Viviparous reptile regarded to have temperature-dependent sex determination has old XY chromosomes

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ABSTRACT

The water skinks *Eulamprus tympanum* and *E. heatwolei* show thermally-induced sex determination where elevated temperatures give rise to male offspring. Paradoxically, *Eulamprus* species reproduce in temperatures of 12-15°C making them outliers when compared to reptiles that use temperature as a cue for sex determination. Moreover, these two species are among the very few viviparous reptiles reported to have thermally-induced sex determination. Thus, we tested whether these skinks possess undetected sex chromosomes with thermal override. We produced transcriptome and genome data for *E. heatwolei*. We found that *E. heatwolei* present XY chromosomes that include 14 gametologs with regulatory functions. The Y chromosomal region is 79-116 million-years-old and shared between water and spotted skinks. Our work provides clear evidence that climate could be useful to predict the type of sex determination systems in reptiles and it also indicates that viviparity is strictly associated with sex chromosomes.

Eulamprus tympanum and *E. heatwolei* reproduce in colder conditions compared to other species with temperature-dependent sex determination

Vertebrates exhibit two major classes of sex determination systems. Genotypic sex determination (GSD), where genetic components guide the development of the gonads, and temperature-dependent sex determination (TSD), where specific incubation temperatures define the sex of the embryos (Bachtrog, et al. 2014). TSD in reptiles is thought to have evolved when external conditions that enhance either male or female offspring fitness could influence the sex of the embryos (Charnov and Bull 1977; Shine 1999). For this reason, the discovery of TSD in a viviparous skink was particularly notable (Robert and Thompson 2001). In viviparous species, the external conditions have little effect because embryonic development and hatchling occur inside the mother's womb in a relatively stable environment.

The viviparous water skinks *Eulamprus tympanum* and *E. heatwolei* (family *Scincidae*) are classified as TSD species (Tree of Sex 2014) because cytogenetic analyses found no evidence of heteromorphic sex chromosomes and female *Eulamprus* skinks give rise to male offspring when they are kept at

warm temperatures (32°C) during pregnancy (Robert and Thompson 2001). Three features, however, make this classification of *Eulamprus* as TSD suspect: 1. These two species inhabit alpine habitats in southeastern Australia (Cogger 1986), whereas most reptiles with TSD systems inhabit lowland areas; 2. Uniquely, while all known viviparous reptiles have genetic sex determination systems, *E. tympanum* and *E. heatwolei* are the only known viviparous reptiles classified as TSD; and 3. Several studies have found 1:1 sex ratios in *E. heatwolei* at mild temperatures, both in the laboratory and in the field (Schwarzkopf and Shine 1991; Robert and Thompson 2001; Allsop, et al. 2006). Taken together, these features implied either a GSD system with thermal override or, although less likely, an atypical TSD system.

We first examined whether ambient temperatures in areas inhabited by *E. tympanum* and *E. heatwolei* during breeding seasons were unusual compared to reptile species with TSD or GSD. For this, we mapped 30 years of ambient temperatures onto the geographic ranges of 101 species with TSD and 99 species with GSD during their breeding season (Figure 1). Average ambient temperatures for *E. heatwolei* and *E. tympanum* during their breeding seasons are 15°C and 12.4°C, respectively (Figure 1). Thus, *E. heatwolei* and *E. tympanum* are clear outliers when considered as TSD species, located at 3 and 4 standard deviations away from the mean of the distribution, respectively (Figure 1). In contrast, *Eulamprus* species are found within the distribution of species with GSD (Figure 1). These results are suggestive of the presence of previously undetected sex chromosomes in these two species.

