Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Suppressing estrogen signaling by a Y-linked nuclear receptor determines sex in Bombina toads

Matthias Stöck

matthias.stoeck@igb-berlin.de

Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB) https://orcid.org/0000-0003-4888-8371

Heiner Kuhl

Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB) https://orcid.org/0000-0001-7623-9227

Leon Obendorf

Freie Universität Berlin https://orcid.org/0000-0002-5641-4961

Christoph Winkler

University of Singapore https://orcid.org/0000-0003-4688-6241

Sophie Leinweber

Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB)

Martin Knytl

Charles University https://orcid.org/0000-0002-6325-6957

Dmitrij Dedukh

Institute of Animal Physiology and Genetics of the CAS

Evgeniya Grigoryeva

Freie Universität Berlin

Sylke Winkler

Max Planck Institute of Molecular Cell Biology and Genetics https://orcid.org/0000-0002-0915-3316

Thomas Brown

Leibniz Institute of Zoo and Wildlife Research https://orcid.org/0000-0001-8293-4816

Martin Pippel

Uppsala University https://orcid.org/0000-0002-8134-5929

Yunjing Goh

National University of Singapore https://orcid.org/0009-0003-7906-2334

Julia Bindl

Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB)

Susan Mbedi

Berlin Center for Genomics in Biodiversity Research (BeGenDiv) https://orcid.org/0000-0002-9633-5045

Markus Harzdorf
Saxon State Company for Environment and Agriculture
Ralph Tiedemann
University of Potsdam https://orcid.org/0000-0002-2604-6336
Sven Wuertz
Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB)
Maria Ogielska
University of Wrocław
Eugene Myers
MPI-CBG, TU Dresden https://orcid.org/0000-0002-6580-7839
Mark Blaxter
Wellcome Sanger Institute https://orcid.org/0000-0003-2861-949X
Jacek Szymura
Jagiellonian University
Petra Knaus
Freie Universität Berlin https://orcid.org/0000-0003-2492-1667

Article

Keywords:

Posted Date: September 21st, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9940999/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: There is **NO** Competing Interest.

Suppressing estrogen signaling by a Y-linked nuclear receptor determines sex in *Bombina* toads

Authors: Heiner Kuhl[1,2,*], Leon Obendorf[3,4,*], Christoph Winkler[1,5,*], Sophie Leinweber[1], Martin Knytl[6,7], Dmitrij Dedukh[8], Evgeniya Grigoryeva[3], Sylke Winkler[9], Thomas Fullerton Brown[9], Martin Pippel[9], Yunjing Goh[5], Julia Bindl[1], Susan Mbedi[10], Markus Harzdorf[11], Ralph Tiedemann[12], Sven Wuertz[1], Maria Ogielska[13], Gene Myers[9,14], Mark L. Blaxter[14], Jacek M. Szymura[15], Petra Knaus[3,§], Matthias Stöck[1,§]

Affiliations:

[1] Leibniz-Institute of Freshwater Ecology and Inland Fisheries, IGB; D-12587 Berlin, Germany.

[2] Ecotoxicological Laboratory, German Environment Agency, 12307 Berlin, Germany.

[3] Institute of Chemistry and Biochemistry; Freie Universität Berlin, Germany.

[4] International Max Planck Research School for Biology and Computation, 14195 Berlin, Germany.

[5] Department of Biological Sciences and Centre for Bioimaging Sciences, National University of 117543 Singapore; Singapore.

[6] Department of Cell Biology, Charles University; 12843 Prague, Czech Republic.

[7] Department of Biology, McMaster University; Hamilton L8S 4K1, Ontario, Canada.

[8] Laboratory of Non-Mendelian Evolution, Institute of Animal Physiology and Genetics of the CAS; 277 21 Liběchov, Czech Republic.

[9] Max Planck Institute of Molecular Cell Biology and Genetics; Dresden, Germany.

[10] Berlin Center for Genomics in Biodiversity Research (BeGenDiv); D-14195 Berlin, Germany.

[11] Saxon State Company for Environment and Agriculture, Görlitz, Germany.

[12] Unit of Evolutionary Biology/Systematic Zoology, Institute of Biochemistry and Biology; University of Potsdam, Potsdam, Germany.

[13] Department of Evolutionary Biology and Conservation of Vertebrates, Faculty of Biological Sciences, University of Wrocław; Wrocław, Poland.

[14] Tree of Life Programme, Wellcome Sanger Institute; Hinxton, Cambridge United Kingdom.

[15] Department of Comparative Anatomy, Institute of Zoology and Biomedical Research, Jagiellonian University; Krakow, Poland.

* These authors contributed equally to this work.

[§] Corresponding authors: matthias.stoeck@igb-berlin.de , petra.knaus@fu-berlin.de

Abstract

Sex determination (SD) in amphibians is poorly understood due to their large genomes, homomorphic sex chromosomes, and unresolved mechanisms. Examining new reference genomes of Eurasian fire-bellied toads (*Bombina*), we inferred *err1Y* as a Y-chromosomal sex determining gene. We mapped male-specific markers to a single-copy region on chromosome 2. This region contained *err1Y*, a duplicate of *err1*, an autosomal estrogen-related receptor. *Err1Y* is male-diagnostic in 24-chromosome *Bombina*, supporting a conserved XY-SD-system. Functional and structural analyses show that *err1Y* is enriched in juvenile testes and antagonizes estrogen signaling through a ligand-binding domain that lacks co-activator and DNA-binding function, promoting male differentiation. This is the first genetic SD-mode directly targeting the estrogen response, revealing a new route to poikilothermic vertebrate SD through neofunctionalization of duplicated nuclear receptor *err1*.

Introduction

Sex determination (SD) in vertebrates is highly diverse. Birds and placental mammals exhibit conserved sex chromosomes and master SD genes within each order[1,2], whereas teleost fishes show the greatest SD-gene diversity, with frequent turnover, multiple SD mechanisms (genetic and environmental), and varied SD genes[3,4]. Reptiles are intermediate, with some clades conserving sex chromosomes and others showing frequent turnover[5-7].

Large gaps of knowledge remain in a major tetrapod group, the amphibians, sister taxon of all other terrestrial vertebrates. Extant amphibians comprise >9,022 species, one-fifth of extant tetrapods[8]. Evidence from sex-reversal, crossings, phylogenetics, cytogenetics, pedigree-based linkage analyses, and reduced-representation sequencing indicates diversity and rapid turnover of sex determination in amphibians[9,10]. Although some species have differentiated sex chromosomes[11], large genomes and mostly homomorphic XX/XY, ZZ/ZW[12] or multiple systems complicate the study of amphibian sex determination[6]. The first amphibian master SD gene was identified in the model species African clawed frog, *Xenopus laevis*, and few relatives[13,14]. In *X. laevis*, the female-specific duplicate *dm-w*, derived from the testes gene *dmrt1* likely following genome duplication by hybridization (allotetraploidization), antagonizes the male pathway[15], and is essential for female development[16]. *Dm-w* is absent or no longer female-specific in other *Xenopus*[17]. Despite extensive research, only a few anuran candidate master SD-genes have been proposed: *dmrt1* in *Hyla arborea* and *Rana temporaria*[18-19], and *sox3* in *Glandirana rugosa*[20]. Recently, we identified a candidate SD locus in Palearctic green toads (*Bufotes viridis* subgroup), involving a male-specific long noncoding RNA (ncRNA-Y) in the 5′-region of *bod1l*[21].

The family Bombinatoridae (Gray 1825), one of the most divergent amphibian lineages, split from other extant anurans ~100 Mya; around 45 Mya, it diverged into the Eurasian *Bombina* and the Southeast-Asian *Barbourula*[22]. *Bombina*, originating from a Miocene ancestor (~20 Mya[23], comprises at least eight extant species, split into taxa with 24 chromosomes (Europe: *B. bombina*, *B. variegata*; East Asia: *B. orientalis*) and with 28 in China and Vietnam: *B. maxima*, *B. microdeladigitora*, and taxonomically debated *B. fortinuptialis* and *B. lichuanensis*[23,24].

The fire-bellied (*B. bombina*) and yellow-bellied toads (*B. variegata* with four nominal subspecies) are widespread in Europe. They differ markedly in morphology, physiology, behavior and genetics. Over 40 years, they have become a textbook system for studying amphibian hybridization and speciation[25,26]. Their distributions mirror distinct habitat preferences: *B. bombina* in large, permanent lowland ponds and *B. variegata* in small, ephemeral mountain pools[25]. Narrow hybrid zones form at range boundaries, shaped by selection and dispersal, with localized genetic

structure[25,27], and limited gene flow[28]. Strong postzygotic isolation[25] is evidenced by genetic incompatibilities, selection with hybrid breakdown[27,29], and habitat mismatch[30]. However, key evidence on sex chromosomes in speciation and reproductive isolation is still missing[31]. In hybrid zones, theory predicts a disproportionate contribution of sex chromosomes to reproductive isolation, consistent with Haldane's rule (i.e. the heterogametic sex, XY males or ZW females, is more often absent, rare, or sterile)[32], the large-X effect[33], and faster-X evolution[34], with reduced gene flow across species/population boundaries via hybridization and backcrossing (introgression) at sex-linked relative to autosomal loci[35]. Yet in *Bombina*, large genome sizes have complicated chromosome-scale assemblies and sex-linked marker development[36], preventing tests of these predictions.

Here, two new high-quality reference genomes enabled the identification of a male-specific hemizygous region in *B. bombina* (9.8 Gbp, GCF_027579735.1) and *B. variegata* (9.4 Gbp, GCA_964205295.1) containing an extra Y-linked copy (*err1Y*) of *err1* (= *esrra*), encoding an estrogen-related receptor, a member of the nuclear receptor superfamily. Although ERRs do not bind estrogen, they regulate similar targets as the estrogen receptor (ERα), through estrogen response elements and thereby influence energy metabolism, mitochondrial function, and gonadal development (Text S1). Sex determination in some poikilothermic vertebrates is highly sensitive to estrogen dosage during a narrow developmental window[3], when even brief hormonal shifts can redirect sexual development. In *Bombina*, our data suggest that *err1Y* acts upstream as a brake on the evolutionarily conserved *foxl2*–aromatase pathway module[1,37], reducing estrogen signaling before *foxl2* reinforces the female programme. Its male-specific presence, timely and gonad-enriched expression in 24-chromosome *Bombina*, and *in vitro*-inhibition of ERα signaling support *err1Y* as a candidate master sex-determining gene. This early action appears to steer the bipotential primordial gonad towards male differentiation and reveals a genetic sex-determining mode that directly targets estrogen response, representing a new route to poikilothermic vertebrate sex determination through neofunctionalization of the duplicated nuclear receptor *err1*. Our findings also provide a framework for studying speciation.

