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The Urogenital System of the Male *Rhyacotriton* and the Evolution of the Male Urogenital Duct System in Salamanders

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Abstract—Between 1945 and 1967 a series of articles on the male urogenital anatomy of salamanders was published in the *Journal of the Tennessee Academy of Science*. These manuscripts covered morphological aspects of all salamander families except for the Dicamptodontidae and Rhyacotritonidae. Here, we provide data on the reproductive biology of *Rhyacotriton* and describe the morphology of the epididymal complex of the genital kidney and the collecting ducts of the pelvic kidney. We then coded characteristics of male salamander urogenital structures and compared the urogenital anatomy of salamanders in a phylogenetic context. The genital kidney of the Rhyacotritonidae is characterized by a number of characteristics most similar to that of Sirenidae (i.e., the testis communicates with the Wolffian duct via a sequential arrangement of vasa efferentia, renal corpuscles, and efferent epididymal ducts; no longitudinal duct or afferent epididymal ducts were observed), many of which have not been described previously in other salamanders. Despite the similarity of the genital kidney to that of sirenids, the pelvic kidney of the Rhyacotritonidae is most similar to that of the Cryptobranchidae and the Hynobiidae (i.e., collecting ducts that communicate individually with the cranial portion of the cloaca), a condition that could be considered homoplastic in the context of our current understanding of salamander phylogeny. In culmination, male urogenital duct structures support a monophyletic Salamandroidea; however, support weakens with the exclusion of male cloacal morphology.

Introduction

Detailed anatomical descriptions exist on the cloaca for all families of salamanders (Sever, 1991a). A similar statement can be made about the male genital and urinary ducts with the exclusion of the Rhyacotritonidae (Chase, 1923; Yamagiwa, 1924; Francis, 1934; Baker, 1945; Baker and Taylor, 1964; Baker, 1965; Ratcliff, 1965; Willett, 1965; Strickland, 1966; Rosenquist and Baker, 1967), although urogenital characteristics of the Dicamptodontidae are also limited (de Marco, 1952). In general, the urogenital ducts of salamanders can be divided into three distinct units: 1) genital kidney, 2) pelvic kidney, and 3) cloaca. In the genital kidney, the testes communicate with the Wolffian ducts through a set of ducts that are modified nephrons with variable morphologies (for review see Williams et al., 1984). The most complex condition, found in the Ambystomatidae, Hynobiidae, Proteidae, Salamandridae, and Sirenidae, consists of the following ducts leading from the testes sequentially: the vasa efferentia, Bidder's duct (longitudinal collecting duct), afferent epididymal ducts, and efferent epididymal ducts. A renal corpuscle is often found in between the afferent and efferent epididymal ducts. The simplest condition utilizes only transverse tubules from the testes to the Wolffian ducts and is present only in the Eurycinae (Williams et al., 1984). Thus, in all salamanders, the nephrons of the genital kidney are associated with sperm transport.

The pelvic kidney is composed of nephrons that function in urine formation, and possibly as secondary sexual characteristics in some salamanders (i.e., the Ambystomatidae, Plethodontidae, and Salamandridae; Aron, 1924; Adams, 1940; Norris, 1987; Siegel et al., 2010; Siegel et al., in press). Nephrons empty into collecting ducts that exit the kidney and communicate with the Wolffian duct or the cloaca. The collecting ducts communicate with the Wolffian ducts along the entire length of the pelvic kidneys in the Proteidae (Chase, 1923; Rosenquist and Baker, 1967) and Sirenidae (Willett, 1965). In all other salamanders the collecting ducts bend caudally during development (Rodgers and Risley, 1938) and either anastomose and enter the cloaca (in the Plethodontidae and some Salamandridae; Francis, 1934; Baker, 1965; Strickland, 1966), anastomose and enter Wolffian ducts (in the Ambystomatidae, Amphiumidae, and some Salamandridae; Baker, 1945; Baker and Taylor, 1964; Baker, 1965), or enter the cloaca individually (in the Cryptobranchidae and Hynobiidae; Ratcliff, 1965; Yamagiwa, 1924).