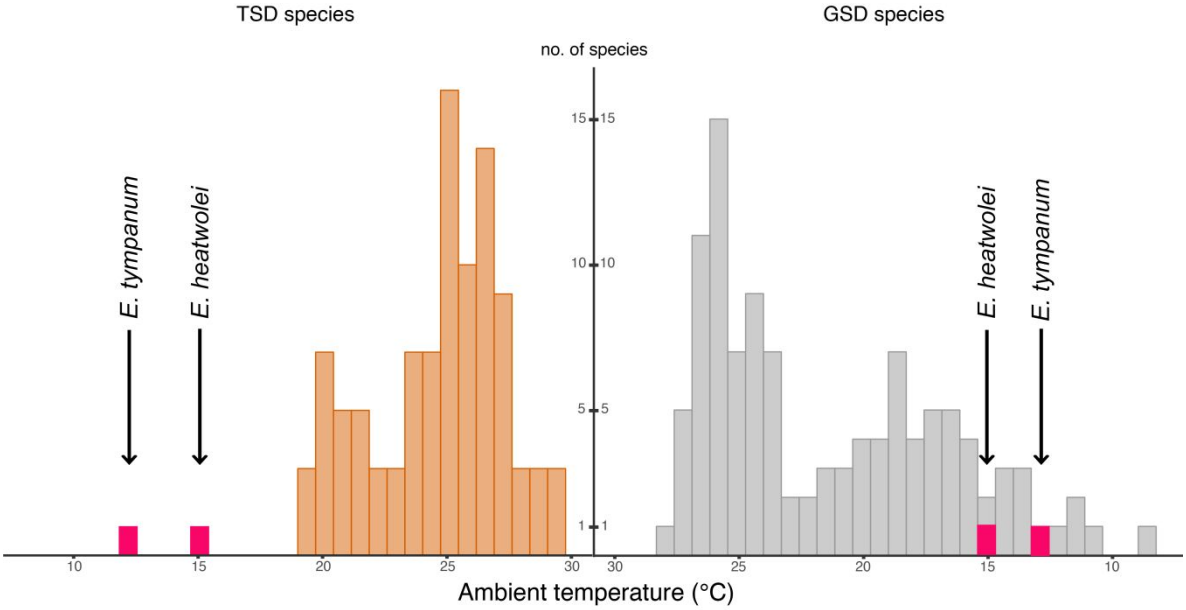

Figure 1. Distribution of average ambient temperature in geographical ranges during breeding seasons for reptile species with TSD (n = 101) and species with GSD (n = 99). Labelled bars in red correspond to average ambient temperature for *E. heatwolei* and *E. tympanum.*

E. heatwolei has XY chromosomes

To test for the presence of previously unidentified sex chromosomes in skinks, RNAseq data were generated from brain, liver, and gonads of one adult male and one adult female *E. heatwolei*. We then applied a subtraction approach (Cortez, et al. 2014; Marin, et al. 2017) to the male and female transcriptomic data of *E. heatwolei*. Specifically, we assembled a male-restricted transcriptome and used male and female genomic reads to uncover Y-linked transcripts (see Methods). We identified Y-linked transcripts from 14 protein-coding genes with known orthologous genes located on a single syntenic block on chromosome 5 in *Anolis carolinensis* and chromosome 1 in chicken (Figure 2a; Supplementary Table 3). Additionally, we performed a male and female genomic read coverage analysis of six chromosomes of *E. heatwolei* (see Methods). We found a region on chromosome 5 where the male shows only half of the coverage (*i.e.* one genomic copy; Figure 2b; Supplementary Figure 1). XY gametologs map to this specific region on chromosome 5 (Figure 2a-b) and analysis of their genomic coverage is consistent with two X gametologs in females but one X and one Y gametolog in males (Supplementary Figure 2). Lastly, we screened the genomes of seven males and seven females using standard PCRs and found that we could only amplify Y-linked sequences in males (Figure 2c; Supplementary Figure 3). In summary, the results reveal the presence of sex chromosomes in *E. heatwolei.*

Functions associated to the identified Y-linked genes (retrieved from the GeneCards database; www.genecards.org) include ubiquitination (*UBE2H* and *CAND1*), signalling pathways (*LEMD3*/*MAN1* and *FRS2*), cell cycle, cell growth and differentiation (*PPP1R12A*, *E2F7*, *RAP1B*, and *BTG1*), transcription regulation (*ZNF384*), ion transport (*ATP2B1*), fatty acid metabolism (*ZDHHC17*), and DNA replication (*NAP1L1*). Many of the identified Y chromosome-linked genes have known regulatory functions. Examining the list of putative Y-linked genes, *PPP1R12A* is of particular interest. The protein coded by this gene is part of the PPP1C protein complex that catalyses many protein dephosphorylation reactions in the cell and is essential for male fertility in mice (Silva, et al. 2014). Another member of the PPP1C complex, the *PPP1CC* gene, is one of the oldest genes on the Y chromosome of pleurodonts (Marin, et al. 2017), a group that diverged from the skink lineage 184.9 million years ago -ma- (data retrieved from the TimeTree database; www.timetree.org/). The convergent co-option of genes forming part of the same molecular pathways (*PPP1R12A* and *PPP1CC* are probably involved in spermatogenesis) on the Y and W chromosomes is a frequent phenomenon in vertebrates (Marshall Graves and Peichel 2010; O'Meally, et al. 2012).