Results

Identification of a male-specific marker in *B. bombina* using ddRADseq

To identify sex-specific markers, double-digest restriction associated DNA-sequencing (ddRADseq) data from a *B. bombina* pedigree (parents plus 90 mostly phenotypically sexed offspring, Table S1) were analyzed using RADsex, yielding 112 candidates (Data S1). Mapping to the 9.8 Gbp reference genome (GCF_027579735.1) revealed the highest marker density on chromosome 2 (LG2; 844,059,191–845,833,806 Mb). The 8 top markers were almost exclusive to phenotypic males, with a few mis-sexed individuals, as later confirmed by the molecular Y-marker (Data S2). Remapping long reads revealed a ~4 Mb hemizygous region in the male genome (Fig. 1), indicating a male-specific chromosome segment, containing a gene annotated as *steroid hormone receptor err1-like* (GenBank XP_053556091), hereafter *err1Y* (Fig. 1). This locus is a duplicate of the locus *err1* (Gene ID: 128665824) on *B. bombina* autosome LG7 (38,745,492-38,820,666 Mb), suggesting a shared evolutionary origin (Table S2).

A cross-amplifying Y-marker in multiple taxa suggests a conserved sex determining gene

To test the male specificity of *err1Y*, we developed multiplex PCR assays. Male-specific primers (BombYonly_300bp_F/R) amplified *err1Y* from LG2 only in males of *B. bombina*, while autosomal primers (Bomb_Auto_397bp_F/R) amplified *err1* from LG7 in both sexes. The male

marker was fully sex-diagnostic (Fig. S1). All phenotypic females amplified only the autosomal marker.

Tests involving multiple specimens of European *Bombina* (*B. variegata variegata*, *B. v. pachypus*, *B. v. scabra*, *B. v. kolombatovici*) and East-Asian *B. orientalis* showed that the Y-marker (BombYonly_300bp) cross-amplified in all males (Fig. 2A, S2), suggesting a conserved male-heterogametic (XY) system across the group of 24-chromosome *Bombina* on LG2. *Bombina* LG2 is syntenic with *Xenopus tropicalis* LG1. The autosomal *err1* resides on *Bombina* LG7, which is partially homologous to several *X. tropicalis* LGs (3, 4, 8, 9; Tables S1, S3).

In both 28-chromosome *Bombina*, *B. maxima* and *B. microdeladigitora*, PCR assays amplified two bands (autosomal and annotated '*err1-like'*) in both sexes without sex-specificity (Fig. S2B). Whole-genome resequencing (male and female *B. maxima*, two male *B. microdeladigitora*) confirmed the PCR product identity for both genes (Fig. S3A, B), but revealed a frameshift in exon 1 of *'err1-like*' (Fig. S4), rendering it likely inactive. In 24-chromosome *Bombina*, male-specific *err1Y* is expressed (see below), whereas its inactivation in the 28-chromosome species suggests loss of sex-determining function and explains the PCR assay's lack of sex-specificity (Fig. S4).

Homology and phylogeny of *err1Y* and *err1* within *Bombina*

To assess interspecies homology and intra-genus relationships, we sequenced the Y-specific (BombYonly_300bp) and autosomal (Bomb_Auto_397bp_F/R) markers in both sexes from each taxon (Data S1, S2) and constructed phylogenetic trees. The *err1Y* and autosomal *err1* tree topologies (Fig. 2B) are largely congruent, indicating a common ancestor for the Y-locus in 24-chromosome *Bombina,* as the gene duplication may have occurred in the common ancestor of the entire genus.

Inheritance of *err1Y* and evidence from interspecies crosses

Molecular sexing in a *B. variegata* family showed the PCR assay was fully sex-diagnostic in the father and all eight male offspring, indicating complete sex-linked transmission (Fig. S5A, B). In an interspecies cross (*B. bombina* ♀ × *B. variegata* ♂), all 17 F_1-sons — but none of 11 daughters — carried paternal *err1Y*, linking it to maleness in hybrids (Fig. S5C).

F_1-hybrids involving 28-chromosome *B. maxima* males generated offspring that were phenotypically male but lacked *err1Y*-sex-specific amplification: (i) *B. microdeladigitora* ♀ × *B. maxima* ♂ and (ii) *B. variegata* ♀ × *B. maxima* ♂. This suggests male development in *B. maxima* relies on a different locus, consistent with the lack of sex-specificity and a likely frameshift in *err1-like*, amplified by BombYonly_300bp_F/R in *B. maxima* and *B. microdeladigitora* (Fig. S5D).

Genomic and cytogenetic evidence for the location of *err1Y* in different *Bombina* taxa

The candidate male sex-determining gene *err1Y* is located on *B. bombina* LG2 (845,166,599–845,362,339). The gene was also found by mapping the *B. bombina* Err1Y protein sequence to the *B.variegata* reference genome (for details Text S1, Data S3). Accordingly, *err1Y* was also detected in a male *B. variegata* draft genome (GCA_905336975.1).

To provide cytogenetic evidence for the chromosomal positions of *err1* and *err1Y*, we performed fluorescence *in situ* hybridization with tyramide signal amplification (FISH-TSA) in both sexes of *B. bombina*, *B. variegata*, and *B. orientalis* (Fig. 3A-F). The *err1Y*-probe of *B. bombina* (LOC128647330) produced a clear signal on one of two copies of LG2 in males of all three species, indicating conserved Y-specificity. It also hybridized to both LG7 homologs in both sexes, near a known secondary constriction[38], marking autosomal *err1*, with signals occasionally appearing on both chromatids.

Organ-specificity and timing of expression of the candidate male sex determining gene

To compare *err1Y* and *err1* expression, we analyzed transcriptomes and exon-boundary–spanning cDNA-PCR products from multiple organs. In testes of *B. variegata* juveniles (Gosner-stage 46, ~4 months post-metamorphosis; Bvarjuv2M), RNAseq recovered a single *err1Y*-contig, spanning 888 bp of the reference mRNA, including 423 bp of 3′-UTR. In two juvenile females (Bvarjuv24, Bvarjuv26) and males (Bvarjuv23, Bvarjuv25) of the same stage, cDNA-PCR detected 5′-*err1Y* in testes and male brains (weakly in other organs (Fig. S6A), whereas autosomal *err1* was expressed in five organs, including testes and ovaries (Fig. S6B), consistent with bulk RNAseq data (PRJNA1282098).

In an older juvenile male *B. orientalis* (Gosner 46; ~6 months post-metamorphosis; Bor_male), RNAseq recovered two *err1Y*-contigs (538 bp), and cDNA-PCR detected 5′-*err1Y* exclusively in testis (Fig. S6B), while autosomal *err1* was expressed in five organs including testis (Fig. S6D), consistent with RNAseq data. Across 53 *Bombina* transcriptomes deposited in INSDC (33 *B. bombina*, 5 *B. variegata*, 15 *B. orientalis*; including adult testes), *err1Y* was rarely detected at meaningful levels, with most apparent hits representing autosomal *err1*. Together, these data indicate that male-specific *err1Y* is preferentially expressed in differentiating, non-adult testes.

To refine understanding spatial expression of *err1Y* in differentiating gonads, we used hybrid chain reaction (HCR) *in situ* hybridization with probes targeting *err1Y* (excluding *err1*) and *vice versa*, alongside the germ cell marker *vasa* (Table S4). In early gonads at metamorphosis (Gosner 46, *B. variegata*), *err1Y* was expressed in genetic males (Bvarjuv14M, 16M, 21M, 22M) in and around *vasa*-positive germ cells (Fig. 4B, B'), while genetic females (Bvarjuv19F, 20F) were *err1Y*-negative (Fig.4F). In testes including gonocytes, autosomal *err1* exhibited only moderate expression at this stage (Fig. 4C, C'), whereas ovaries showed strong *err1*-levels within oocytes, matching levels of *vasa* expression (Fig. 4F, G; Figs. S7, S8).

Phylogeny across amphibians and amino acid changes in *err1Y*

Phylogenetic analyses of *err1Y* nucleotide (Fig. 2B) and amino acid sequences place it closest to paralogous *err1*, yet it shows ~11% amino acid divergence, distinguishing it from the autosomal *Bombina err1* and positioning it as a sister lineage to all anuran *err1* (Fig. S9). Conserved domain analysis (NCBI) further indicates that *err1Y* retains characteristic features of estrogen-related receptors (ERRs).

A truncated isoform of the *err1Y* mRNA

Mapping transcriptomes to the *B. bombina* genome revealed discrepancies with the original gene model of *err1Y* (LOC128647330), while reads aligned cleanly to autosomal *err1* (LOC128665824), despite its relatively lower expression (Fig. S10). This suggested genuine alternative err1Y transcripts, generating a highly expressed truncated isoform initiated from a novel promoter and transcription start site within intron 4, incorporating a new first exon joined to exons 5–7 of the original model (Fig. 5A). In contrast, the full-length transcript was only basally expressed with few reads spanning the original first exons (Fig. S10). *Err1Y* expression was 5–6 × higher than *err1* or *erα*, and *de novo* assembly recovered only this truncated isoform. The transcript contains an intact open reading frame (ORF) with an ATG start in exon 5, 94 bp downstream of the new transcription start site (efficient 5′-UTR ≥40–50 bp; human mean: 100–200 bp), encoding a protein that retains the ligand-binding (LBD) but lacks the DNA-binding domain (DBD) for estrogen response elements (ERE) (Fig. S10).

