of the PCR and cytogenetic markers to sex seems to be very strong as they are sex-linked not only in *P. vitticeps* but also in other amphibolurine dragon lizards. The same molecular and cytogenetic W-specific markers developed for *P. vitticeps* were observed to be W-linked also in *P. henrylawsoni* and *P. minor* (this study), and previously, parts of the content of *P. vitticeps* sex chromosomes mapped to sex chromosomes in *P. barbata* and *Diporiphora nobbi* [Ezaz et al., 2009; Quinn et al., 2010]. All of these observations point to the stability and common origin of *Pogona* and *Diporiphora* sex chromosomes, which according to the estimated divergence times, evolved around 25 million years ago [Tonini et al., 2016]. Considering this large evolutionary stability of the linkage of the markers to sex, we speculated that the male with the W-specific markers is a reversed ZW individual. We tested this hypothesis by determination of the phenotypic sex in crosses with ZZ and ZW females. However, the results show that the chromosome assigned by us as W^m does not operate in the same way as the W in sex determination: most importantly, the individuals with the ZW^m genotype are males.

C-banding and FISH detecting the accumulation of the AAGG motif gave different results. Whereas the conspicuous heterochromatin block is located exclusively on the female-specific W chromosome (and is thus exclusively present in females with either a ZW or WW^m genotype), the AAGG accumulation was detected in both sexes – on the female-specific W and on the W^m present in part of males and females. The physical mapping of the sex chromosome content revealed that the Z chromosome is smaller than the W in *P. vitticeps* [Ezaz et al., 2013]. The W^m chromosome is also obviously smaller than the W chromosome. Therefore, we suggest that the W^m likely originated from the Z by an addition of a small part including the PCR W-specific marker and a part of the AAGG accumulation from the W chromosome. The absence of heterochromatin on the W^m chromosome might reflect different epigenetic dynamics of the Z chromosome, or simply the method of heterochromatin visualization, which is relatively crude and the detection of heterochromatin in a smaller block of repeats might be below the detection limit in comparison with the sensitive PCR and FISH analyses. We cannot exclude that the genetic material was translocated from the W to another microchromosome other than the Z. The W^m would then actually be an autosome. In any case, the linkage between the cytogenetic and PCR markers is loose, as the progeny of the ZW^m male had various combinations of presence/absence of these markers (Fig. 1, 2; online suppl. Fig. 2). Additional experiments would be needed to decipher the position of the otherwise W-specific markers in the genome in the family studied by us.

Our finding has important consequences as *P. vitticeps* has a prominent status among amniote vertebrates. It is the only amniote with a well-supported transition from the ancestral GSD (in amphibolurids stable for around 25 million of years) to ESD. Despite the cytogenetic difference of sex chromosomes and their age, it has been shown in the laboratory that high incubation temperatures override the genotype and produce ZZ females in *P. vitticeps* [Quinn et al., 2007]. Offspring sex ratio of these sex-reversed females depends on the incubation temperature, suggesting that one generation is sufficient for a GSD-to-ESD transition and the loss of the W chromosome [Holleley et al., 2015]. It was demonstrated that the lack of W chromosome and/or extreme incubation conditions affect morphological and behavioural traits of sex-reversed ZZ females [Holleley et al., 2015; Li et al., 2016; Jones et al., 2020]; however, the influence of sex reversal on fitness under natural conditions is not known. In the laboratory, the females are fully viable and fertile [Holleley et al., 2015]. Sex-reversed animals identified by molecular and cytogenetic markers were reported in the bearded dragon also in the field, and their slightly higher incidence in recent years was put into connection with global warming [Holleley et al., 2015]. In the recent study [Castelli et al., 2020], the mismatch between phenotypic and genotypic sex was assigned by the same molecular marker [Quinn et al., 2010; Holleley et al., 2015; Ehl et al., 2017] (all these studies used the same contig as the sex-specific marker although they differed in the primers used for amplifications of fragments from the same W-linked regions) and was found in 5% out of 534 examined individuals of *P. vitticeps* covering the whole species range. Notably, all 28 animals with the mismatch were phenotypic females and they were recorded only in the south-western part of the

species range. While this clustered distribution is consistent with non-random occurrence of sex reversals in certain environmental conditions at the edge of the species distribution as interpreted by Castelli et al. [2020], the spatial clustering can also reflect the geographic spread of a mutation, recombination, or other rearrangement concerning the region of the W chromosome containing the otherwise female-specific marker used for identification of the individuals with the mismatch. The current study discloses that the given marker might not be as reliable as thought for identification of sex-reversed individuals in the field and that the causes of the mismatch between the phenotypic and genotypic sex should be investigated more rigorously. Holleley et al. [2015] tested the reliability of the molecular marker by a congruence with cytogenetics, but our current work demonstrates that neither presence of the accumulation of AAGG repeats is reliable. Crossbreeding experiments with the animals assigned as sex-reversed in the field based on genetic markers were not performed. Our study indicates that it can be premature to assign an animal with a mismatch between the phenotypic and genotypic sex as sex-reversed without the necessary experimental crosses. As demonstrated here, even molecular markers linked to sex for many millions of years can be misleading in this respect.

Various techniques were used for genotypic sexing in vertebrates, including analysis of karyotypes, anonymous sex-linked markers, (derived for example from reduced-representation sequencing and microsatellites), and comparison of copy number variation between sexes by quantitative PCR [Cooper et al., 1997; Halverson and Spelman, 2002; Sulandari et al., 2014; Gamble et al., 2015; Rovatsos et al., 2017b]. As the transition from GSD to ESD requires an intermediate step of sex reversal, the reliable detection of sex-reversed individuals is crucial for studies of a turnover of sex determination mechanisms. Individuals with a mismatch between the genotypic and phenotypic sex were quite often reported in the field and were interpreted as sex-reversed individuals and thus as evidence for turnover in the sex determination system (from GSD to ESD or for a turnover in sex chromosomes), a potential for such a turnover or a co-occurrence of GSD and ESD in a population [Shine et al., 2002; Barske and Capel, 2008; Holleley et al., 2015; Hundt et al., 2019; Lambert et al., 2019]. We think that these studies should be taken as good evidence for mismatches between molecular markers and phenotypic sex, not as the documentation of the frequency of environmentally-induced sex reversals in the field. This might seem like a subtle shift but it is important for interpretations and formulations of hypotheses about transitions from GSD to ESD, which require sex reversals. We warn that there are also other mechanisms producing such a mismatch between phenotypic sex and genetic markers, for example, a recombination between sex chromosomes in otherwise non-recombining or rarely recombining regions, translocation of a sex-specific marker to autosomes, translocation of sequences between non-recombining regions of sex chromosomes, or a mutation in a sex-determining gene [Hawkins et al., 1992; Murata et al., 2016; Furman et al., 2020; Xu et al., 2020]. We should learn more about the frequency of events leading to a mismatch between gonadal and genotypic sex and evaluate to what extend are the reports of sex reversals based on given markers reliable. We conclude that individuals should not be assigned as sex-reversed by environmental conditions unless their genotype is carefully examined or a functional analysis of their genetic constitution is evaluated in breeding experiments.

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Statement of Ethics

All breeding and experimental procedures were carried out under the supervision and with the approval of the Ethics Committee of the Faculty of Science, Charles University, followed by the Ministry of Education, Youth and Sports (permissions No. 35484/2015–14 and 23,852/2014–11).

Conflict of Interest Statement

We declare no competing interests in relation to the work.

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Author Contribution

All 3 authors designed the project and wrote and approved the manuscript. J.E. performed the work with animals, and M.A. performed the cytogenetic analyses.

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