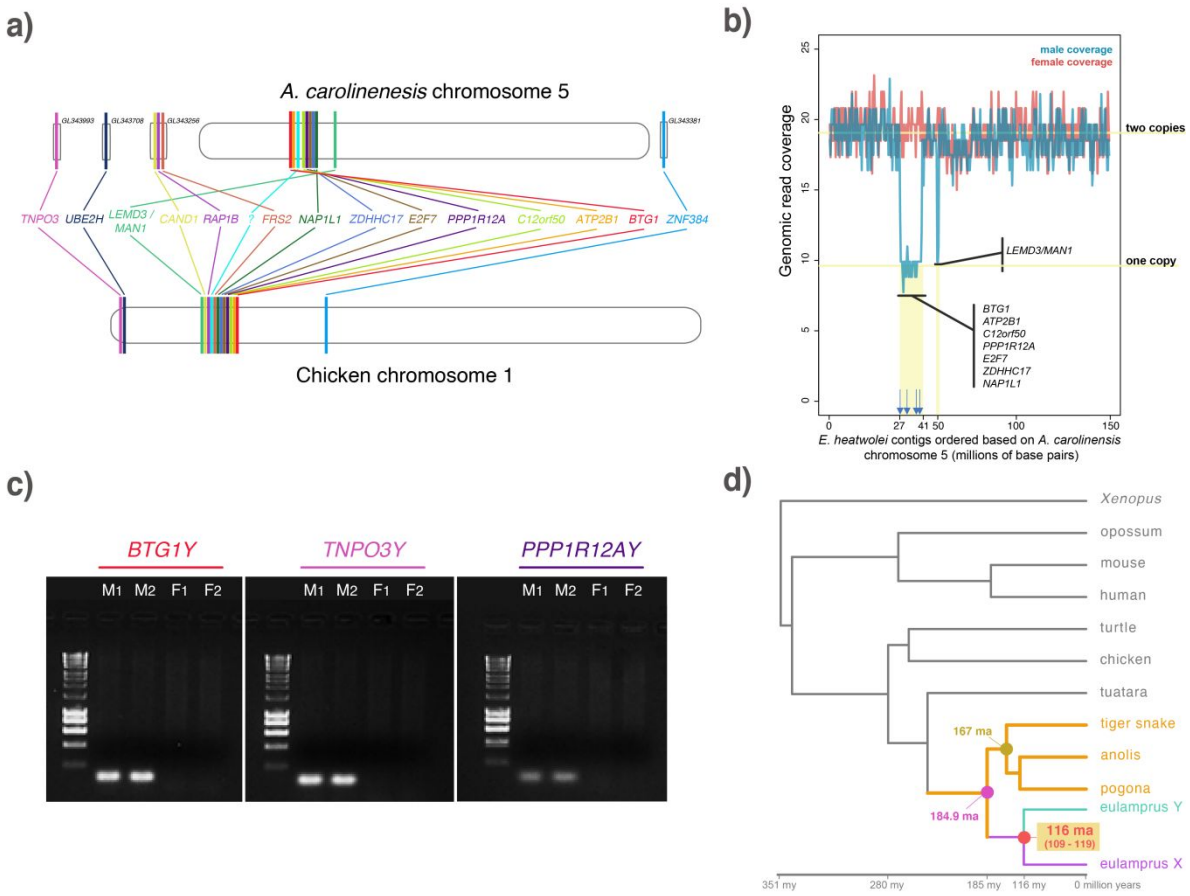

Figure 2. a) Synteny of the 14 XY gametologs in other species. **b)** Male (blue) and female (red) genomic coverage along the chromosome 5 of *E. heatwolei*. A syntenic region shows half of the coverage in males (one copy) but regular coverage in females (two copies). XY gametologs map to this region. Blue arrows show the matching locations of Y-linked markers from *N. ocellatus*. **c)** PCR screenings of two males and two females using primers designed to amplify three Y-linked genes (seven males and seven females were screened in total; see Supplementary Figure 3). **d)** Time-calibrated synonymous substitution tree used to estimate the age of the XY chromosomes in *E. heatwolei*. Branch lengths represent millions of years.