Truncated Err1Y inhibits Err1 and Erα signaling in functional reporter gene assays
To investigate the function of the male-specific truncated Err1Y, we compared predicted protein structures of long and truncated forms and their transcriptional activity in ERE reporter gene assays alongside autosomal Err1 and Erα in human embryonic kidney (HEK) cells (Figs. 5B, C). Because Err1 and Erα pathways overlap at estrogen-response elements (ERE), we also included Erα to unravel potential regulation. We designed and validated expression constructs for Err1Y, truncated Err1Y, Err1 and Erα, tagged N-terminally with small peptides (Tables S5, S6, Fig. S11). Both Err1Y variants alone show no reporter activation above background (Fig. 5B, yellow bars). To investigate whether Err1Y modulates Erα- or Err1-dependent transcriptional activation in a dose-dependent manner, we titrated increasing amounts of an Err1Y overexpression construct to either Erα or Err1. While long Err1Y had no significant effect on Err1 or Erα signaling, the truncated form repressed Err1- and Erα-dependent reporter activation dose-dependently (Fig. 5B) at ratios similar to expression-levels of RNAseq. We further examined a putative ERE motif in the *foxl2*-promoter of *B. bombina*, a key regulator of ovarian differentiation and female development, exhibiting a very similar inhibition profile when titrating Err1 and Erα with truncated Err1Y (Fig. 5D).
This shows that both Err1Y forms are functionally distinct from each other: the truncated variant acts as a dominant inhibitor of Err1/-Erα-responsive transcription, while the full-length form cannot activate ERE-dependent transcription or modulate ERR-like signaling. Structural predictions using Boltz-2[39] suggest that Err1Y disrupts co-activator binding through two independent features: a unique C-terminal tail, potentially blocking the co-activator interface, and the absence of a 20-amino acid N-terminal segment universally required for co-activator recruitment across nuclear receptors (Fig. 5E)[40]. Rosetta docking analyses further suggest that receptor-receptor interactions of the LBD are most stable in heterodimeric Err1-Err1Y compared to either homodimer (Fig. 5F). Together, these data support a model in which truncated Err1Y inhibits ER signaling through three major processes of Erα/Err1 signaling (Fig. 5G): 1) Dimerization: Relatively high expression of truncated *err1Y* might favor the formation of inactive protein heterodimers with either Erα or Err1, through the energetically favored LBD-interface, thereby reducing the formation of active Erα homodimers. 2) DNA-binding: The lack of the DBD in the heterodimers likely impairs efficient binding to ERE transcription factor binding sites. 3) Co-activator recruitment: In case of DNA-binding by heterodimers, co-receptors cannot be recruited to the truncated Err1Y due to missing and blocking parts in the co-activator binding site.

Discussion
Amphibian sex determination is typically genetic, with either male or female heterogamety. However, large genomes and often undifferentiated sex chromosomes[9,14] have hindered identification of master sex-determining genes[21,41]. Although several anuran candidates have been proposed, only a few have been functionally validated[13,17,20 23].
Recent developments in genome sequencing technologies have enabled us to assemble the large *Bombina bombina* and *B. variegata* genomes to reference quality (Table S7). This helped us to identify a Y-specific copy of an orphan steroid hormone receptor gene (*err1Y*) in *Bombina* as a strong novel candidate master sex-determining gene. Physical and genomic mapping, including FISH-TSA and synteny analyses, localized *err1Y* to the Y-chromosome (LG2; Table S3). The locus likely arose through duplication and translocation in the common ancestor of 24- and 28-chromosome *Bombina* over 20 Mya. The timing of *err1-like* recruitment and adaptation for sex determination in 24-chromosome toads remains unclear; in the 28-chromosome lineage, such recruitment as SD-gene may either never have evolved or has been secondarily lost.

The *Bombina* Y-chromosome (LG2) is homologous to *X. tropicalis* LG1, which harbors key sexual development genes (*dmrt1, amh*) and has evolved as a sex chromosome in multiple anurans[10,21,42,43]. Our assignment differs from earlier linkage mapping to LG5[36](Chr4 in latest *B. bombina* assembly; Table S1), possibly due to a different pedigree design involving an interspecific cross and F_2-recombinants.

Err1Y functions in a conserved male heterogametic (XY) system across Eurasian 24-chromosome *Bombina* and is expressed in differentiating testes 0–6 months post-metamorphosis, as shown by cDNA-PCR, RNAseq in *B. orientalis* and *B. variegata,* and HCR at metamorphosis in *B. variegata*, indicating focal expression in male gonads.

The truncated *err1Y* expression pattern and its capacity to modulate Err1- and Erα-signaling via lack of co-activator and DNA-binding interface support Err1Y-LBD in facilitating male differentiation via early cell reprogramming.

The SD candidate *err1Y* encodes an estrogen-related receptor (ERR), a nuclear receptor evolutionarily related to estrogen receptors (ERs) and conserved across vertebrates[44,45](Text S2). Unlike ERs, ERRs are orphan receptors that regulate metabolism without binding estrogen[46](Text S2), yet can activate transcription via estrogen response elements (EREs), estrogen-related response elements (ERREs), and SF-1 response elements (SFREs) in a ligand-independent manner. ERR1 is broadly expressed, including in mouse testis[46], and is constitutively active through coactivator binding. It can also modulate estrogen signaling by competing with Erα for DNA binding sites and shared co-regulators, thereby influencing ER target gene expression[47,48].

Due to the absence of DNA- and co-activator-binding domains and the resulting lack of transcriptional response on ERE motifs, truncated *err1Y* likely modulates Err1/Erα signaling rather than directly activating transcription. Unlike in some other vertebrates, where aromatase is the candidate master sex-determining trigger (e.g.[49]), we hypothesize that *err1Y* acts ontogenetically upstream to suppress the conserved *foxl2*–aromatase pathway[50]. This reduces estrogen signalling before *foxl2* reinforces female development and thereby promotes male differentiation through early repression of ovarian pathways rather than activation of testis-specific programs. Accordingly, truncated *err1Y* likely functions as a Y-linked inhibitory receptor derivative. This reveals a previously unknown mechanism of vertebrate sex determination that directly modulates estrogen signaling/response, representing a new route to poikilothermic vertebrate sex determination by neofunctionalization of the duplicated nuclear receptor *err1*.

Beyond its biological significance, the identification of a molecular Y-marker will allow the integration of *Bombina* hybrid zones into speciation research under a framework of Haldane's rule, offering a new analytical dimension to this textbook model of amphibian speciation[31,32,35,51].

References and Notes

1. J. A. M. Graves, *Sex, Genes and Chromosomes: Their Discovery, Function and Evolution*. Cambridge University Press, Cambridge, UK, 2026. ISBN: 978-1-009-68801-7.

2. B. Capel, Vertebrate sex determination: Evolutionary plasticity of a fundamental switch. *Nat. Rev. Genetics*, **18**, 675–689 (2017). doi: 10.1038/nrg.2017.60; pmid: 28804140.

3. Y. Nagahama, T. Chakraborty, B. Paul-Prasanth, K. Ohta, M. Nakamura, Sex determination, gonadal sex differentiation, and plasticity in vertebrate species. *Physiological Reviews* **101**, 1237–1308 (2021). doi: 10.1152/physrev.00044.2019; pmid: 33180655.

4. J. Kitano, S. Ansai, S., Y. Takehana, Y. Yamamoto, Diversity and convergence of sex-determination mechanisms in teleost fish. *Annual Rev. Animal Biosci.* **12**, 233–259 (2024). doi: 10.1146/annurev-animal-021122-113935; pmid: 37863090.

5. M. Rovatsos, J. Vukić, M. Altmanová, M. Johnson Pokorná, J. Moravec, L. Kratochvíl, Conservation of sex chromosomes in lacertid lizards. *Mol. Ecol.* **25**, 3120–3126 (2016). doi: 10.1111/mec.13635; pmid: 27037610.

6. M. Stöck, L. Kratochvíl, H. Kuhl, M. Rovatsos, B. J. Evans, A. Suh *et al.*, A brief review of vertebrate sex evolution with a pledge for integrative research: towards "sexomics". *Philosophical Transactions of the Royal Society B* **376**, 20200426 (2021). doi: 10.1098/rstb.2020.0426; pmid: 34247497.

7. B. J. Pinto, T. Gamble, C.H. Smith, M.A. Wilson, A lizard is never late: squamate genomics as a recent catalyst for understanding sex chromosome and microchromosome evolution. *J. Heredity* **114**, 445–458 (2023). doi: 10.1093/jhered/esad023; pmid: 37212108.

8. AmphibiaWeb; https://amphibiaweb.org.

9. I. Miura, Sex determination and sex chromosomes in Amphibia. *Sex. Dev.* **11**, 298–306 (2018). doi: 10.1159/000485270; pmid: 29241181.

10. D. L. Jeffries, G. Lavanchy, R. Sermier, *et al*., A rapid rate of sex-chromosome turnover and non-random transitions in true frogs. *Nat. Comm.* **9**, 4088 (2018). doi: 10.1038/s41467-018-06517-2; pmid: 30291233.

11. M. Schartl, M. Schmid, I. Nanda, Dynamics of vertebrate sex chromosome evolution: from equal size to giants and dwarfs. *Chromosoma* **125**, 553–571 (2016). doi: 10.1007/s00412-015-0569-y; pmid: 26715206

12. Á. S. Roco, A.W. Olmstead, S.J. Degitz, T. Amano, L.B. Zimmerman, *et al*., Coexistence of Y, W, and Z sex chromosomes in *Xenopus tropicalis*. *Proceedings of the National Academy of Sciences U.S.A.* **112**, E4752–E4761 (2015). doi: 10.1073/pnas.1505291112; pmid: 26216983.

13. S. Yoshimoto, E. Okada, H. Umemoto, K. Tamura, Y. Uno *et al*., A W-linked DM-domain gene, DM-W, participates in primary ovary development in *Xenopus laevis*. *Proceedings of the National Academy of Sciences U.S.A.* **105**, 2469–2474 (2008). doi: 10.1073/pnas.0712244105; pmid: 18268317.

14. M. Ito, Sex determination and differentiation in frogs. In *Reproductive and Developmental Strategies: The Continuity of Life*, K. Kobayashi, T. Kitano, Y. Iwao, and M. Kondo, eds. (Tokyo: Springer Japan, 2018), pp. 349–366. doi: 10.1007/978-4-431-56609-0_17.

15. S. Yoshimoto, N. Ikeda, Y. Izutsu, T. Shiba, N. Takamatsu, M. Ito, Opposite roles of DMRT1 and its W-linked paralogue, DM-W, in sexual dimorphism of *Xenopus laevis*: implications of a ZZ/ZW-type sex-determining system. *Development* **137**, 2519–2526 (2010). doi: 10.1242/dev.048751; pmid: 20573695.

