PERSPECTIVES AND HYPOTHESIS

Title: Reversals in complex traits uncovered as reticulation events: lessons from the evolution of parity-mode, chromosome morphology, and maternal resource transfer

Running title: Reticulation and trait reversals

Authors: Jose L. Horreo^{1,2,3}, Teresa Suarez⁴, Patrick S. Fitze^{1,2,*}.

¹Department of Biodiversity and Evolutionary Biology, Museo Nacional de Ciencias Naturales (MNCN-CSIC), José Gutiérrez Abascal 2, 28006 Madrid, Spain.
horreojose@gmail.com.

²Department of Ecology and Evolution (DEE), University of Lausanne, 1015 Lausanne, Switzerland.

³UMIB Research Unit of Biodiversity (UO, CSIC, PA), C/Gonzalo Gutiérrez Quirós s/n, 33600, Oviedo University - Campus Mieres, Spain

⁴Centro de Investigaciones Biológicas (CIB-CSIC), Calle Ramiro de Maetzu 9, 28040 Madrid Spain. teresa@cib.csic.es.

***Corresponding author:** Dr. Patrick S. Fitze, Department of Biodiversity and Evolutionary Biology, Museo Nacional de Ciencias Naturales (MNCN-CSIC), José Gutiérrez Abascal 2, 28006 Madrid, Spain. Phone: +34 914 11 13 28, ext. 981225. E-mail: patrick.fitze@mncn.csic.es

Abstract

Complex traits include, among many others, the evolution of eyes, wings, body forms, reproductive modes, human intelligence, social behaviour, diseases, and chromosome morphology. Dollo's law states that the evolution of complex traits is irreversible. However, potential exceptions have been proposed. Here, we investigated whether reticulation, a simple and elegant means by which complex characters may be reacquired, could account for suggested reversals in the evolution of complex characters using two datasets with sufficient genetic coverage and a total of five potential reversals. Our analyses uncovered a potential reversal in the evolution of parity mode and a potential reversal in the evolution of placentotrophy of fish (Cyprinodontiformes) as reticulation events. Moreover, in a reptile that exhibits a potential reversal from viviparity to oviparity (*Zootoca vivipara*), reticulation provided the most parsimonious explanation for sex chromosome evolution. Therefore, three of the five studied potential reversals were unravelled as reticulation events. This constitutes the first evidence that accounting for reticulation can fundamentally influence the interpretation of the evolution of complex traits, that testing for reticulation is crucial for obtaining robust phylogenies, and that complex ancestral characters may be reacquired through hybridization with a lineage that still exhibits the trait. Hybridization, rather than re-appearance of ancestral traits by means of small evolutionary steps, may thus account for suggested exceptions to Dollo's law. Consequently, ruling out reticulation is required to claim evolutionary reversal of complex characters and potential exceptions to Dollo's rule.

Keywords: reticulation, complex traits, evolution, placentotrophy, parity mode evolution, evolution of chromosome morphology.

The evolution of complex traits has fascinated evolutionary biologists for over a century (Darwin, 1859). Dollo (Dollo, 1893) proposed that “organisms cannot return, even partially, to a previous state already realized in its ancestral series”. However, this idea has been highly criticized since its proposition (Gould, 1970). Debate has intensified over the last 15 years following spectacular claims of reversion (Stiassny, 1992) to complex states in the evolution of arthropod eyes (Oakley & Cunningham, 2002), stick insect wings (Whiting, Bradler, & Maxwell, 2003), parasites (Cruickshank & Paterson, 2006), self-incompatibility in plants (Ferrer & Good-Avila, 2007), digit loss in squamate reptiles (Brandley, Huelsenbeck, & Wiens, 2008; Kohlsdorf & Wagner, 2006), coiled shells in gastropods (Collin & Cipriani, 2003), and parity-mode evolution (Griffith et al., 2015; Helmstetter et al., 2016; Pyron & Burbrink, 2014; Surget-Groba et al., 2006). These studies suggested reappearance of long-lost ancestral traits, thus evidence against Dollo’s claim.

Discussion about reversals and Dollo’s law centred around parity-mode evolution (e.g. Blackburn, 2015; Griffith et al., 2015; Helmstetter et al., 2016; Pyron & Burbrink, 2014; Surget-Groba et al., 2006) and it is suggested that the re-appearance of oviparity by means of small evolutionary steps is difficult and highly unlikely (Griffith et al., 2015; Helmstetter et al., 2016; Pyron & Burbrink, 2014; Surget-Groba et al., 2006) once viviparity has evolved, which is in line with Dollo’s claim. The evolution of viviparity is complex because it requires changes in the timing of reproductive events (e.g., embryonic retention) and the evolution of structures that facilitate intrauterine gas exchange between the mother and her offspring. In squamate reptiles, which evolved viviparity many times (e.g. > 100 times in reptiles; Sites, Reeder, & Wiens, 2011), this process also involves the loss of features required to produce eggshells (Griffith et al., 2015), and in mammals the evolution of sophisticated placentas (Moffet & Loke, 2006). The reversal from viviparity

to oviparity is equally complex: reversal to oviparity may have existed because the machinery necessary for oviparous reproduction may have been retained during viviparity and thereafter re-activated, or alternatively, reversal may exist due to the re-appearance of oviparity by means of micro-evolutionary steps (Griffith et al., 2015). However, there is little evidence for the existence of reversals to oviparity (Griffith et al., 2015).

Original claims for reversals from viviparity to oviparity have been made based on the presence of oviparous lineages embedded in viviparous lineages (e.g. deFraipont Clobert & Barbault, 1996; Pyron & Burbrink, 2014), but subsequent work refuted these claims based on analytical problems, data problems (e.g. Lee & Shine, 1998; Shine & Lee, 1999; Blackburn, 1999), and other grounds (Blackburn, 2015; Duchêne & Lanferar, 2015; Griffith et al., 2015; King & Lee, 2015; Mank, Promislow, & Avise, 2005; Parenti LoNostro, & Grier, 2010; Pyron, 2015; Shine, 2015; Wright et al., 2015).