To obtain an estimate for the origin of the male-specific region on the Y chromosome (MSY) in the *E. heatwolei* lineage, we used d_S trees based on the nucleotide sequences of the XY gametologs in *E. heatwolei* and orthologous sequences from other species (see Methods). From the synonymous substitution rates of the concatenated sequences of the XY gametologs we estimated that *E. heatwolei* sex chromosomes originated approximately 116 million years ago (Ma; 95% confident intervals: 109.45 Ma – 119.28 Ma; values derived from 100 bootstrap rounds; Figure 2d). Moreover, estimates obtained using BEAST resulted in a sex chromosome age of ~93 Ma (Supplementary Figure 4). Next, we retrieved Y-linked markers reported for the spotted skink, *Niveoscincus ocellatus* (Hill, et al. 2018). These sequences are short (17-70bp) and likely represent repeated, intergenic or intronic regions of the MSY. Only nine Y markers aligned to the *E. heatwolei* and *A. carolinensis*

genomes; four mapped to multiple genomic locations (i.e. likely repeated sequences), one mapped to chromosome three and four mapped to chromosome five, exactly within the MSY of *E. hetawolei* (Figure 2, blue arrows; Supplementary Table 3). This association is highly significant (Fisher-exact test, $P < 0.001$) and indicative that water and spotted skinks share a common MSY, which originated >79 million years ago (divergence time between the two groups of skinks; data retrieved from TimeTree; http://www.timetree.org/).

Conclusions

Our work identified the MSY locus in *E. heatwolei*'s chromosome five and, importantly, it provided evidence that climate could be a good predictor of sex determination systems in reptiles. We can now reclassify *E. heatwolei* (and probably *E. tympanum*) as a viviparous skink showing GSD with thermally-induced sex reversal at elevated temperatures (Shine, et al. 2002; Quinn, et al. 2007; Radder, et al. 2008; Holleley, et al. 2015). In the past, also the viviparous skink, *N. ocellatus* was assumed to have TSD on a lowland population (Pen, et al. 2010). Here, we found that *E. heatwolei* and *N. ocellatus* share Y-linked sequences. We estimated that the sex-linked locus originated around 79-116 million years ago. Note that other species in the *Scincidae* family also have XY chromosomes (Supplementary Figure 5), so perhaps all skink species share the same GSD system.

Formerly, reptiles were thought to either have GSD or TSD systems. However, various studies have shown that in several species, including the viviparous *E. heatwolei* (Robert and Thompson 2001) (and this work), the viviparous *N. ocellatus* (Hill, et al. 2018), the oviparous *Pogona vitticeps* (Quinn, et al. 2007; Holleley, et al. 2015) and the oviparous *Bassiana duperreyi* (Shine, et al. 2002; Radder, et al. 2008), certain incubation temperatures can override the signalling cascade initiated by sex-linked genes and influence the fate of the embryonic gonads. These thermally-induced sex reversal mechanisms may represent retained elements of ancestral TSD systems. Further analyses in *E. heatwolei* and related species could help answer this question.

We know that viviparity has evolved from oviparity more than 100 times (Sites, et al. 2011; Pyron and Burbrink 2014) and it is strongly correlated with the colonisation of cold alpine environments (Lambert and Wiens 2013). The *Eulamprus* species were the last viviparous reptiles classified as TSD (Tree of Sex 2014). Our results indicate, for the moment, that viviparity in reptiles is strictly associated with GSD systems.