16. C. M. Cauret, D. C. Jordan, L. M. Kukoly, S.R. Burton, E.U. Anele, J.M. Kwiecien, *et al*., Functional dissection and assembly of a small, newly evolved, W chromosome-specific genomic region of the African clawed frog *Xenopus laevis*. *PLoS Genet.* **19**, e1010990 (2023). doi: 10.1371/journal.pgen.1010990; pmid: 37792893.

17. B. J. Evans, V. Gvoždík, M. Knytl, C.M. Cauret, A, Herrel, *et al*. Rapid sex chromosome turnover in African Clawed Frogs (*Xenopus*) and the origins of new sex chromosomes. *Mol. Biol. Evol.*, **41**, msae234 (2024). doi: 10.1093/molbev/msae234; pmid: 39665151.

18. A. Brelsford, C. Dufresnes, N. Perrin, Trans-species variation in *Dmrt1* is associated with sex determination in four European tree-frog species. *Evolution*, **70**, 840-847 (2016). doi: 10.1111/evo.12891; pmid: 26920488.

19. N. Rodrigues, N., T. Studer, C. Dufresnes, W.J.Ma, P. Veltsos, N. Perrin, *Dmrt1* polymorphism and sex-chromosome differentiation in *Rana temporaria*. *Mol. Ecol*., **26**, 4897-4905 (2017). doi: 10.1111/mec.14222 ; pmid: 28675502.

20. I. Miura, Y. Hasegawa, M. Ito, T. Ezaz, M. Ogata, Disruption of sex-linked Sox3 causes ZW female-to-male sex reversal in the Japanese frog *Glandirana rugosa*. *Biomolecules* **14**, 1566 (2024). doi: 10.3390/biom14121566; pmid: 39766273.

21. H. Kuhl, W. H. Tan, C. Klopp, W. Kleiner, B. Koyun, M. Ciorpac, *et al.*, A candidate sex determination locus in amphibians, evolved by structural variation between X- and Y-chromosomes. *Nature Comm.* **15**, 4781 (2024). doi: 10.1038/s41467-024-49025-2; pmid: 38839766.

22. D. C. Blackburn, J.A. Gray, E. L. Stanley, The only “lungless” frog has a glottis and lungs. *Curr. Biol.* **34**, R492–R493 (2024). doi: 10.1016/j.cub.2024.03.017; pmid: 38772334.

23. M. Pabijan, A. Wandycz, S. Hofman, K. Węcek, M. Piwczyński, J. Szymura, Complete mitochondrial genomes resolve phylogenetic relationships within *Bombina* (Anura: Bombinatoridae). *Mol. Phylogen. Evol*. **69**, 63-74, doi: 10.1016/j.ympev.2013.05.007; pmid: 23707701.

24. J. M. Szymura, T. Passakas-Szymczak, A new chromosome number for *Bombina* (Anura, Discoglossidae). *Experientia* **44**, 521–523 (1988). doi: 10.1007/BF01958936.

25. J. M. Szymura, Analysis of hybrid zones with *Bombina*. In Harrison, R. G., ed., *Hybrid Zones and the Evolutionary Process*. Oxford University Press, New York (1993), pp. 261–289.

26. C. M. Zacho, I. Van Riemsdijk, B. De Cahsan, J. Vörös, M.E. Allentoft, M. The *Bombina* hybrid zone: a review of the legacy and future research directions. *Amphibia-Reptilia* **46** 141-167 (2025). doi: 10.1163/15685381-bja10225.

27. T.H. Vines, S.C. Koehler, M. Thiel, M., *et al.*, The maintenance of reproductive isolation in a mosaic hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*. *Evolution* **57**, 1876–1888 (2003). doi: 10.1111/j.0014-3820.2003.tb00595.x; pmid: 14503629.

28. J. Vörös, M. Alcobendas, M., I. Martínez-Solano, M. García-París, M., Evolution of *Bombina bombina* and *Bombina variegata* (Anura: Discoglossidae) in the Carpathian Basin: a history of repeated mtDNA introgression across species. *Mol. Phylogen. Evol.* **38**, 705–718 (2006). doi: 10.1016/j.ympev.2005.08.010; pmid: 16214377.

29. L. E. B. Kruuk, J.S. Gilchrist, N.H. Barton, Hybrid dysfunction in fire-bellied toads (*Bombina*). *Evolution* **53**, 1611–1616 (1999). doi: 10.1111/j.1558-5646.1999.tb05425.x; pmid: 28565554.

30. C. J. MacCallum, B. Nürnberger, N.H. Barton, J.M. Szymura, Habitat preference in the *Bombina* hybrid zone in Croatia. *Evolution* **52**, 227–239 (1998). doi: 10.1111/j.1558-5646.1998.tb05156.x; pmid: 28568140.

31. B. A. Payseur, D.C. Presgraves, D.A. Filatov, Sex chromosomes and speciation. *Mol. Ecol.* **27**, 3745–3747 (2018). doi: 10.1111/mec.14828; pmid: 30086196.

32. M. Schilthuizen, M.C. Giesbers, L.W. Beukeboom, Haldane's rule in the 21st century. *Heredity* **107**, 95–102 (2011). doi: 10.1038/hdy.2010.170, pmid: 21224879.

33. B. Charlesworth, J.A. Coyne, J. A., N.H. Barton, The relative rates of evolution of sex chromosomes and autosomes. *Am. Nat.* **130**, 113–146 (1987). doi: 10.1086/284701.

34. B. Charlesworth, J.L. Campos, B.C. Jackson, Faster-X evolution: theory and evidence from *Drosophila*. *Mol. Ecol.* **27**, 3753–3771 (2018). doi: 10.1111/mec.14534; pmid: 29431881

35. C. Dufresnes, T. Majtyka, S.J. Baird, *et al*., Empirical evidence for large X-effects in animals with undifferentiated sex chromosomes. *Sci. Rep.* **6**, 21029 (2016). doi: 10.1038/srep21029; pmid: 26868373.

36. B. Nürnberger, S.J. Baird, D. Čížková, A. Bryjová, A.B. Mudd, M.L. Blaxter, J. Szymura, A dense linkage map for a large repetitive genome: discovery of the sex-determining region

in hybridizing fire-bellied toads (*Bombina bombina* and *Bombina variegata*). *G3: Genes, Genomes, Genetics* **11**, jkab286 (2021). doi: 10.1093/g3journal/jkab286; pmid: 34849761.

37. Bertho, S., Pasquier, J., Pan, Q., Le Trionnaire, G., Bobe, J., Postlethwait, J. H., *Foxl2* and its relatives are evolutionarily conserved players in gonadal sex differentiation. *Sex. Dev.* **10**, 111–129 (2016). doi: 10.1159/000447611.

38. M. Schmid, L. Vitelli, R. Batistoni, Chromosome banding in Amphibia XI. Constitutive heterochromatin, nucleolus organizers, 18S+28S and 5S ribosomal RNA genes in Ascaphidae, Pipidae, Discoglossidae (incl. *Bombina*), and Pelobatidae. *Chromosoma* **95**, 271–284 (1987). doi: 10.1007/BF00294784, pmid: 3622081.

39. S. Passaro, G. Corso, J. Wohlwend, M. Reveiz, S. Thaler, V. R. Somnath*, et al*., Boltz-2: Towards accurate and efficient binding affinity prediction. *bioRxiv* (2025). doi: 10.1101/2025.06.14.659707[Preprint] (2025).

40. B. D. Darimont, R. L. Wagner, J. W. Apriletti, M. R. Stallcup, P. J. Kushner, J. D. Baxter, et al., Structure and specificity of nuclear receptor–coactivator interactions. *Genes Dev.* **12**, 3343–3356 (1998). doi: 10.1101/gad.12.21.3343; pmid: 9808622; pmid: 9808622.

41. S. Xie, J. Li, W. Chen, L. J. Fong, C. Huang, Y. Feng, et al., Extreme heterochiasmy and high rates of sex-reversed recombination result in large yet homomorphic sex chromosomes in the Emei moustache toad. *Genome Res.* **35**, 1325–1336 (2025). doi: 10.1101/gr.280161.124; pmid: 40274362.

42. I. Miura, An evolutionary witness: the frog *Rana rugosa* underwent change of heterogametic sex from XY male to ZW female. *Sex. Dev*. **1**: 323–331 (2008). doi: 10.1159/000111764 ; pmid: 18391544.

43. A. Brelsford, M. Stöck, C. Betto-Colliard, S. Dubey, C. Dufresnes, H. Jourdan-Pineau, *et al*., Homologous sex chromosomes in three deeply divergent anuran species. *Evolution* **67**, 2434–2440 (2013). doi: 10.1111/evo.12151; pmid: 23888863.

44. V. Papadogiannis, D. Hockman, S. Mercurio, C. Ramsay, M. Hintze, C. Patthey, *et al.*, Evolution of the expression and regulation of the nuclear hormone receptor ERR gene family in the chordate lineage. *Dev. Biol.* **504**, 12–24 (2023). doi: 10.1016/j.ydbio.2023.09.003; pmid: 37696353.

45. M. Bondesson, R. Hao, C. Y. Lin, C. Williams, J.-Å. Gustafsson, Estrogen receptor signaling during vertebrate development. *Biochim. Biophys. Acta Gene Regul. Mech.* **1849**, 142–151 (2015). doi: 10.1016/j.bbagrm.2014.06.005; pmid: 24954179.

46. M. Vanacker, K. Pettersson, J.-Å. Gustafsson, V. Laudet, Transcriptional targets shared by estrogen receptor-related receptors (ERRs) and estrogen receptor (ER) α, but not by ERβ. *EMBO J.* **18**, 4270–4279 (1999). doi: 10.1093/emboj/18.15.4270; pmid: 10428965.

47. R. J. Kraus, E. A. Ariazi, M. L. Farrell, J. E. Mertz, Estrogen-related receptor α1 actively antagonizes estrogen receptor-regulated transcription in MCF-7 mammary cells. *J. Biol. Chem.* **277**, 24826–24834 (2002). doi: 10.1074/jbc.M202952200; pmid: 11986328.