Potential reversals have been detected in other complex traits (Oakley & Cunningham, 2002; Whiting et al., 2003), but discussion about their existence was less intense and for some traits discussion was almost absent (e.g. chromosome morphology, maternal resource transfer). For example, the combination of phylogenetic studies (Horreo et al., 2018a) and studies on the chromosome morphology of *Zootoca vivipara* (Kupriyanova, Kuksin, & Odierna, 2008), suggests that there may exist a reversal in the morphology of the W chromosome, but this hypothesis has not been tested so far. Similarly, phylogenetic analyses of Cyprinodontiformes suggest reversals to oviparity (Helmstetter et al., 2016) and reversals in maternal resource transfer from placentotrophy to lecithotrophy (Pollux, Meredith, Springer, Garland, & Reznick, 2014). In general, reversal of complex traits is considered to be highly unlikely, and it is even less likely that the re-evolution of a long lost character by means of small micro-evolutionary steps would produce the same character state as the one observed in the ancestor (Cruickshank

& Paterson, 2006; Teotónio & Rose, 2000). Alternatively, although never previously investigated, reticulation (i.e., natural hybridization and introgression; Arnold, 1997) could offer an explanation for the observed potential reversals from viviparity to oviparity, the reversal in chromosome morphology, and reversals to lecithotrophy. In the present study, we investigated whether reticulation could explain potential reversals from viviparity to oviparity as well as potential reversals in two other complex characters: W chromosome morphology and maternal resource transfer.

Methods

We searched phylogenetic trees of sauropsids (Pyrón & Burbrink, 2014), amphibians, mammals, and fish (Helmstetter et al., 2016) for potential reversals from viviparity to oviparity, and selected species groups with at least six homologous gene fragments to detect reticulation. While no suitable species groups were found for mammals, amphibians, or sauropsids, the order Cyprinodontiformes fulfilled our search criteria and sequences were obtained from GenBank (Supporting Information Table S1). In addition, an exhaustive search in Cyprinodontiformes provided also information on another complex trait, the capacity of viviparous Cyprinodontiformes to transfer resources to offspring in utero. Placentotrophy allows for post-fertilization maternal provisioning by means of a placenta, while lecithotrophic species lack placentas and allocate all resources necessary for the embryo development to the egg (Pollux et al., 2014).

We additionally selected *Zootoca vivipara*, since models of parity-mode evolution suggest the existence of a reversal from viviparity to oviparity (Surget-Groba et al., 2006) and since the combination between phylogenetic and morphological studies suggest that a reversal may exist in the evolution of chromosome evolution (Horreo et al., 2018a;

(Kupriyanova et al., 2008). *Zootoca vivipara* is the terrestrial squamate species with the largest natural distribution, ranging from northwest Spain and Ireland in the West to the Peninsula of Sakhalin (Russia) and Hokkaido (Japan) in the East, and from the southern Balkan (Bulgaria) and the Pyrenees in the South to the Arctic Circle. It has seven well-defined clades (Surget-Groba et al., 2006), a bimodal reproductive pattern, and is a model species for the study of evolution, population dynamics, behaviour, coloration, and physiology (Fitze & Le Galliard, 2011; Gonzalez-Jimena & Fitze, 2012; Horvathova, et al., 2013; San-Jose, Peñalver-Alcázar, Milá, Gonzalez-Jimena, & Fitze, 2014). Since the existing genetic coverage was not sufficient for the envisaged analyses, we amplified three nuclear (nZV1, nZV2, and nZV3; Horreo, Peláez, Suárez, & Fitze, 2018b) and three mitochondrial gene fragments (16S rDNA, 16S: Clary & Wolstenholme, 1985; cytochrome B, cytB: Smith & Patton, 1991; and NADH dehydrogenase 2, ND2: Macey, Larson, Ananjeva, Fang, & Papenfuss, 1992) in 24 individuals belonging to all extant clades (three individuals per clade; Surget-Groba et al., 2001), except for clade B, from which 9 individuals were used, three per subclade; Horreo et al., 2018a; Mila, Surget-Groba, Heulin, Gosá, & Fitze, 2013) and two outgroups (Supporting Information Tables S2, S3) following original protocols (Horreo et al., 2018b). One specimen per population was used (Supporting Information Table S2). Tissue samples were obtained from previous studies (e.g. Mila et al., 2013) and from collaborators (see acknowledgements). Nuclear fragments exhibited between four and ten haplotypes (Supporting Information Table S4), haplotype diversity ranged from 0.222 (nZV1) to 0.775 (nZV2) and nucleotide diversity from 0.005 (nZV1) to 0.010 (nZV2). Mitochondrial gene fragments (16S, cytB, and ND2) exhibited 11, 18, and 22 haplotypes (Supporting Information Table S4), haplotype diversity ranged from 0.911 (16S) to 0.988 (ND2), and nucleotide diversity from 0.028 (16S) to 0.068 (ND2).