Materials and Methods

Data generation

One adult male (Euhea_18_05) and one adult female individual (Euhea_18_03) of *E. heatwolei* species were captured from a population that inhabits Woods Reserve, Corin Road, ACT, Australia (-35.480751, 148.940398). Both individuals were sacrificed by intraperitoneal injection of pentobarbitone following the standard operating procedures specified by the animal ethics committee of the University of Canberra. We generated DNA-seq libraries for a male and female *E. heatwolei* from liver tissue using the Illumina TruSeq DNA protocol for short insert size (400–450 nt). We generated strand-specific RNA-seq libraries (using the Illumina TruSeq Stranded mRNA Library protocol) for a total of 6 samples obtained from brain, liver, and gonads for a male and female *E. heatwolei*. All libraries were sequenced on Illumina HiSeq 2500 sequencers at the University of Canberra. We generated 262-269 million 150-nt paired-end DNAseq reads. We generated 82-95 million 125-nt paired-end RNAseq reads. Further details in Supplementary Table 4. Quality of the reads was verified using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and the remaining adaptors were removed with Trimmomatic (Bolger, et al. 2014).

Assembly of Y-linked transcripts

To assemble Y-linked transcripts in *E. heatwolei* we used a subtraction approach based on male and female RNAseq data (Cortez, et al. 2014; Marin, et al. 2017; Acosta, et al. 2019). Briefly, male RNA-seq reads were aligned onto the *de novo* reconstructed female transcriptome from *E. heatwolei* using Hisat2 (v2.0.2) (Kim, et al. 2015); no mismatches allowed; reads not mapping were selected. We also removed male RNA-seq reads sharing k-mers with the female transcriptome (Akagi, et al. 2014). The selected reads were passed to Trinity (v2.0.2, default k-mer of 25 bp) (Grabherr, et al. 2011) to assemble transcripts that were only present in male tissues. We obtained 21,249 transcripts that were subsequently aligned to the male and female genomic reads using blastN (Altschul, et al. 1990); at a 100-99% identity threshold. We selected those transcripts showing 4X-14X of averaged coverage of male genomic reads and zero averaged coverage of female genomic reads (Supplementary Table 3). To establish Y gene identity, we searched NCBI GenBank (Reptile

taxa only; http://www.ncbi.nlm.nih.gov/genbank) with blastN and blastX for the closest homologs and identified transcripts that coded for 14 proteins (Supplementary Table 3). BlastX searches also allowed the identification of CDS regions. For these 14 Y-linked protein-coding genes, we performed blastN searches against the *de novo* reconstructed female transcriptome from *E. heatwolei* to find the X gametologs (best match over the entire sequence; 95-97% identity). We verified the X gametologs identity using coverage analyses of male and female genomic reads and GenBank searches (same gene identity as Y gametologs). XY gametologs in *E. heatwolei* were searched against the *A. carolinensis* and chicken genomes using the sequence search engine at the ENSEMBL webpage (https://www.ensembl.org/Multi/Tools/Blast) to establish whether they formed a syntenic block. We validated the presence of a Y chromosome by PCR screenings using genomic DNA obtained from tails snips of seven males and seven females. Additional information can be found in the extended Methods in the Supplementary Material. We retrieved the Y-linked markers in *N. ocellatus* (Hill, et al. 2018) and used blastN (e-value < 0.01) to map these sequences onto the reconstructed *E. heatwolei* chromosomes and the *A. carolinenesis* reference genome downloaded from the Ensembl database (https://www.ensembl.org/; v.97). More details in Supplementary Table 3.

Genomic coverage analyses

We followed a methodology previously published (Vicoso, et al. 2013). Briefly, the male and female genomic reads were assembled into contigs. The contigs were subsequently aligned and ordered based on the *A. carolinensis* reference genome. We used bowtie2 (Langmead and Salzberg 2012) to align the DNA-seq reads from the male and female *E. heatwolei* onto the reconstructed chromosomes. Coverage along the chromosomes was calculated using BEDtools (Quinlan and Hall 2010), bins of 100,000 nucleotides. Additional information can be found in the extended Methods in the Supplementary Material.

Data collection

Full list of reptiles with known TSD system was obtained from the Tree of Sex database (Tree of Sex 2014) and literature searches. We searched the literature and dedicated databases for the duration and month intervals of the breeding seasons. We collected information for 101 species with TSD (Supplementary Tables 1 and 2). Temperature data from the entire surface of the planet was downloaded from the Climatic Research Unit (http://

http://catalogue.ceda.ac.uk/uuid/3df7562727314bab963282e6a0284f24; version 3.24.01). Additional information can be found in the extended Methods in the Supplementary Material.