As observed in the gonoducts, great variation exists in the male cloaca of salamanders, and this variation is mainly due to the presence or absence of different cloacal glands. Cloacal gland morphology has been examined extensively (Sever, 1991a, b; 1992a–d). In general terms, male salamanders with external fertilization (i.e., the Cryptobranchidae, Hynobiidae, and Sirenidae) have few or no cloacal glands whereas salamanders with internal fertilization (all other salamanders; i.e., Salamandroidea) have abundant cloacal glands. Cloacal glands have been implicated in the production of different components of the male spermatophore (Sever and Houck, 1985).

In this investigation we provide data on the urogenital tract of the Rhyacotritonidae. We also compare the morphological variation observed in the male salamander urogenital duct system in a phylogenetic context. Willett (1965) believed that the urogenital tract of salamanders would be useful in testing hypotheses about the higher-level relationships of salamanders because the urogenital tract would always develop to adulthood and prevent any inaccurate phylogenetic assessments that could be misconstrued due to retention of larval characteristics. Furthermore, it has been well documented that larval features of adult salamanders have interfered with our ability to reconstruct the evolutionary history of salamanders (Wiens et al., 2007; Struck, 2007). Thus, here, we also test the hypothesis that male urogenital duct anatomy will support a more traditional salamander phylogeny with a monophyletic Salamandroidea over a more controversial phylogeny with a paraphyletic Salamandroidea.

Materials and Methods

Urogenital Anatomy of Rhyacotriton—Male specimens of *Rhyacotriton olympicus* and *R. cascadae* were obtained from the museum collection at the University of Missouri. Specimens were preserved in 70% ethanol. Urogenital tracts were removed from each specimen and dehydrated via ascending concentrations of ethanol, cleared with toluene, and embedded in paraffin. The right testis, kidney, and cloaca from each specimen were oriented for sagittal sectioning, whereas the left testis, kidney, and cloaca were oriented for transverse sectioning. Serial sections were obtained at 7 μm, affixed to albumenized slides, and stained with hematoxylin and eosin for general histological examination following the protocol of Kiernan (1990). Representative micrographs were obtained via a Quicam 12-Bit Mono Fast 1394 Cooled digital camera (Qimaging Corporation, British Columbia, Canada) attached to a Leica DM4500 microscope (Leica Microsystems, Wetzlar, Germany). Micrographs were oriented into plates and labeled in Adobe Creative Suite (Adobe Systems, San Jose, CA).

Evolution of the Urogenital Duct Anatomy of Salamanders—Discrete characters with variable states were coded for the different salamander families from an in-depth literature review (see introduction for citations) and from the anatomical descriptions of *Rhyacotriton*. Morphological data from Gymnophiona and Anura were utilized to assess urogenital duct features of salamander relatives, and thus, character polarity. The states utilized for the ancestor to salamanders are provided in the results section, along with evidence for the coding scheme. States for the ancestor to salamanders for cloacal characters were taken from Sever (1991a). Although it is recognized that cloacal glands exist in some anuran taxa (Van Dijk, 1959), we followed Sever's (1991a) hypothesis that these glands are probably not homologous with those of salamanders. Character states were subsequently optimized to the phylogenies of Wiens et al. (2005) and Struck (2007). We recognize that many other higher-level salamander phylogenies have been reconstructed with varying results, dependent largely on the use of non-adult characteristics for paedomorphic salamander lineages or molecular versus morphological character sets (e.g., Duellman and Trueb, 1986; Larson and Wilson, 1989; Hillis, 1991; Larson, 1991; Sever, 1991a; Larson and Dimmick, 1993; Hay et al., 1995; Gao and Shubin, 2001; Larson et al., 2003; Weisrock et al., 2005; Frost et al., 2006; Zhang and Wake, 2009). However, the topologies from Wiens et al. (2005) and Struck (2007) were chosen to generalize a comparison between topologies recovering a monophyletic or paraphyletic Salamandroidea (i.e., a hypothesized grouping of internal fertilizing salamanders). The topology recovered by Struck (2007) was reconstructed with the use of molecular and morphological characteristics with all non-adult characteristics utilized for paedomorphic lineages (Struck 2007:6, Fig. 1). The topology recovered by Wiens et al. (2005) was reconstructed with the use of molecular and morphological characteristics with all non-adult characteristics coded as unknown for paedomorphic lineages (Wiens et al. 2005:102, Fig. 7). The major difference between the two topologies was the recovery of the Sirenidae as the basal salamander lineage (results in a monophyletic Salamandroidea; Wiens et al., 2005) or the recovery of the Cryptobranchidae + Hynobiidae clade as the basal salamander lineage and the Sirenidae as the sister taxon to Proteidae (results in a paraphyletic Salamandroidea; Struck, 2007). Descriptive statistics were compared between optimizations to garner support for one topology over the other and included tree length (e.g., number of evolutionary steps), retention index (RI; a measure of synapomorphic value; Farris, 1989a, b), and consistency index (CI; a measure of homoplasy; Kluge and Farris, 1969). The resulting optimization with fewer steps (fewer evolutionary transitions), greater synapomorphic value (value closer to 1.0), and less homoplasy (value closer to 1.0) was considered a more parsimonious recovery of higher-level salamander evolutionary history in terms of the variation observed in salamander urogenital duct morphology.