Nuclear and mitochondrial gene fragments of *Z. vivipara* and genes of Cyprinodontiformes were concatenated into a single alignment of 2621 (nuclear genes: 1266 base pairs; mitochondrial genes: 1355 base pairs, for details see Supporting Information Table S4 and alignment) and 5349 base pairs, respectively, following a total evidence approach. Phylogenetic trees were reconstructed in BEAST v.2.3.1 using Bayesian inference assuming a Random Local Clock (RLC) or strict clock model (Bouckaert et al., 2014), under the Hasegawa-Kishino-Yano (HKY) substitution model (in *Z. vivipara*) and the Generalised Time Reversible (GTR) model (in Cyprinodontiformes), both corresponding to the best-fit model evaluated using MetaPiga v.2.0 (Helaers & Milinkovitch, 2010). Molecular calibration of the *Z. vivipara* tree was achieved using the date of the node between the oviparous clade A of *Z. vivipara* and the other clades (4.5 Mya ago, CI95% = 6.1–2.6 Mya) based on geological calibration (Cornetti, Menegon, Giovine, Heulin, & Vernesi, 2014). Several runs, including 20 and 50 million Markov chain Monte Carlo (MCMC) repetitions were needed in the case of Cyprinodontiformes and *Z. vivipara*, respectively, for ensuring convergence, which was tested with Tracer v.1.6 (<http://tree.bio.ed.ac.uk/software/tracer>). In both cases, reticulation was tested using TriLoNet (Oldman, Wu, van Iersel, & Moulton, 2016), which infers reticulated events from constructing rooted phylogenetic level-1 networks from sequence alignments (Supporting Information Text S1). TriLoNet employs trinets and it compares well with Lev1athan (Huber, Moulton, & Wu, 2016), which tends to “overestimate the number of reticulated events as compared with those generated by TriLoNet” (Oldman et al., 2016).

To understand whether reticulation or incomplete lineage sorting (ILS) led to the observed patterns, we estimated the time to most recent common ancestor (TMRCA, time needed to observe complete reciprocal monophyly) and compared it with dating from the

phylogenetic tree (Supporting Information Table S3). To this end we estimated the effective population size (N_e), using the software BEAST under the Coalescent Bayesian Skyline Plot (BSP; Drummond, Rambaut, Shapiro, & Pybus, 2005) inference, and calculated TMRCA under the assumption that ancestral polymorphisms coalesce within approximately $5 N_e$ generations (Degnan & Rosenberg, 2009; Rosenberg, 2003) as well as within a range of $9 N_e g - 12 N_e g$ (being “g” the generation time, which in this species is ≥ 2 years; San-Jose et al., 2014). Incomplete lineage sorting was tested in *Z. vivipara*, since in this intra-specific dataset N_e could be estimated, but not in the inter-specific Cyprinodontiforme dataset.

Results

The topology of the Bayesian phylogenetic tree for Cyprinodontiformes (Fig. 1A), was similar to that previously published by Helmstetter and coworkers (Helmstetter et al., 2016). This tree revealed robust statistical evidence (Bayesian posterior probabilities ≥ 0.95 , Fig. 1) for a potential reversal from viviparity to oviparity in the ancestor of *Tomeurus gracilis* and for reversals from lecithotrophy to placentotrophy in the ancestor of *Pamphorichthys hollandi* and the ancestor of *Poeciliopsis elongate*. The phylogenetic positions of *Phalloceros caudimaculatus* and *Oxyzygonectes dovii* were unresolved (no significant statistical support in Fig.1, and no reversal according to Helmstetter et al., 2016) and thus no statistical support existed for a reversal to placentotrophy and oviparity, respectively.

Consequently, two possible scenarios of parity mode evolution exist: (i) a single evolution of viviparity (in the ancestor of Anableps and the violet cluster in Fig. 1A) and two potential reversals from viviparity to oviparity, in the ancestor of *Tomeurus gracilis*

and in the ancestor of *Oxyzygonectes dovii*, or (ii) multiple evolution of viviparity (i.e. at least three times, in the ancestor of *Jenynsia* and *Anableps*, in the ancestor of *Xenodexia*, and in the ancestor of the other viviparous species – the ancestor of the violet cluster in Fig. 1A). Testing for reticulation using TriLoNet (Oldman et al., 2016: see Supporting Information Text S1 for detailed methodological details) revealed three clusters in the level-1 network (Fig. 1B): the grey cluster including *Cyprinodon* and *Jordanella*, the green cluster including *Fluviphylax*, *Aplocheilichthys*, *Oxyzygonectes*, *Jenynsia*, *Anableps*, and *Xenodexia*; and the violet cluster including the remaining species (Fig. 1B). These clusters coincided with the clusters of the Bayesian phylogeny (Fig. 1A). The level-1 network further identified two reticulated sequences belonging to *Tomeurus gracilis* and *Pamphorichtys hollandi* (Fig. 1B).

The former, has direct implications for the evolution of viviparity because the RCL tree shows that *T. gracilis* is an oviparous species placed within viviparous species (Fig. 1A), which suggests a reversal to oviparity, while the reticulation scenario suggests that viviparity evolved once (in the ancestor of *Jenynsia* and the other viviparous species) and the reversal constitutes a hybridization between the viviparous and an oviparous lineage (Fig. 1B).

The latter, has direct implications for the evolution of lecithotrophy because the phylogenetic tree (Fig. 1A) suggests (i) a single evolution of lecithotrophy (in the ancestor of the violet cluster in Fig. 1A) and two to three reversals to placentotrophy (in the ancestors of *P. hollandi* and *P. elongate*, and potentially in the ancestor of *P. caudimaculatus*), or alternatively, (ii) multiple evolution of lecithotrophy (i.e. at least seven times: in the ancestor of *G. metallicus*, *C. decemmaculatus*, *M. picta*, *L. dominicensis*, the ancestor of *Priapella* and *Xiphophorus*, *N. tridentiger*, and the ancestor of *Pseudopecilia* and *Phallichthys*, Fig. 1A). In contrast, the reticulation scenario suggests

that *P. hollandi* originates from reticulation between a placentotrophic and a lecithotrophic lineage (Fig. 1B). Consequently, the observed patterns may be explained by (i) a single evolution of lecithotrophy and one potential reversal to placentotrophy (in the ancestor of *P. elongata*), or (ii) multiple evolution of lecithotrophy (i.e. four times: in the ancestor of *Cnesterodon* and *Limia*, in the ancestor of *Priapella* and *Xiphophorus*, in the ancestor of *Pseudopoecilia* and *Phallichthys*, and in the ancestor of *Neoheterandria*, Fig. 2B).