48. Z. Zhang, C. T. Teng, Estrogen receptor α and estrogen receptor-related receptor α1 compete for binding and coactivator. *Mol. Cell. Endocrinol.* **172**, 223–233 (2001). doi: 10.1016/S0303-7207(00)00372-5; pmid: 11165056.

49. Fan, B., Guo, J., Lei, C., Yang, S., Meng, Z., *et al.*, Haplotype-resolved genomics identifies *cyp19a1a* as a candidate master sex-determining gene in golden trevally (*Gnathanodon speciosus*). *iScience*, **28**, 113493 (2025). doi: 10.1016/j.isci.2025.113493; pmid: 41019367.

50. Y. Oshima, Y. Uno, Y. Matsuda, T. Kobayashi, and M. Nakamura, Molecular cloning and gene expression of *Foxl2* in the frog *Rana rugosa*. *Gen. Comp. Endocrinol.* **159**, 170–177 (2008). doi: 10.1016/j.ygcen.2008.08.013; pmid: 18805419.

51. C. Dufresnes, P.-A. Crochet, B. Rozenblut-Kościsty, S. N. Litvinchuk, N. Rodrigues, *et al*., The heterogametic transition in European *Bufo* toads switches the sex linkage of key vertebrate sex determination genes and associates with a large sex chromosome effect. *Mol. Biol. Evol.* **42**, msaf142 (2025). doi: 10.1093/molbev/msaf142; pmid: 40521715.

52. L. Obendorf, L. Kuhl_Obendorf_Winkler_*et-al*._structural_computational_analyses. Zenodo. doi: 10.5281/zenodo.20247687.

53. A. Fijarczyk, K. Nadachowska, S. Hofman, S. N. Litvinchuk, W. Babik, M. Stuglik, et al., Nuclear and mitochondrial phylogeography of the European fire-bellied toads *Bombina bombina* and *Bombina variegata* supports their independent histories. *Mol. Ecol.* **20**, 3381–3398 (2011). doi: 10.1111/j.1365-294X.2011.05184.x; pmid: 21749513.

54. A. Brelsford, C. Dufresnes, and N. Perrin, High-density sex-specific linkage maps of a European tree frog (*Hyla arborea*) identify the sex chromosome without information on offspring sex. *Heredity* **116**, 177–181 (2016). doi: 10.1038/hdy.2015.87; pmid: 26374238.

55. A. Rhie, McCarthy, S.A., Fedrigo, O. et al. Towards complete and error-free genome assemblies of all vertebrate species. *Nature* **592**, 737–746 (2021). doi:10.1038/s41586-021-03451-0; pmid: 33911273.

56. C.S. Chin, P. Peluso, F. Sedlazeck, *et al*. Phased diploid genome assembly with single-molecule real-time sequencing. *Nat Methods* **13**, 1050–1054 (2016). Doi: 10.1038/nmeth.4035; pmid: 27749838.

57. D. Guan, S.A. McCarthy, J. Wood, K. Howe, Y. Wang, R. Durbin, Identifying and removing haplotypic duplication in primary genome assemblies. *Bioinformatics*, **36**, 2896-2898 (2020). doi: 10.1093/bioinformatics/btaa025; pmid: 31971576.

58. https://github.com/wtsi-hpag/Scaff10X/

59. J. Ghurye, A. Rhie, B.P. Walenz, A.Schmitt, S. Selvaraj, M.Pop, M., *et al*., Integrating Hi-C links with assembly graphs for chromosome-scale assembly. *PLoS Computational Biol.*, **15**, e1007273 (2019). doi: 10.1371/journal.pcbi.1007273; pmid: 31433799.

60. https://github.com/PacificBiosciences/gcpp

61. https://github.com/freebayes/freebayes

62. https://github.com/10XGenomics/longranger

63. H. Cheng, G.T. Concepcion, X. Feng, H.Zhang, H. Li, Haplotype-resolved de novo assembly using phased assembly graphs with hifiasm. *Nat Methods* **18**:170–175 (2021). doi:10.1038/s41592-020-01056-5; pmid: 33526886.

64. C. Zhou C, S.A. McCarthy, R. Durbin, YaHS: yet another Hi-C scaffolding tool. *Bioinformatics* **39**:btac808 (2023). doi:10.1093/bioinformatics/btac808; pmid: 36525368.

65. M. Uliano-Silva, J.G.R.N. Ferreira, K. Krasheninnikova, *et al.*, MitoHiFi: a python pipeline for mitochondrial genome assembly from PacBio high fidelity reads. *BMC Bioinformatics* **24**:288 (2023). doi:10.1186/s12859-023-05385-y; pmid: 37464285.

66. D.L. Pointon, W. Eagles, Sims, M. Muffat, M. Binzer-Panchal *et al.*, Tree of Life service account. 2026. sanger-tol/treeval: 1.4.7 - Ancient Hippaforalkus (H7) (1.4.7). Zenodo. doi:10.5281/zenodo.19631188 .

67. Feron, R. et al. RADSex: a computational workflow to study sex determination using restriction site-associated DNA sequencing data. *Mol. Ecol. Resour.* **21**, 1715–1731 (2021). doi:10.1111/1755-0998.13360; pmid: 33590960.

68. H. Li, Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics* **34**, 3094–3100 (2018). doi: 10.1093/bioinformatics/bty191; pmid: 29750242.

69. A. R. Quinlan, I. M. Hall, BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **26**, 841–842 (2010). doi: 10.1093/bioinformatics/btq033; pmid: 20110278.

70. H. Li, Protein-to-genome alignment with miniprot. *Bioinformatics* **39**, btad014 (2023). doi: 10.1093/bioinformatics/btad014; pmid: 36648328.

71. H. Li, B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, *et al.*, and the 1000 Genome Project Data Processing Subgroup, The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009). doi: 10.1093/bioinformatics/btp352; pmid: 19505943.

72. J. T. Robinson, H. Thorvaldsdóttir, W. Winckler, M. Guttman, E. S. Lander, G. Getz, J. P. Mesirov, Integrative Genomics Viewer. *Nat. Biotechnol.* **29**, 24–26 (2011). doi: 10.1038/nbt.1754; pmid: 21221095.

73. Y. Peng, H. C. M. Leung, S. M. Yiu, F. Y. L. Chin, IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics* **28**, 1420–1428 (2012). doi: 10.1093/bioinformatics/bts174; pmid: 22495754.

74. K. Katoh, K. Misawa, K.-I. Kuma, T. Miyata, MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* **30**, 3059–3066 (2002). doi: 10.1093/nar/gkf436; pmid: 12136088.

75. T. A. Hall, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**, 95–98 (1999).

76. B. Q. Minh, H. A. Schmidt, O. Chernomor, D. Schrempf, M. D. Woodhams, A. von Haeseler, and R. Lanfear, IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. *Mol. Biol. Evol.* **37**, 1530–1534 (2020). doi: 10.1093/molbev/msaa015; pmid: 32011700.

77. M. Knytl, N. R. Fornaini, Measurement of chromosomal arms and FISH reveal complex genome architecture and standardized karyotype of model fish, genus *Carassius*. *Cells* **10**, 2343 (2021). doi: 10.3390/cells10092343; pmid: 34571992.

78. H. C. Macgregor and J. Varley, *Working with Animal Chromosomes*, 2nd ed. (John Wiley & Sons, New York, 1988).

79. V. Krylov, T. Tlapakova, J. Macha, Localization of the single copy gene *Mdh2* on *Xenopus tropicalis* chromosomes by FISH-TSA. *Cytogenet. Genome Res.* **116**, 110–112 (2007). doi: 10.1159/000097427; pmid: 17268187.

80. M. Knytl, T. Tlapakova, T. Vankova, V. Krylov, *Silurana* chromosomal evolution: a new piece to the puzzle. *Cytogenet. Genome Res.* **156**, 223–228 (2019). doi: 10.1159/000501611; pmid: 30537723.

81. S. Guindon, J.-F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk, O. Gascuel, New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* **59**, 307–321 (2010). doi: 10.1093/sysbio/syq010; pmid: 20525638.

82. S. Picelli, O. Faridani, Å. Björklund, et al., Full-length RNA-seq from single cells using Smart-seq2. *Nat. Protoc.* **9**, 171–181 (2014). doi: 10.1038/nprot.2014.006; pmid: 24385147.

83. Y. Peng, H. C. M. Leung, S.-M. Yiu, M.-J. Lv, X.-G. Zhu, and F. Y. L. Chin, IDBA-tran: a more robust de novo de Bruijn graph assembler for transcriptomes with uneven expression levels. *Bioinformatics* **29**, i326–i334 (2013). doi: 10.1093/bioinformatics/btt219; pmid: 23813001.

84. L. K. Gosner, A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16**, 513–543 (1960).

85. R. Ibarra-García-Padilla, A. G. A. Howard IV, E. W. Singleton, and R. A. Uribe, A protocol for whole-mount immuno-coupled hybridization chain reaction (WICHCR) in zebrafish embryos and larvae. *STAR Protoc.* **2**, 100709 (2021). doi: 10.1016/j.xpro.2021.100709; pmid: 34401776.

86. J. Jatzlau, S. N. Do, R. A. Mees, et al., Rare but specific: 5-bp composite motifs define SMAD binding in BMP signaling. *BMC Biol.* **23**, 79 (2025). doi: 10.1186/s12915-025-02183-1; pmid: 40082964.

87. R Core Team, R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (2021). https://www.R-project.org/.

88. S. Passaro, G. Corso, J. Wohlwend, M. Reveiz, S. Thaler, V. R. Somnath, et al., Boltz-2: Towards accurate and efficient binding affinity prediction. *bioRxiv* (2025). doi: 10.1101/2025.06.14.659707[Preprint] (2025).

89. S. J. Fleishman, A. Leaver-Fay, E. J. Corn, E.-M. Strauch, S. D. Khare, N. Koga, et al., RosettaScripts: a scripting language interface to the Rosetta macromolecular modeling suite. *PLoS One* **6**, e20161 (2011). doi: 10.1371/journal.pone.0020161; pmid: 21731610.

90. Schrödinger, LLC, PyMOL Molecular Graphics System, version 3.1.0 (2024). https://pymol.org/ .

91. S. D. Johnston, X. Liu, F. Zuo, T. L. Eisenbraun, S. R. Wiley, *et al.*, Estrogen-related receptor α1 functionally binds as a monomer to extended half-site sequences including ones contained within estrogen-response elements. *Mol. Endocrinol.* **11**, 342–352 (1997). doi: 10.1210/mend.11.3.9897; pmid: 9058380.