Results

Urogenital Anatomy of Rhyacotriton—Through a midsagittal ventral incision the urogenital organs are observed dorsal to the gastrointestinal tract and urinary bladder. The testes are elongate and lie slightly ventrolateral to the cranial epididymal and Wolffian ducts of the genital kidney (Figs. 1A, B). The Wolffian ducts travel caudally, lateral to the pelvic kidney until they approach the caudal portion of the kidney where they migrate ventromedially and cross over the ventral surface of the pelvic kidney. A common serosa that possesses abundant melanophores surrounds the Wolffian ducts and the rest of the urogenital organs. Due to the small size of *Rhyacotriton*, the urogenital duct associations were difficult to delineate with gross examination. Thus, the remainder of the descriptions was accomplished with light microscopy.

A longitudinal canal passes through the testes (Figs. 1C and 2A). This canal is not central but located toward the lateroventral portion of the testis; however, we term this canal a central testicular canal to maintain consistency with previous salamander urogenital morphology studies. Lobules of the testis communicate with this canal along its entire length by means of medial canal branches. The canal empties into the vasa efferentia (4–5 sequentially arranged tubules along the length of the canal; Figs. 1C and 2B, C), which could most accurately be described as inconspicuous ducts (Figs. 2B, C). The lumina of the vasa efferentia are very narrow and difficult to observe, and the epithelium lining the tubules is comparable to that of the endothelium of a capillary (i.e., the cytoplasm is reduced and the observation of the small nuclei of the individual squamous epithelial cells is the epithelium's only observable characteristic; Figs. 2B–D). The tunic of the vasa efferentia is reduced and composed of only a single layer of collagenous fibers and appears highly elastic, similar to that of a capillary.

The lumina of the vasa efferentia expand into renal corpuscles as they approach the Wolffian ducts (Figs. 1C and 2C, D). The epithelium remains squamous, and glomeruli fill the expanded lumina (Figs. 1C and 2C, D). Distal to the renal corpuscles, the lumina decrease in size due to the increase in size of the epithelium lining the epididymal ducts (Figs. 2C–E). This increase in epithelial height delineates the proximal portion of the efferent epididymal ducts. The epithelium of the most proximal region is columnar with all