The topology of the *Z. vivipara* phylogenetic tree derived from Bayesian inference and a RLC model (Fig. 2A) was identical to that previously published (Surget-Groba et al., 2006). In contrast to previous studies, posterior probabilities were maximal for all nodes (Fig. 2A) and all samples belonging to a given clade belonged to the same lineage (Fig. 2A, lineages are indicated with colours). This tree reveals two possible scenarios of parity mode evolution: (i) a single evolution of viviparity (node C) and a potential reversal from viviparity to oviparity (node F), in the ancestor of clade B, or (ii) multiple evolution of viviparity (i.e. three times, in nodes H, I, J; Fig. 2A). The level-1 network revealed seven clusters (Fig. 2B, clusters are represented with different colours), corresponding to the outgroup, clade A, B, C, D, E, and F (Fig. 2B). These clusters are congruent with the lineages detected by the Bayesian phylogeny (Fig. 2A). The level-1 network further unravelled three reticulation events (Fig 2B): a) the entire clade F resulted from reticulation between a parent belonging to a subtype of the viviparous clade D and a parent belonging to a subtype of the oviparous clade A; reticulation existed b) within clade A (green cluster) and c) within the Ibero-Pyreanean clades (note: excluding the reticulated species B1_09_503; Oldman et al., 2016 led to two clades within the violet Ibero-Pyreanean clade, consisting of a cluster containing B2 and a cluster containing B3 and B1; which is congruent with the Bayesian phylogeny; see also Supporting

Information Text S1). The network thus suggests that viviparity evolved once and that clade B might be a reversal.

A literature search on *Z. vivipara* returned information for all extant clades on two additional complex traits: egg retention stage, and the female sex chromosome (W) morphology, which is linked to the sex chromosome system. Egg retention stage was uninformative since all viviparous clades exhibit egg retention stage 40. However, variability was observed for the W chromosome morphology. Clades A and F exhibit a ZW system with 34 autosomes, while clades B, C, D, and E exhibit a Z_cZ_cW system with 32 autosomes, caused by fusion of the ninth autosome with the W chromosome (Kupriyanova et al., 2008). The evolution of the W chromosome morphology was reconstructed using both the RLC tree topology (Figs. 3A,B) and the topology resulting from the level-1 network (Fig. 3C). Applying the RLC topology, the most parsimonious scenario requires five evolutionary shifts, with two possible alternatives (Figs. 3A,B). The first involves fusion of the ninth autosome with the W chromosome and subsequent un-fusing of the ninth autosome from the W chromosome in the same region in which it previously fused in an ancestor of the current clade F (Fig. 3A). The second possibility requires two identical fusions of the ninth autosome with the W chromosome (Fig. 3B). The topology resulting from the level-1 network requires four evolutionary shifts (Fig. 3C), and includes a single fusion of the ninth autosome with the W chromosome. The RCL tree thus suggests (i) fusion of the ninth autosome with the W chromosome and a reversal to the ancestral chromosome morphology (Fig. 3A), or alternatively, (ii) two, identical fusions of the ninth autosome with the W chromosome (Fig. 3B). In contrast, the reticulation scenario suggests a single fusion of the ninth autosome with the W chromosome, and a reticulation between a lineage with fused autosome and a lineage, which conserved the ancestral W-chromosome morphology (Fig. 3C).

To understand whether reticulation or incomplete lineage sorting (ILS) may have led to the observed W-chromosome morphology in clade F, the TMRCA was estimated. According to coalescence theory, ancestral polymorphisms are likely to coalesce within approximately $5N_e$ generations (Rosenberg, 2003), leading to a TMRCA of node C (Fig. 2A) of 18.52 Mya (6.14-92.69; 95% CI), i.e. of the node including the reticulation event (Fig. 2B). Less conservative coalescence time estimates ($18N_e$ and $24N_e$; Hudson & Coyne, 2002) estimated a TMRCA of 66.67 Mya (22.09-333.79; 95% CI) and 88.90 Mya (29.45- 444.92; 95% CI). Consequently, 95% CI of TMRCA did not overlap with the dating of node C (2.52 Mya, 2.11-2.55; 95% CI; Supporting Information Table S3).

Testing heterotachy, provided evidence for its existence in Cyprinodontiformes and *Z. vivipara* (Figs. 1, 2). Heterotachy was detected in all but one branches in which reticulation existed (see Supporting Information Text S2 for methodological details).

Discussion

Here we tested, whether reticulation may explain potential reversals of complex traits, using two datasets with potential reversals in parity mode evolution that exhibited sufficient genetic coverage and reversals in other complex traits (W chromosome morphology and maternal resource transfer). Contrary to Dollo's law (1983), which states "that organisms cannot return, even partially, to a previous state already realized in its ancestral series", the here derived phylogenetic trees for Cyprinodontiformes (Fig. 1A) and *Z. vivipara* (Fig. 2A) point to three reversals with statistical support in Cyprinodontiformes (a reversal to oviparity in the ancestor of *T. gracilis* and two reversals to placentotrophy in the ancestors of *P. hollandi* and *P. elongate*; Fig. 1), which are congruent with conclusions from previous studies (Helmstetter et al., 2016; Pollux et al., 2014), and two reversals in *Z. vivipara* (one reversal to oviparity in the

ancestor clade B and a reversal to the ancestral chromosome morphology in clade F; Fig. 2A), which is also congruent with previous conclusions (Horreo et al., 2018a).