92. G. Deblois, J. A. Hall, M. C. Perry, J. Laganière, M. Ghahremani, *et al*., Genome-wide identification of direct target genes implicates estrogen-related receptor α as a determinant of breast cancer heterogeneity. *Cancer Res.* **69**, 6149–6157 (2009). doi: 10.1158/0008-5472.CAN-09-1251; pmid: 19622763.

93. M. Vanacker, E. Bonnelye, S. Chopin-Delannoy, C. Delmarre, V. Cavaillès, V. Laudet, Transcriptional activities of the orphan nuclear receptor ERRα (estrogen receptor-related receptor-α). *Mol. Endocrinol.* **13**, 764–773 (1999). doi: 10.1210/mend.13.5.0281; pmid: 10319326.

94. R. L. Ladek, J. A. Bader, and V. Giguère, The orphan nuclear receptor estrogen-related receptor α is a transcriptional regulator of the human medium-chain acyl coenzyme A dehydrogenase gene. *Mol. Cell. Biol.* 17, 5400–5409 (1997). doi: 10.1128/MCB.17.9.5400; pmid: 9271417.

95. GeneCards: the human gene database. ESRRG (estrogen related receptor gamma) gene summary and annotations. Weizmann Institute of Science, Israel. https://www.genecards.org/cgi-bin/carddisp.pl?gene=ESRRG (accessed 2026).

96. E. Bonnelye, J. M. Vanacker, N. Spruyt, S. Alric, B. Fournier, et al., Expression of the estrogen-related receptor 1 (ERR-1) orphan receptor during mouse development. *Mech. Dev.* **65**, 71–85 (1997). doi: 10.1016/S0925-4773(97)00059-2; pmid: 9256346.

97. S. Misra, A. K. Pandey, S. Gupta, A. Kumar, P. Khanna, et al., Estrogen related receptor is required for the testicular development and for the normal sperm axoneme/mitochondrial derivatives in *Drosophila* males. *Sci. Rep.* **7**, 40372 (2017). doi: 10.1038/srep40372; pmid: 28094344.

98. W. Xie, H. Hong, N. N. Yang, R. J. Lin, C. M. Simon, *et al*., Constitutive activation of transcription and binding of coactivator by estrogen-related receptors 1 and 2. *Mol. Endocrinol.* **13**, 2151–2162 (1999). doi: 10.1210/mend.13.12.0381; pmid: 10598588.

99. Z. Zhang, C. T. Teng, Estrogen receptor-related receptor α1 interacts with coactivator and constitutively activates the estrogen response elements of the human lactoferrin gene. *J. Biol. Chem.* **275**, 20837–20846 (2000). doi: 10.1074/jbc.M001880200; pmid: 10779508.

100. A. P. B. Seneviratne, Z. Turan, A. Hermant, P. Lecine, W. O. Smith, J. P. Borg, et al., Modulation of estrogen related receptor alpha activity by the kinesin KIF17. *Oncotarget* **8**, 50359 (2017). doi: 10.18632/oncotarget.18104; pmid: 28881568.

Acknowledgments

AI (Perplexity.ai, Claude.ai and ChatGPT.ai) was used for scientific English polishing, condensing own drafts, and literature mining. We used BioRender to design Fig. 5 (https://BioRender.com/bg8abhh). We are very grateful for help with initial contacts to sample wild *B. bombina*: Konrad Kürbis (Natural History Museum Erfurt); for further information, enabling field sampling and permit applications: Dirk Weis (Biosphere Reserve 'Oberlausitzer Heide- und Teichlandschaft', i.e. 'Upper Lusatian heath and pond landscape'); for issuing the field work permit for *B. bombina* (C45_DD-8493/343/1; 19 Jan. 2024): Niels Hansen (State Directorate of Saxony); Romain Ferron for a preliminary analysis of ddRADseq data prior to the availability of a reference genome; for help with field work on *Bombina bombina*: Annett Hertweck (NGO - Nature conservation station 'Naturschutzstation Östliche Oberlausitz e.V.'); for help in the molecular lab: Wibke Kleiner, Nathalie Granath, Eva Kreuz, Julianna Yeliz Camurdas, Made Murniati Annik Tengg, Sascha Behrens, and Baturalp Koyun; for toad transfer: Daniel Langenhaun; for technical assistance during swab sampling of captive toads: Amélie Fenzel.

Funding:

German Research Foundation (DFG) STO493/8-1 and IGB funds to MS

Singapore Ministry of Education (AcRF A-8002499-00-00) to CW

Programme Johannes Amos Comenius, CZ.02.01.01/00/22_010/0002902 MSCA Fellowships CZ-UK to MK

Author contributions:

Conceptualization MS, HK, PK, JMS, CW

Methodology: MS, CW, PK, DD, HK, EG, JB, JMS, LO, MB, SWi, TFB, MP, GM, MH, MK, RT, SL, SM, SWü

Investigation: HK, MS, CW, LO, PK, MO

Visualization: MS, HK, MH, LO, GY, CW, MK

Funding acquisition: MS, CW, MK, PK, GM, MB

Projekt administration: MS, PK, CW, HK, SWi

Supervision: MS, PK, CW, LO, SWü

Writing original draft: MS, HK, CW, LO

Writing – review & editing: all co-authors

Competing interests:

Authors declare that they have no competing interests.

Data, code, and materials availability:

DdRAD data single-end sequencing of the genetic family of *Bombina bombina* data: BioProject PRJNA1142776; gonadal transcriptomes of the present project: *B. orientalis* and *B. variegata*: BioProject PRJNA1282098; short-read whole genome sequencing data for female and male *B. maxima* and two males of *B. microdeladigitora*: BioProject PRJNA1307253.
The reference genomes of *B. bombina* (GCA_027579415.1, male) and *B. variegata* (GCA_964205295.1, maternal haplotype; GCA_964205305.1; paternal haplotype) are available from GenBank.
Long-read sequencing raw data of a male *B. bombina* (GCA_027579415.1) are available from the GenomeArk repository:
https://genomeark.s3.amazonaws.com/species/Bombina_bombina/aBomBom1/genomic_data/pacbio/fasta/m54345U_201015_200926.fasta.gz
https://genomeark.s3.amazonaws.com/species/Bombina_bombina/aBomBom1/genomic_data/pacbio/fasta/m54345U_201016_114938.fasta.gz
https://genomeark.s3.amazonaws.com/species/Bombina_bombina/aBomBom1/genomic_data/pacbio/fasta/m54345U_201017_032949.fasta.gz
https://genomeark.s3.amazonaws.com/species/Bombina_bombina/aBomBom1/genomic_data/pacbio/fasta/m54345U_201028_055951.fasta.gz
https://genomeark.s3.amazonaws.com/species/Bombina_bombina/aBomBom1/genomic_data/pacbio/fasta/m54345U_201028_213910.fasta.gz
https://genomeark.s3.amazonaws.com/species/Bombina_bombina/aBomBom1/genomic_data/pacbio/fasta/m54345U_201030_115900.fasta.gz) and mapped it back to the genome assembly.

Scripts for computational structural predictions using Boltz-2 and Rosetta Docking analyses are available on Github[52]

Reviewer links for NCBI Bioprojects (to be removed after review process):
https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1142776
https://dataview.ncbi.nlm.nih.gov/object/PRJNA1282098?reviewer=jq3bsdgttrk5ihgthedcn4ti19
https://dataview.ncbi.nlm.nih.gov/object/PRJNA1307253?reviewer=p0amhnqu01i36igonubfh81k8d

Supplementary Materials

Materials and Methods

Supplementary Texts S1 to S2.

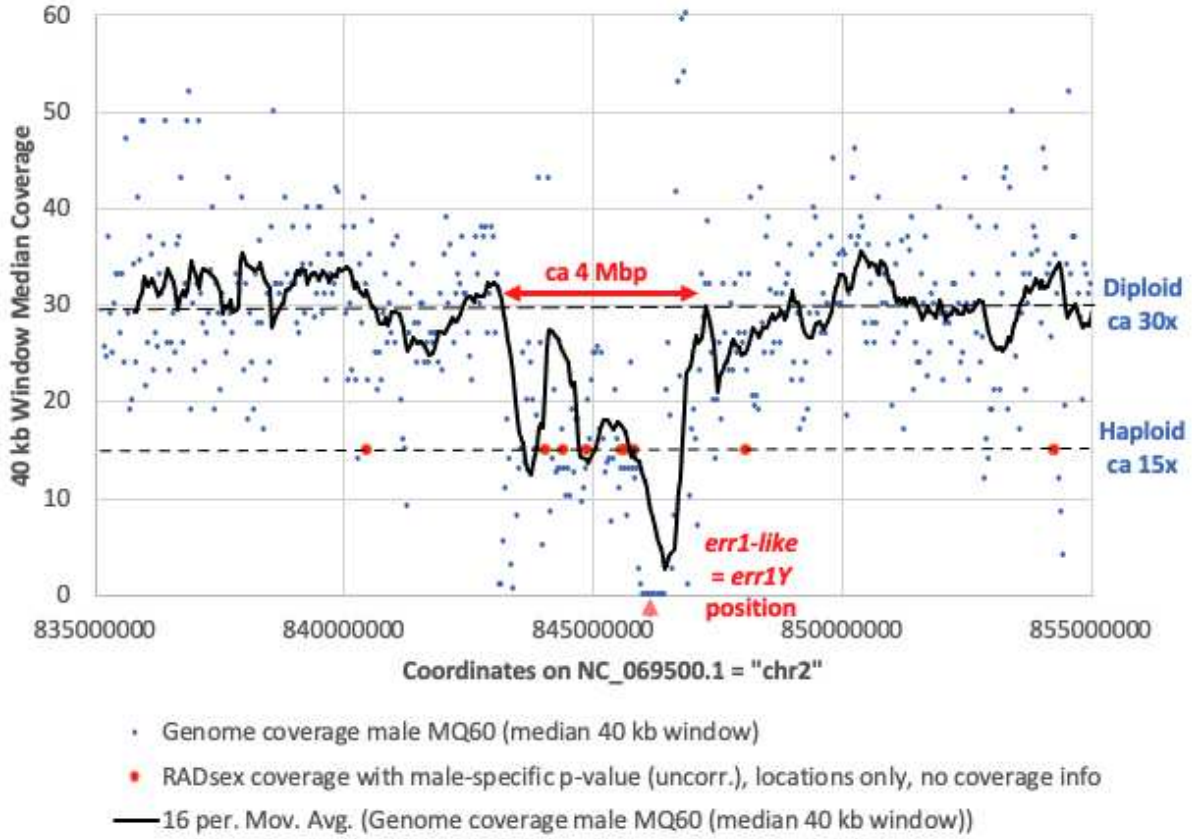
Figs. S1 to S11

Tables S1 to S7

References (53-100)