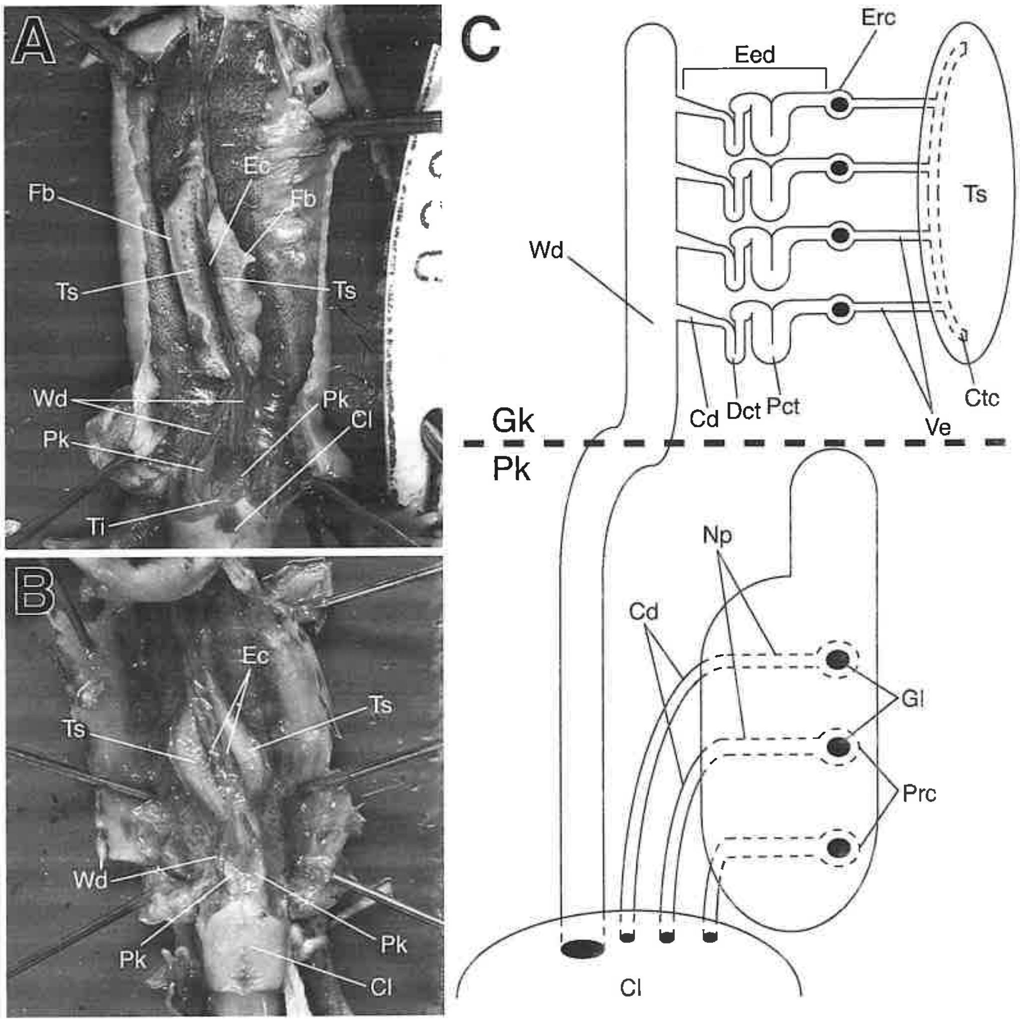

Fig. 1. Gross urogenital anatomy of Rhyacotritonidae (A, *Rhyacotriton olympicus*; B, *Rhyacotriton cascadae*), and microanatomy reconstructed from serial histological sections (C; the testis and pelvic kidney nephrons have been simplified to draw attention to the genital kidney epididymal ducts and pelvic kidney collecting ducts). Compare the schematic of the urogenital ducts with Figs. 2 and 3. Cd, collecting ducts; Cl, cloaca; Ctc, central testicular canal; Ec, epididymal complex; Eed, efferent epididymal ducts; Erc, renal corpuscle of epididymis; Fb, fat body; Dct, distal convoluted tubule; Gk, genital kidney; Gl, glomerulus; Np, nephrons; Pk, pelvic kidney; Pct, proximal convoluted tubule; Pk, pelvic kidney; Prc, renal corpuscle of pelvic kidney; Ti, terminal intestine; Ts, testis; Ve, vasa efferentia; Wd, Wolffian duct.

cells possessing basal nuclei and apical microvillus-like projections. This region of the epididymal ducts is equivalent to the proximal convoluted tubule of the nephron (Fig. 1C). The proximal convoluted tubule opens into the distal convoluted tubule of the nephron (Fig. 1C), which has a more cuboidal epithelium with centrally located nuclei. The distal convoluted tubule opens into the collecting duct, which possesses a columnar epithelium with basal nuclei. Thus, all regions of a typical salamander nephron (see Siegel et al., 2010) exist in the efferent epididymal ducts of the Rhyacotritonidae except for a ciliated neck and intermediate segment. The efferent epididymal ducts are highly convoluted giving the appearance of many more ducts than actually exist (Figs. 2C–F).