Geographical ranges

Shapefiles for 29 species where downloaded from the RedList database (http://www.iucnredlist.org/; version 3; Supplementary Table 1). For 72 additional species (Supplementary Table 2) we generated geographic ranges using the ecological niche modelling routines applying the maximum entropy algorithm in Maxent (Phillips, et al. 2006) using the R package kuenm (Cobos, et al. 2019). Additional information can be found in the extended Methods in the Supplementary Material.

Mapping climate data to the species distribution

We matched the climate data with the species shapefiles using a dedicated R package built by Dr. Anna Krystalli as part of the Newton Advanced Fellowship program (https://github.com/annakrystalli/IUCNextractR). We recovered the median temperature (ambient temperature) of all months comprised in the breeding season. Additional information can be found in the extended Methods in the Supplementary Material.

Synonymous substitution analyses

To assess the age at which the XY system was originated in *E. heatwolei*, we followed a previous procedure (Cortez, et al. 2014; Marin, et al. 2017; Acosta, et al. 2019). Briefly, we aligned using PRANK (Loytynoja and Goldman 2005) the coding sequences of XY gametologs in *E. heatwolei* and coding sequences of 1-1 orthologous in other reptiles, mammalian and *Xenopus* species downloaded from the Ensembl database (https://www.ensembl.org/; v.97). We obtained the species' tree from the TimeTree database (http://www.timetree.org/). We concatenated the alignments and calculated synonymous substitution rates (d_S) using codeml (Yang 1997) and a bootstrap approach. Branch lengths on the species' tree were used to obtain an ultrametric, time-calibrated, tree using the *chronos* library (*ape* package in R, v5.0) (Paradis and Schliep 2019). The age of the sex chromosomes was obtained from the calibrated branch lengths just before and after the split of the XY gametologs and the time since *E. heatwolei* diverged from the *Snake-Pogona-Anolis* lineage (divergence data retrieved from TimeTree; http://www.timetree.org/). We also

calculated the age of the sex chromosomes using BEAST v1.10.4 (http:/beast.bio.ed.ac.uk/), which resulted in an age estimate of ~93 million years ago. We used the relaxed clock and calibrated the tree based on the reptile/mammalian divergence time. We ran the analyses two independent times for 100,000,000 generations, sampling every 1,000 generations. Additional information can be found in the extended Methods in the Supplementary Material.

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Data deposition

This project has been deposited at NCBI-SRA database (www.ncbi.nlm.nih.gov/sra) under the accession BioProject PRJNA573688. Y-linked sequences found in *E. heatwolei* are available in Supplementary Material. Shapefiles of geographical ranges of reptiles are available in the figshare platform at the following link https://figshare.com/articles/Reptile_shapefiles/7416638. The program to map climatic data onto geographical ranges is available in the GitHub platform at the following link https://github.com/annakrystalli/IUCNextractR.

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Authors' contributions

DC, AOU, TS, and AG designed the study. DSBD and AG performed the fieldwork, tissue collection, DNA and RNA extractions, and PCR screenings. PCP, DSBD, ALN, MLMP, AA, and DC performed the analyses. CRS preformed additional analyses. FRMC contributed to the analyses, discussion and ecological data collection. All authors contributed to the interpretation of the results. AOU, AG, TS, and DC wrote the manuscript. All authors read and approved the final manuscript.

Competing financial interests

The authors declare that they have no competing interests.

Supplementary Material

Supplementary Figure 1: Male and female genomic read coverage for six reconstructed chromosomes in *E. heatwolei*.

Supplementary Figure 2: Male and female genomic read coverage for XY gametologs in *E. heatwolei*.

Supplementary Figure 3: PCR screening of Y-linked genes in seven males and seven females *E. heatwolei*.

Supplementary Figure 4: Age of sex chromosomes based on analysis using BEAST.

Supplementary Figure 5: Phylogenetic tree of 132 genera from the *Scincidae* family.

Supplementary Table 1: Data for 128 reptile species with known breeding season and Redlist shapefiles used in this study.

Supplementary Table 2: Data for 72 reptile species with known breeding season and shapefiles generated for this study.

Supplementary Table 3: Details regarding the Y-linked sequences in *E. heatwolei*.

Supplementary Table 4: Details regarding RNAseq and DNAseq libraries.

Supplementary Material: Extended methods, code and command-line used, Y-linked transcripts, and sequencing quality reports.