In both datasets, testing for reticulation unravelled several reticulations (Figs. 1B, 2B). In Cyprinodontiformes, the level-1 network unravelled *T. gracilis* and *P. hollandi* as a reticulation. The former, resulting from a hybridization between a viviparous and an oviparous parent, which may explain why it shares reproductive characters with both oviparous and viviparous taxa (Parenti, LoNostro, & Grier, 2010), and the latter resulting from a hybridization between a placentotrophic and lecithotrophic parent. In *Z. vivipara*, the level-1 network unravelled clade F as a reticulation (Fig. 2C) between a lineage with fused autosome and a lineage, which conserved the ancestral W-chromosome morphology (Fig. 3C). The reticulate origin of clade F coincides with a recently published study pointing to important past migration events in clade F (Recknagel, Kamenos, & Elmer, 2019; Fig S4D-F see CV2: central viviparous clade, which corresponds to clade F, Surget-Groba et al., 2006).

Since reticulation creates a short-cut with respect to the commonly assumed evolution by means of small evolutionary steps (Ridley, 2004), the evolutionary rate of character displacement will differ in the reticulated branches. In both Cyprinodontiformes and *Z. vivipara*, heterotachy was detected in all branches with evidence for reticulation (Figs. 1A,2A; Supporting Information Text S2 for methodological details), being in line with predictions from reticulation. Alternatively, the suggested reticulations might be explained by incomplete lineage sorting (ILS; Linder C. & Rieseberg, 2004). The estimation of TMRCA showed that the TMRCA estimated under ILS was significantly longer. In other words, ILS would need much more time to generate the observed genetic diversity. Consequently, it is improbable that ILS instead of reticulation explains the observed patterns.

Our analyses thus support that three of the five reversals may exist due to reticulation with lineages that conserved the ancestral trait. For the other two reversals (reversal to placentotrophy in the ancestor of *P. elongata*, and reversal to oviparity in the ancestor of clade B of *Z. vivipara*), our analyses do not provide evidence for reticulation. However, analyses based on a single homologous genomic region, even in the case of representative genes (Horreo, 2012), or based on a few genes are unlikely to reveal reticulation, and detection of reticulation depends on the amount of genetic information and the analysed regions (Edwards, Potter, Schmitt, Bragg, & Moritz, 2016). For the studied datasets, only six homologous gene fragments were available, and thus the probability to detect reticulation was low. Thus, in these two reversals reticulation cannot be ruled out unless genome-wide analyses with high coverage provide no evidence for reticulation (Edwards et al., 2016). While for the Cyprinodontiformes no genomic analyses were available, for *Z. vivipara* genomic analyses based on 1.3 million base pairs (approx. 200'000 SNP loci) were congruent with a reversal from viviparity to oviparity in clade B (Recknagel et al., 2019) and they did not unravel important past migration (Fig. S4 in Recknagel et al., 2019; WOV corresponds to clade B). Consequently, only one of the here studied potential reversals may have originated by means of small evolutionary steps (reversal to oviparity in clade B), three reversals may exist due to reticulation, and one reversal is of unknown origin and requires future investigation (*P. elongate*). These results have important implications for Dollo's law and the evolution of complex characters, since they suggest that less violations of Dollo's law may exist than previously thought (e.g. Pollux et al., 2014). Moreover, oviparity of *Z. vivipara*'s clade B may not necessarily reflect a reversal to "a previous state already realized in its ancestral series" (Dollo, 1893), since it exhibits important differences in eggshell thickness (Heulin, Ghiemi, Vogrin, Surget-

Groba, & Guillaume, 2002) and embryonic developmental stage at egg laying (Heulin, Arrayago, Bea, & Braña, 1992; Heulin et al., 2002) compared to the ancestral oviparous clade A, which is in line with the low likelihood that the re-evolution of long lost characters by means of small micro-evolutionary steps would produce the same character state as the one observed in the ancestor (Cruickshank & Paterson, 2006; Teotónio & Rose, 2000).

The reticulation scenarios also point to less complex evolutionary events, namely: reticulation instead of reversal by means of small evolutionary steps in the ancestors of *T. gracilis* and *P. hollandi*; and reticulation and a single fusion of the ninth autosome with the W chromosome, instead of two almost identical fusions, or fusion of the ninth autosome and unfusion in the same region (Fig. 3A,B). While transitions from oviparity and viviparity or from viviparity back to oviparity require anatomical, physiological, biochemical and endocrinological changes (Griffith et al., 2015), reticulation does not require the complex process of re-appearance of ancestral traits by means of small evolutionary steps or retaining of the genetic machinery by the same specimens. In contrast, reticulation abbreviates this complex process because the transition is acquired through hybridization, which facilitates bigger changes over small evolutionary time intervals (e.g. step-wise evolution; Ross et al., 2014).

Conclusions

Hybridization with a lineage that still exhibits the ancestral trait can lead to the re-appearance of ancestral traits in a descendant. Our analyses provide evidence that three of the five studied reversals may constitute a hybridization with a lineage that still exhibited the ancestral trait. Interestingly, the two lineages involved in the reticulation leading to *T. gracilis* and *P. hollandi* and the two lineages involved in the reticulation

giving raise to *Z. vivipara*'s clade F split several million years (>10 and > 2 million years) before the reticulation event happened, and the first mentioned lineages belong to different genera and families (Helmstetter et al., 2016). This indicates that reticulation may not only be possible among recently diverged species (Mallet, Besansky, & Hahn, 2016). Moreover, the exceeding rarity of phylogenetically distant reversals (Coyne & Orr, 1997; Griffith et al., 2015), might be explained by the fact that reticulation is more likely among closely related species than among more distant species (Mallet et al., 2016), and also among species exhibiting bigger differences in complex traits. These results provide evidence that reticulation may have played an important role in the evolution of complex characters, and in the evolution of vertebrates. Moreover, they stress that reticulation needs to be considered when evaluating evolutionary reversals and evidence for or against Dollo's law. Finally, accepting the existence of evolutionary reversals requires ruling out reticulation by means of datasets with high genomic coverage.

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Data accessibility statement

Original data used in this study will be archived in a public repository upon acceptance.