Data S1 to S3

712



713

714

715 **Fig. 1. Long-read coverage (blue) across the single-copy (hemizygous) male-specific region**
716 **of the otherwise diploid *Bombina bombina* reference genome, identified using ddRAD**
717 **markers (red) from a large pedigree.** Genomic positions of male-specific ddRADseq markers
718 (red dots) from a *Bombina bombina* family comprising the parents and 90 anatomically sexed
719 offspring, overlaid with long-read coverage (blue dots) on chromosome 2 of the male reference
720 genome (GCF_027579735.1). The ~4 Mbp region coinciding with the 8 top male-specific ddRAD
721 markers shows hemizygous long-read coverage and contains the steroid receptor annotated as
722 "*err1-like*" (*err1Y*). Coverage was calculated as median x-fold values in 40 kb windows; the black
723 line shows a moving average based on 16 neighboring data points. Stringent mapping-quality
724 filtering (MQ 60) produced a few windows with very low or zero coverage because of high repeat
725 content. The x-axis shows chromosomal positions from 835,000,000 to 855,000,000 bp on
726 chromosome 2.

727

728

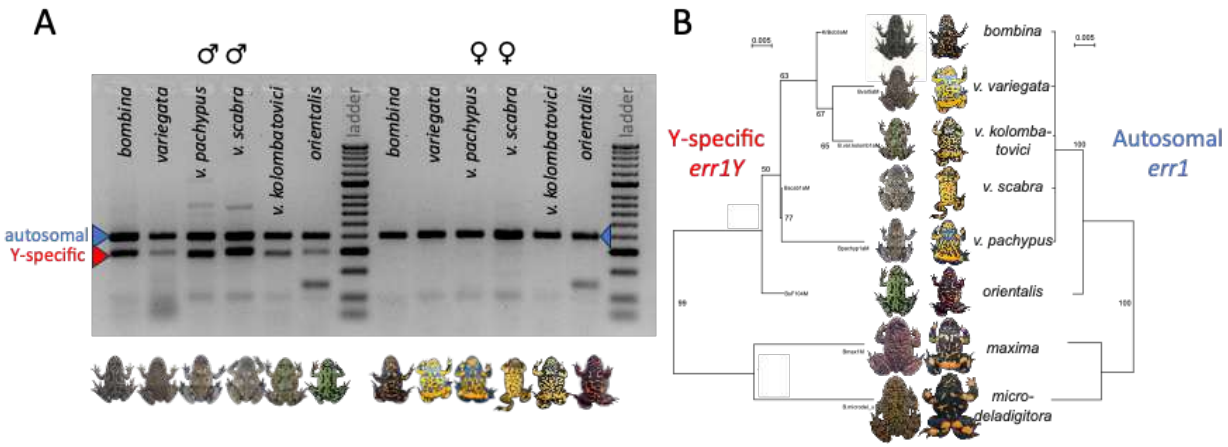


Fig. 2. Molecular markers for male-specific *err1Y* (= *err1-like*) and autosomal *err1* (*essra*) genes in a multiplex PCR in five Eurasian *Bombina* taxa and phylogenetic trees of both markers across the genus *Bombina*. A: Males (♂♂) left, females (♀♀) right; primers *BombYonly_300bp_F/_R* amplify a 300 bp *err1Y* (red arrow) band in males only; *Bomb_Auto_397bp_F/R* amplify a 397 bp *err1* (autosomal; blue arrows) band in both sexes. PCR products run on 1.5% agarose gel (5 µL sample + 1 µL 6× dye, 80 min, 45 V); marker: 5 µL HyperLadder 50 bp (Bioline): 'ladder'. Some additional non-specific bands appeared in *B. v. scabra*, *B. v. pachypus*, and *B. orientalis*. **B:** Phylogenetic trees of eight *Bombina* taxa involving a fragment of the candidate sex determination (SD) gene *err1Y* (= *err1-like*) and the autosomal *err1 (=essra)*. *Bombina bombina, B. variegata variegata, B. v. kolombatovici, B. v. scabra, B. v. pachypus,* and *B. orientalis*, all have 2n = 24 chromosomes and share *err1Y* as the male-determining gene. In *Bombina maxima* and *B. microdeladigitora*, with 2n = 28 chromosomes, homologous *err1-like* is not sex-specific. The entire *Bombina* radiation is estimated to have a most recent common ancestor about 20 Mya[23]. Trees were calculated using maximum likelihood with 1000 bootstraps. Phenotypic diversity is not sex-specific but illustrated in each subfigure (A, B) by dorsal (left) and ventral (right) views. Toad photos: Markus Harzdorf ©

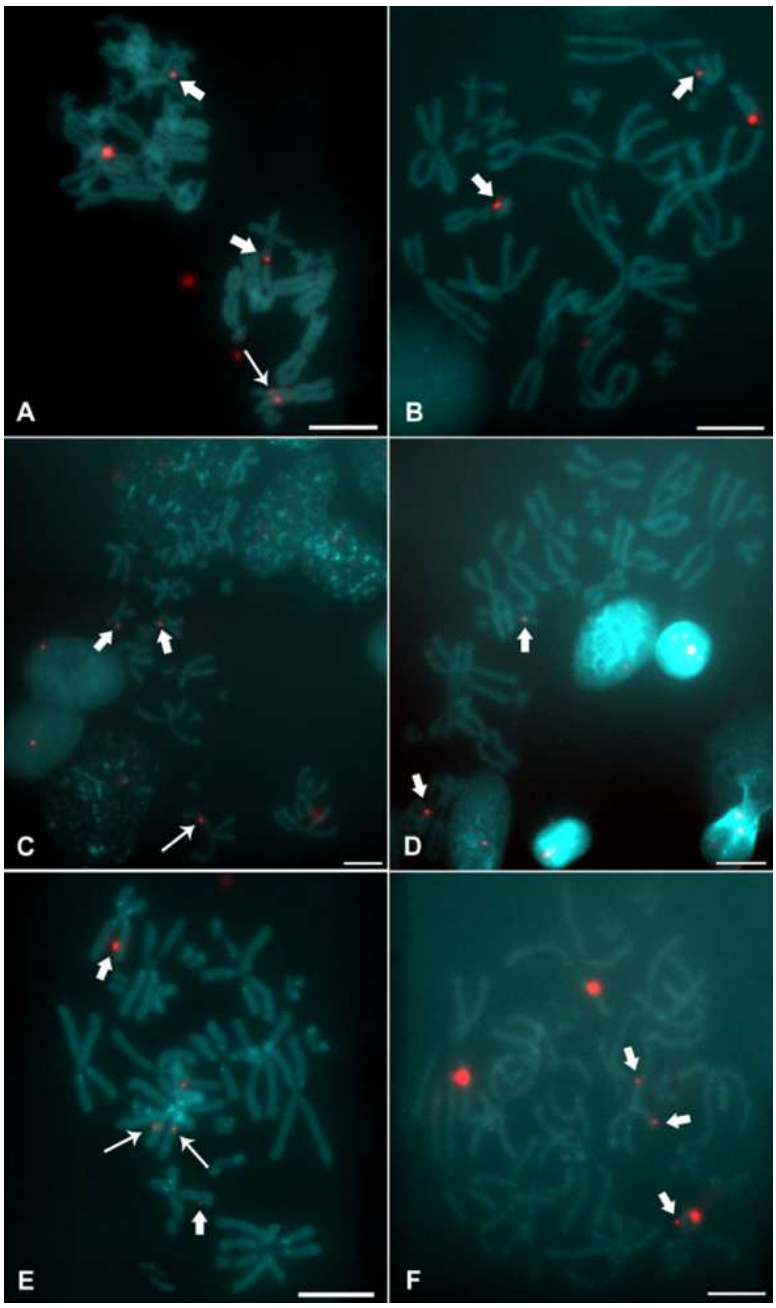


Fig. 3. Cytogenetic mapping of the steroid hormone orphan receptor gene *err1Y* (LOC128647330) in *B. bombina* revealed a conserved Y-linked locus on LG2 (homologous to LG1 in *Xenopus tropicalis*) in males across three *Bombina* species. FISH-TSA on metaphase chromosome spreads of **A, B:** *B. bombina* (male, female; kidney), **C, D:** *B. variegata* (male, intestine; female, kidney), and **E, F:** *B. orientalis* (male, intestine; female, kidney) showing the *B. bombina-err1Y* probe hybridizing to the long arm of a single chromosome 2 homolog in males of all three species (red dots, slender arrows), indicating gene location homology and Y-specificity. The probe also mapped near a secondary constriction on autosomal LG7 in both sexes (red dots, bold arrows), corresponding to the autosomal paralogous gene *estrogen related receptor α* (*err1 = essra*). Some signals appeared on both chromatids of a chromosome (A, F). Images B-C show interphase nuclei. DAPI staining (blue/green) revealed all 24 chromosomes. Scale bars present 10 µm.