The collecting ducts of the efferent epididymal ducts subsequently open into the Wolffian duct, which carries sperm to the cloaca (Figs. 1C and 2C). The tunic of the Wolffian duct is composed of 2–3 layers of collagen fibers and fibroblasts, and the epithelial lining is irregular. A thin layer of smooth muscle encompasses the tunic caudally. Cranially, the epithelium is simple columnar with few ciliated cells observed. Distal to the termination of the testes, the number of ciliated cells increases. We term the epithelium of the Wolffian

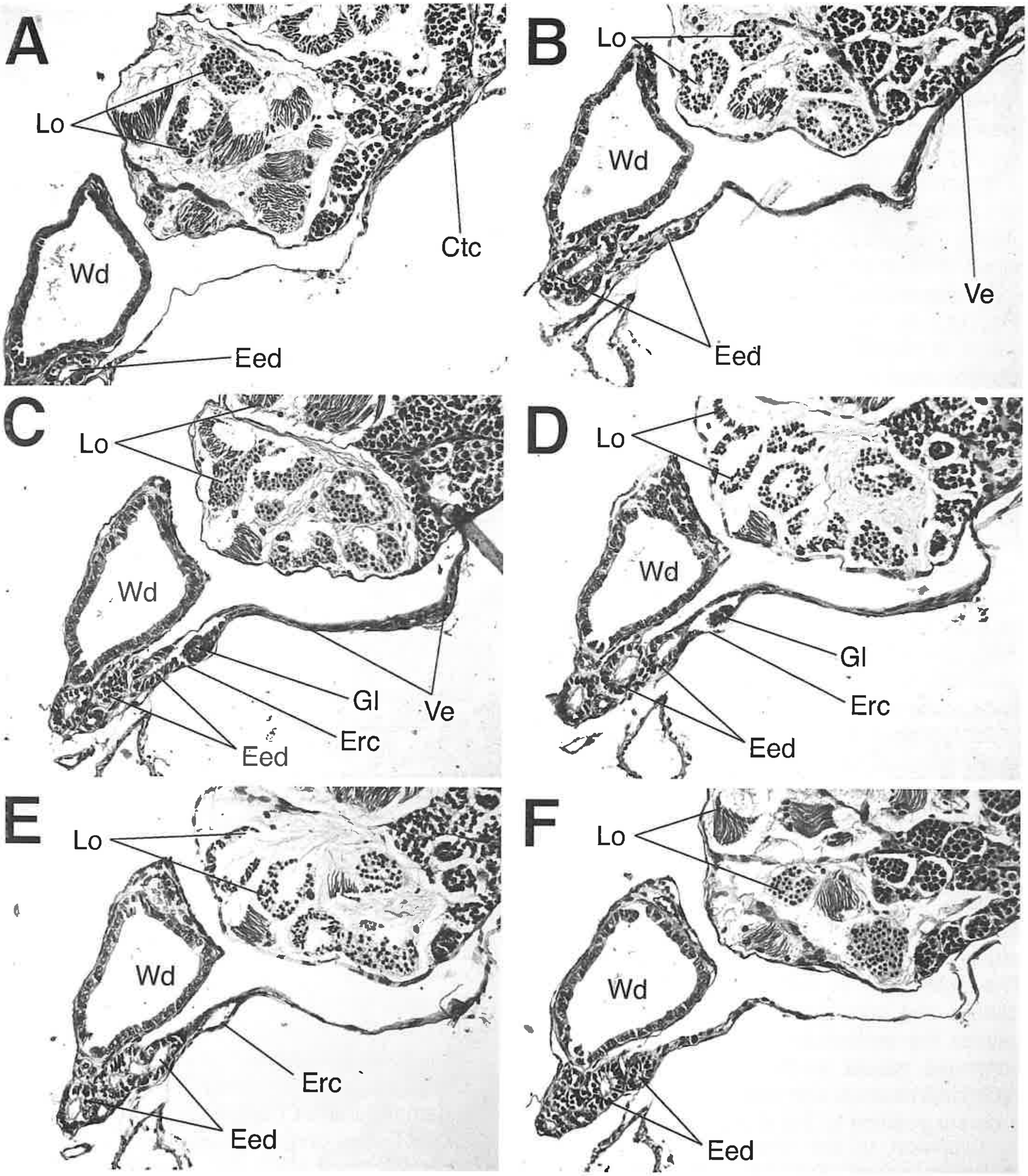

Fig. 2. Serial transverse histological sections from the left testis and genital kidney of *Rhyacotriton olympicus*. Micrographs are arranged sequentially from cranial (A) to caudal (F). Ctc, central testicular canal; Eed, efferent epididymal ducts; Erc, renal corpuscle of epididymis; Gl, glomerulus; Lo, lobule of the testis; Ve, vasa efferentia; Wd, Wolffian ducts.

ducts irregular because the basal plasma membrane of the ciliated cells does not come into close contact with the basal lamina of the Wolffian duct. Instead, the ciliated cells are held away from the basal lamina in an apical position between the majority non-ciliated cells.