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Figure legends

Figure 1. Phylogenetic relationship assuming absence (A) and potential presence (B) of reticulation in Cyprinodontiformes. A) Bayesian phylogeny generated in BEAST v.2.3.1, assuming a random local clock, a birth-death model, and a general GTR sequence evolution model. Node numbers indicate posterior probabilities. Stars indicate branches with significant heterotachy ($pp \geq 0.98$). B) Phylogenetic level-1 network inferred by TriLoNet.

Differently shaded areas delimitate species belonging to different clusters. Non-shaded species correspond to reticulated species. Eggs and fishes to the right of the species name correspond to oviparous and viviparous species, respectively. Red and blue dots to the right of the species name represent lecithotrophic species with a matrotrophy index (MI) <1 and placentalotrophic species with an MI >1 , respectively (Knight, Lombardi, Wourms, & Burns, 1985; Turner, 1933). Eggs and fishes and red dots at ancestral nodes correspond to significant ($pp \geq 0.98$) ancestral state reconstructions using BayesTraits 2.0 (Pagel, Meade, & Barker, 2004).

Figure 2. Phylogenetic relationship assuming absence (A) and potential presence (B) of reticulation for specimens belonging to all extant *Z. vivipara* clades and the outgroups *L. bilineata* and *P. muralis*. A) Bayesian phylogeny, assuming a random local clock with node numbers indicating posterior probabilities. Stars indicate branches with significant heterotachy ($pp \geq 0.98$). The most important nodes are indicated with letters A to G, and their estimated age is provided in the Supporting Information Table S3. Node A corresponds to the ancestor of *Z. vivipara* and the outgroups. The scale bar corresponds to time before present in million years (Mya ago). B) Phylogenetic level-1

network inferred by TriLoNet with the reticulated individual VAU6A removed (see Supporting Information Text S1).

Differently shaded areas delimitate species belonging to different clades and clusters. Eggs and lizards to the right of or above the sample code correspond to oviparous and viviparous specimens and clades, respectively. Sample codes refer to the *Z. vivipara* clade (green: A; violet: B1, B2, and B3; orange: C; light blue D; dark blue: E; and pink: F), followed by the sample name. ZW and Z₁Z₂W denominate the sex chromosome system.

Figure 3. Schematic reconstruction of the evolution of the W chromosome morphology in *Z. vivipara*, assuming that reticulation does not (A, B) or does exist (C). Figure (A, B) shows the evolution of the female's C-banded W chromosome according to the phylogenetic topology generated using the Bayesian-RLC approach (Fig. 2A) and (C) the topology when accounting for reticulation (Fig. 2B). The clades' W chromosome morphology was derived from the literature (Kupriyanova et al., 2008). Ancestral states were reconstructed based on the parsimony principle and all equally parsimonious scenarios are plotted (A, B). Clades/subclades and parity mode are indicated above the chromosomes. Parity mode is represented by eggs (oviparity) and lizards (viviparity). All evolutionary steps required to explain the W chromosome morphology according to the tree topology are indicated. *A. eryth* depicts the outgroup and refers to the closest species with known W chromosome morphology: *Acanthodactylus erythrurus*.

667 **Figure 1**

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670 **Figure 2**

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Supporting Information

Text S1. Testing for reticulation.

Text S2. Testing for heterotachy in Cyprinodontiformes and *Zootoca vivipara*.

Table S1. GenBank accession numbers for the 6 genomic regions (enc1, myh6, ND2, pomgnt2, rag1, and x-src) used to test the effects of reticulation on complex character evolution in Cyprinodontiformes. Accession numbers and reproductive modality (Rep: ovi, oviparity; vivi, viviparity) of the fish species studied are provided.

Table S2. List of the studied *Zootoca vivipara* specimens. Clade, subclade, reproductive mode (Repro_Mode), sample name, population name, and geographic location (country) are shown for each specimen. Populations and clades are named as in Surget-Groba et al. (20016) and Mila et al. (2013), and subclades as in Horreo et al. (2018a).

Table S3. Molecular dating of nodes shown in Fig 2. Average age, 95% confidence intervals (CI95%), and ancestral state for each node are shown in Mya.

Table S4. Genetic variability of the selected nuclear sequences measured in the studied *Zootoca vivipara* specimens. The number of haplotypes (Nh), haplotype diversity (Hd), and nucleotide diversity (Nd), as determined using DNAsp v.5.10.1 are shown. bp, sequence length in base pairs; S, number of variable sites; Pos: position (in bp) of the gene within the alignment.

Fig. S1. Phylogenetic level-1 network inferred by TriLoNet including all sampled *Z. vivipara* specimens.

Fig. S2. Bayesian phylogeny, assuming a strict clock, for the Cyprinodontiformes

shown in Figure 1. Node numbers correspond to posterior probabilities, and species

names are followed by reproductive mode (OVI, oviparous; VIVI, viviparous).

Fig. S3. RLC Beast tree including all non-reticulated Cyprinodontiformes species.

Node numbers correspond to posterior probabilities, and species names are followed by

reproductive mode (OVI, oviparous; VIVI, viviparous).

Fig. S4. Bayesian phylogenetic tree, assuming a strict clock, for specimens

belonging to all extant *Z. vivipara* clades and the outgroups *L. bilineata* and *P.*

***muralis*.** Node numbers correspond to posterior probabilities. Sample codes refer to *Z.*

vivipara clades (A, B, C, D, E and F) and subclades (B1, B2, B3), followed by the

sample name.