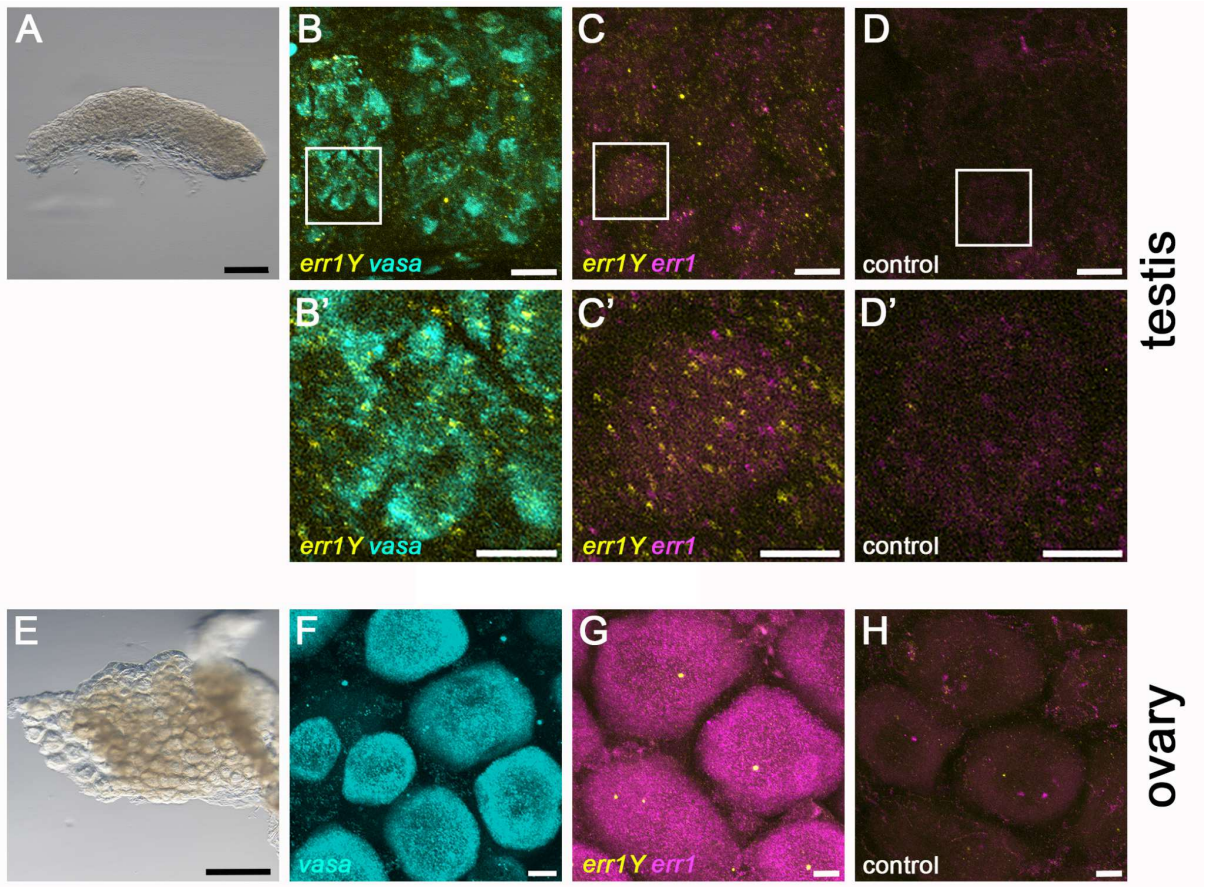


Fig. 4. Expression of the candidate sex-determining gene *err1Y* in early *Bombina* gonads at metamorphosis (Gosner 46, B-C'), visualized via hybrid chain reaction (HCR) in comparison with autosomal *err1*, and labelled gonocytes (*vasa*). **A**: Primordial testis of a genetic male *B. variegata*; **B**: *Err1Y* (yellow) and *vasa* (cyan) expression; **B'**: Enlarged view of area boxed in **B**, *vasa* labels gonocytes. **C**: sex-chromosomal *err1Y* (yellow) and autosomal *err1* (magenta) expression; **C'**: Enlarged view of area boxed in **C**; **D**: Negative control, no HCR initiator oligos added, no signal, only background. **D'**: enlarged view of area boxed in **D**. For **A-D**, testes dissected from male individuals Bvarjuv14M, 16M, 21M and 22M were used. **E:** Developing ovary of a genetic female *B. variegata*; **F**: *Vasa* (cyan) expression in early diplotene oocytes **G**: *Err1* expression (magenta) in early diplotene oocytes, only background signal for *err1Y* (yellow); **H**: Negative control, no HCR initiator oligos added, no signal, only background. For **E-H**, ovaries were dissected from female individuals Bvarjuv 7F, 19F and 20F. Images C, C', D, D', G and H were captured with the same intensity settings to allow comparison of expression levels. All fluorescent images are maximum intensity projections, merged channels (546 and 647 nm), except for F (546 nm). Single channel images are shown in Figs. S7, S8. Scale bars are 200 µm (A, E), 20 µm (B-D, F-H) and 10 µm (B'-D').

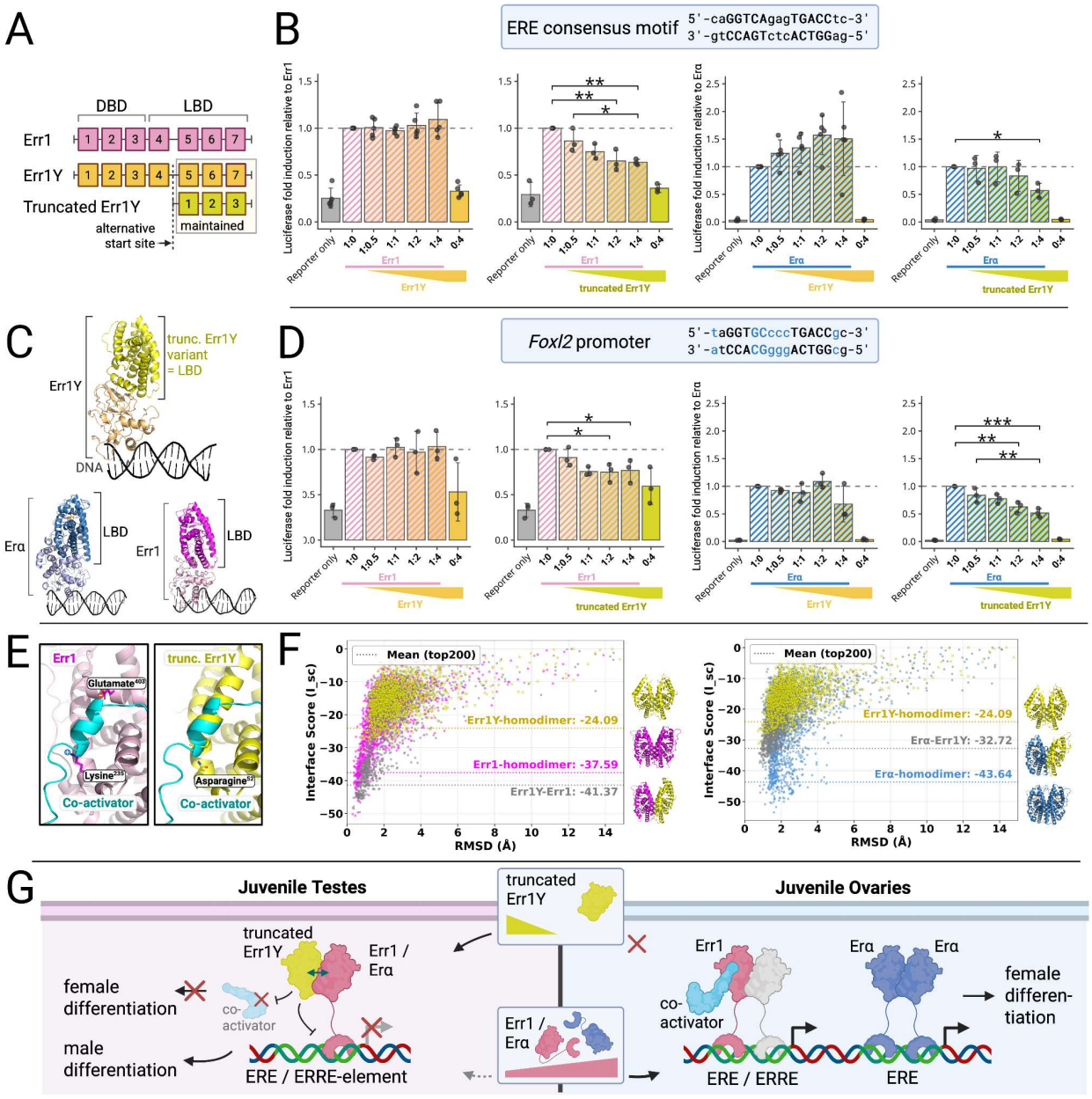


Fig. 5. Functional investigation of Err1Y using computational modelling and reporter gene assays. A: Schematic representations of the exons 1-7 encoding autosomal Err1 (magenta) and sex-chromosomal Err1Y (orange) highlighting the alternative transcriptional start of the more abundant truncated Err1Y (exons 1-3, yellow; Fig. S10 RNAseq mapping data). **B**: Dual-luciferase assay in human embryonic kidney (HEK) cells using a minimal ERE reporter. Cells were transfected with 50 ng myc-tagged Err1 or FLAG-tagged Erα together with increasing amounts (25, 50, 100, 200 ng, shown as ratios to Err1/Erα) of HA-tagged Err1Y or truncated Err1Y. For visualization, values were normalized within each biological replicate to the receptor-only condition (1 : 0). Statistical analysis was performed on values normalized to the corresponding 1 : 1 condition using ANOVA with Tukey's post hoc test; expression control (Westernblot) in Fig. S11C. **C:** Structural predictions using Boltz-2 of *B. bombina* Err1Y (LBD-only and full length variant) in yellow, ERα (plus estrogen) in blue, and Err1 (magenta) in complex with a DNA fragment containing an estrogen response element (ERE). **D:** Dual-luciferase assay analogous to

812 panel B using the top scoring ERE motif from the *B. bombina foxl2* promoter region as reporter.
813 **E**: Structural comparison of the ligand-binding domains of Err1 and Err1Y highlighting the co-
814 activator binding region with nuclear-receptor specific binding residues (Glutamate403 and
815 Lysine235 in Err1, left) mutated or not present in Err1Y (right). **F**: Rosetta docking analysis of
816 homo- and heteromeric interfaces formed by the ligand binding domains (LBDs) of Err1, Err1Y,
817 and Erα, shown as interface score (REU) versus iRMSD (Å). **G:** Model summarizing the proposed
818 role of truncated Err1Y in primordial gonads and juvenile testes (left) where relatively high
819 expression of truncated Err1Y leads to male differentiation while in absence of truncated Err1Y
820 the female pathway can be promoted via Err1 / Erα.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplDataS1ToS3BombinaNatGen.zip
- BombinaNatGensupplementarymaterials4June2026FINAL.pdf