S1 Text. Testing for reticulation. Reticulation was tested using TriLoNet (Oldman, Wu, van Iersel, & Moulton, 2016). TriLoNet constructs rooted phylogenetic level-1 networks using sequence alignments (important for the theory of phylogenetic networks; Yu, Dong, Liu, & Nakhleh, 2014), which aim to represent reticulated events that correspond to generalizations of evolutionary trees (Oldman et al., 2016), TriLoNet renders results similar to those generated in Lev1athan (Huber, Moulton, & Wu, 2016), which tends to “overestimate the number of reticulated events as compared with those generated by TriLoNet” (Oldman et al., 2016). However, some care needs to be taken when interpreting clusters with sequences involved in a reticulation event (Oldman et al., 2016), especially in the presence of reticulate vertices; i.e. vertices with two parents in a cycle. In this case, removal of the reticulated sequence is required to unravel cluster affiliation of the different specimens (Oldman et al., 2016); in our case of the sampled specimens to different clades (Fig. 2B). The level-1 network including all specimens revealed 5 clusters (S1 Fig), corresponding to the outgroup (grey cluster), to clade A (green), to clades D and E and one specimen belonging to clade C (blue), to the Ibero-Pyrenean oviparous clades and one sample belonging to clade C (violet), and to clade F (pink cluster; S4 Fig). The level-1 network revealed a reticulate vertex with C-VAU6A being a recombinant sequence resulting from reticulation between a parent belonging to the oviparous Ibero-Pyrenean lineage (violet cluster) and a parent belonging to the viviparous lineage (blue cluster consisting of clade C, D and E; S1 Fig). Moreover, C appeared as a leaf in two different clusters (blue and violet cluster). To unravel the cluster affiliation of clades B1, B2, B3, C, D, and E, removal of the reticulated sequence C-VAU6A is required (Oldman et al., 2016). This new analysis unraveled 7 clusters (Fig. 2B). Three clusters were identical in both analyses: (cluster 1: corresponding to the outgroup, 2: corresponding to clade A, and 5: corresponding to clade F). The blue clade (S1 Fig, cluster 3), split into

clade D (light blue) and clade E (dark blue; Fig. 2B), and the specimens belonging to clade C clustered together (Fig. 2B, orange cluster), which is congruent with the clades detected by the Bayesian phylogeny (Fig. 2A).

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S2 Text. Testing for heterotachy in Cyprinodontiformes and *Zootoca vivipara*. As demonstrated by King & Lee (2015), Bayesian analyses must account for rate heterogeneity; failure to account for variable evolutionary rates can result in highly anomalous and even incorrect results (King & Lee, 2015). Specifically, failure to consider heterotachy can lead to differences in tree topology and/or node support (King & Lee, 2015). We assessed the effects of heterotachy on phylogenetic relationships by comparing a Bayesian phylogenetic tree with a strict clock model (Supplementary Figs. S1, S3) with the corresponding RLC tree (Figs. 1, 2). Accounting for heterotachy in Cyprinodontiformes revealed 4 nodes with posterior probabilities < 0.99 , and thus little or no statistical support (Fig. 1). Not accounting for heterotachy revealed 6 nodes with little or no statistical support (S2 Fig). Moreover, excluding both reticulated species from the analyses resulted in full support ($pp = 1$) of all nodes (S3 Fig) and a topology similar to that of the RLC tree including the reticulated species (Fig. 1). Models directly testing for heterotachy (Pagel & Meade, 2008) revealed heterotachy in all terminal branches of species where reticulation has been detected by TriLoNet (*Tomeurus gracilis* and *Pamphorichthys hollandi*), and in many other branches of the tree (Fig. 1).

In *Z. vivipara*, models accounting for heterotachy rendered full support for all nodes ($pp = 1$, Fig. 2), not accounting for heterotachy provided no statistical support for the node of the ancestor of clades B and C (S4 Fig). Models directly testing for heterotachy (Pagel & Meade, 2008) revealed heterotachy in the branch leading to the ancestor of clade F (*i.e.*, the branch in which reticulation existed), and in the branches leading to the ancestor of clade E, the ancestor of clade D, and the ancestor of clade B2 (Fig. 2).

More evidence existed for heterotachy than for reticulation (Fig. 1, 2), and the tree topology close to the reticulation event (but not in other areas of the tree) changed when heterotachy was taken into account. Thus, for Cyprinodontiformes the topology changed

close to the root (Fig. 1 and S2 Fig), and for *Z. vivipara* the topology changed close to node D (Fig. 2 and S4 Fig.). This indicates that method-dependent topology changes can be indicative of reticulation, but the mere existence of heterotachy does not enable pinpointing of reticulation events. However, in Cyprinodontiformes, the second reticulation event (producing *Pamphorichthys hollandi*) did not alter tree topology (Fig. 1, S2 Fig) and no heterotachy was detected within clade A (Fig. 2). Consequently, reticulation should be tested in all regions of all trees. The tree topology changes also stress that testing for reticulation is inevitable to produce robust phylogenetic trees.

References

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S1 Table. GenBank accession numbers for the 6 genomic regions (enc1, myh6, ND2, pomgnt2, rag1, and x-src) used to test the effects of reticulation on complex character evolution in Cyprinodontiformes. Accession numbers and reproductive modality (Rep: ovi, oviparity; vivi, viviparity) of the fish species studied are provided.

Species	Rep	enc1	myh6	ND2	pomgnt2	rag1	x-src
<i>Jordanella floridae</i>	ovi	KJ696901	KJ697121	AP006778	KJ697011	KF141266	KJ697600
<i>Cyprinodon variegatus</i>	ovi	KJ696885	KJ697105	AF449344	KJ696995	KF141215	KJ697585
<i>Oxyzygonectes dovii</i>	ovi	KJ696922	KJ697142	AF449340	KJ697032	EF017407	KJ697621
<i>Anableps anableps</i>	vivi	KJ696870	KJ697089	AF449341	KJ696979	EF017405	KJ697572
<i>Jenynsia lineata</i>	vivi	KJ696899	KJ697119	EF017559	KJ697009	EF017406	KJ697598
<i>Aplocheilichthys normani</i>	ovi	KJ696873	KJ697093	KJ697200	KJ696983	KJ697272	KJ697575
<i>Fluviphylax simplex</i>	ovi	KJ696888	KJ697108	KJ697210	KJ696998	KJ697280	KJ697587
<i>Tomeurus gracilis</i>	ovi	KJ696969	KJ697189	EF017607	KJ697079	EF017455	KJ697668
<i>Carllhubbsia stuarti</i>	vivi	KJ696879	KJ697099	EF017581	KJ696989	EF017430	KJ697580
<i>Cnesterodon decemmaculatus</i>	vivi	GU179168	GU179243	EF017579	GU179197	EF017427	GU179152
<i>Girardinus metallicus</i>	vivi	KJ696895	KJ697115	EF017593	KJ697005	EF017441	KJ697594
<i>Limia dominicensis</i>	vivi	GU179170	GU179245	EF017582	GU179199	EF017431	GU179154
<i>Micropoecilia picta</i>	vivi	GU179177	GU179251	EF017586	GU179206	EF017435	GU179161
<i>Neoheterandria tridentiger</i>	vivi	KJ696920	KJ697140	EF017576	KJ697030	EF017423	KJ697619
<i>Pamphorichthys hollandi</i>	vivi	HQ857470	HQ857458	EF017587	HQ857464	HQ857446	HQ857434
<i>Phallichthys amates</i>	vivi	KJ696923	KJ697143	EF017563	KJ697033	EF017410	KJ697622
<i>Phalloceros caudimaculatus</i>	vivi	KJ696926	KJ697146	EF017578	KJ697036	EF017426	KJ697625
<i>Poeciliopsis elongata</i>	vivi	KJ696944	KJ697164	AF412172	KJ697054	KJ697327	KJ697643
<i>Priapella compressa</i>	vivi	KJ525891	KJ525851	EF017603	KJ525871	EF017451	KJ525911
<i>Priapichthys annectens</i>	vivi	KJ696961	KJ697181	EF017591	KJ697071	EF017439	KJ697660
<i>Pseudopoecilia festae</i>	vivi	KJ696965	KJ697185	EF017592	KJ697075	EF017440	KJ697664
<i>Xenodexia ctenolepis</i>	vivi	KJ696971	KJ697191	EF017606	KJ697081	EF017454	KJ697670
<i>Xenophallus umbratilis</i>	vivi	KJ696973	KJ697193	EF017577	KJ697083	EF017424	KJ697672
<i>Xiphophorus hellerii</i>	vivi	KJ525879	KJ525839	EF017597	KJ525859	EF017445	KJ525899

S2 Table. List of the studied *Zootoca vivipara* specimens. Clade, subclade, reproductive mode (Repro_Mode), sample name, population name, and geographic location (country) are shown for each specimen. Populations and clades are named as in Surget-Groba et al., (2006) and Mila et al., (2013), and subclades as in Horreo et al., 2018).

Clade	Subclade	Repro_Mode	Sample	Population	Country
A	1	Oviparous	OI13A	ALB	Italy
A	1	Oviparous	OI27A	VLT	Italy
A	1	Oviparous	OI40A	SAN	Italy
B	3	Oviparous	09-585	Burgos	Spain
B	3	Oviparous	AG09201	Irun	Spain
B	3	Oviparous	BUG1	BUG	France
B	2	Oviparous	SS1	SS1	France
B	2	Oviparous	OF30A	PIN	France
B	2	Oviparous	CET1	CET	France
C		Viviparous	VAU2B	WEI	Austria
C		Viviparous	VAU6A	SEM	Austria
C		Viviparous	ZM2	Moosbrunn	Austria
D	1	Viviparous	VR2A	ALT	Russie
D	1	Viviparous	S26	Överkalix	Sweden
D	3	Viviparous	VH5E	BRT	Hungary
E	1	Viviparous	VP1	Wurzen	Germany
E	1	Viviparous	VF27A	AUT	France
E	1	Viviparous	VI18A	PRA	Italy
F	2	Viviparous	VU3	Izsak	Hungary
F	2	Viviparous	VH4D	OSC	Hungary
F	2	Viviparous	VH4E	OSC	Hungary
B	1	Oviparous	09-503	Lugo	Spain
B	1	Oviparous	09-532	Somiedo	Spain
B	1	Oviparous	OE8B	ANC	Spain

S3 Table. Molecular dating of nodes shown in Fig. 2. Average age, 95% confidence intervals (CI95%), and ancestral state for each node are shown in Mya.

Node	Average Age	Upper CI95%	Lower CI95%	Ancestor of Clades
A	5.04	4.63	19.31	
B	4.41	4.19	4.72	<i>Z. vivipara</i>
C	2.88	2.51	3.04	B-F
D	2.52	2.11	2.55	B, C, F
E	2.28	1.96	2.41	B, C
F	1.70	1.24	2.01	B
G	0.93	0.70	1.01	B3 & B1
H	0.72	0.34	0.88	C
I	0.33	0.14	0.40	F
J	2.52	2.01	2.83	D and E
K	0.91	0.68	1.09	A

S4 Table. Genetic variability of the selected nuclear sequences measured in the studied *Zootoca vivipara* specimens. The number of haplotypes (Nh), haplotype diversity (Hd), and nucleotide diversity (Nd), as determined using DNAsp v.5.10.1, are shown. bp, sequence length in base pairs; S, number of variable sites. Pos: position (in bp) of the gene within the alignment.

Gene	bp	S	Nh	Nd	Hd	Pos
nZV1	286	14	4	0.00445	0.222	1-286
nZV2	512	37	10	0.00987	0.775	287-798
nZV3	468	22	8	0.00729	0.662	799-1266
16S	439	80	11	0.02761	0.911	1267-1705
cytB	379	99	18	0.04449	0.972	1706-2084
ND2	537	196	22	0.068	0.988	2085-2621

893 **Figure S2**

897 **Figure S3**

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