The Müllerian duct is noticeable at the level of the mid-testis (Figs. 3A, B) and has a simple non-ciliated cuboidal epithelium. This duct is connected to the Wolffian duct by a thin mesentery and is encompassed by 1–2 layers of collagenous fibers (Fig. 3A). As the Müllerian duct approaches the cloaca, it migrates into the lamina propria of the Wolffian duct (Figs. 3B, C). The Müllerian duct and Wolffian duct communicate with the cloaca separately through the ventral/lateral wall of the urodaeum, with the Wolffian duct in a more lateral position (Fig. 3D). No prominent papilla is formed through which the Müllerian duct and Wolffian duct communicate with the cloaca.

The Wolffian duct is only responsible for the transportation of sperm from the testes to the cloaca, as 7–8 collecting ducts that do not communicate with the Wolffian duct drain the nephrons of the pelvic kidney. These collecting ducts originate from the lateral aspect of the pelvic kidney individually, bend ventrally, become encompassed in a common tunic with the Wolffian and Müllerian ducts, and then empty into the cloaca individually (Figs. 1C and 3C–E). Collecting ducts that exit the kidney more caudally, communicate with the urodaeum more caudally. All of the collecting ducts communicate with the urodaeum more lateral and caudal to where the Wolffian ducts communicate (Fig. 3E). The epithelium of the collecting ducts is non-ciliated and cuboidal, and a thin tunic encompasses the epithelium. The body of the kidney continues caudal to the communication of the Wolffian/Müllerian and collecting ducts and sits in a dorsal position to the cloaca.

Evolution of the Urogenital Duct Anatomy of Salamanders—Through a thorough literature search we identified 15 male urogenital duct characters that exhibit variation between salamander families. Proposed characters and associated states within different salamander families are organized into a data matrix (Table 1). Urogenital duct characters with observed variation within a family are coded as polymorphic, while polymorphic character states for male cloacal characters were resolved following Sever (1991a). Thus, male cloacal characters that lost phylogenetic signal (e.g., variation at the family level) were not included (see Sever, 1991a). Unknown character states were coded as "?" and highlight areas that require further investigation. Caecilian and anuran urogenital duct characters were utilized to assess character polarity, and when states for these taxa are known, they were coded as "state 0" indicating the hypothesized pleisiomorphic condition.

Pelvic kidney characters and states

- *Character 1: Collecting duct morphology:* The collecting ducts of the kidney exit the kidney laterally and communicate with the Wolffian duct individually along the entire length of the Wolffian duct that runs adjacent to the pelvic kidney (state 0). The collecting ducts of the kidney exit the kidney laterally, travel ventrally, and either communicate with the cloaca individually, anastomose and then communicate with the cloaca, or anastomose and communicate with the Wolffian duct (state 1). We concluded that state 0 was the state possessed by the ancestor to salamanders, as this morphology is identical to that described by Wake (1970) for Gymnophiona. The majority of anurans lack an extended pelvic region of the kidney; however, those that do (see Bhaduri and Basu, 1957) have an identical morphology to state 0.
- *Character 2: Collecting duct interaction:* The collecting ducts do not anastomose before subsequent communication with the Wolffian duct or cloaca (state 0). The collecting ducts do anastomose (state 1). We concluded that state 0 was the state possessed by the ancestor to salamanders, as collecting ducts that anastomose before communication with the Wolffian duct or cloaca have never been described in Gymnophiona (Wake, 1970) or Anura (Bhaduri, 1953; Bhaduri and Basu, 1957).
- *Character 3: Collecting duct interaction with the cloaca/Wolffian duct:* Collecting ducts communicate with the Wolffian duct (state 0). Collecting ducts communicate with the cloaca

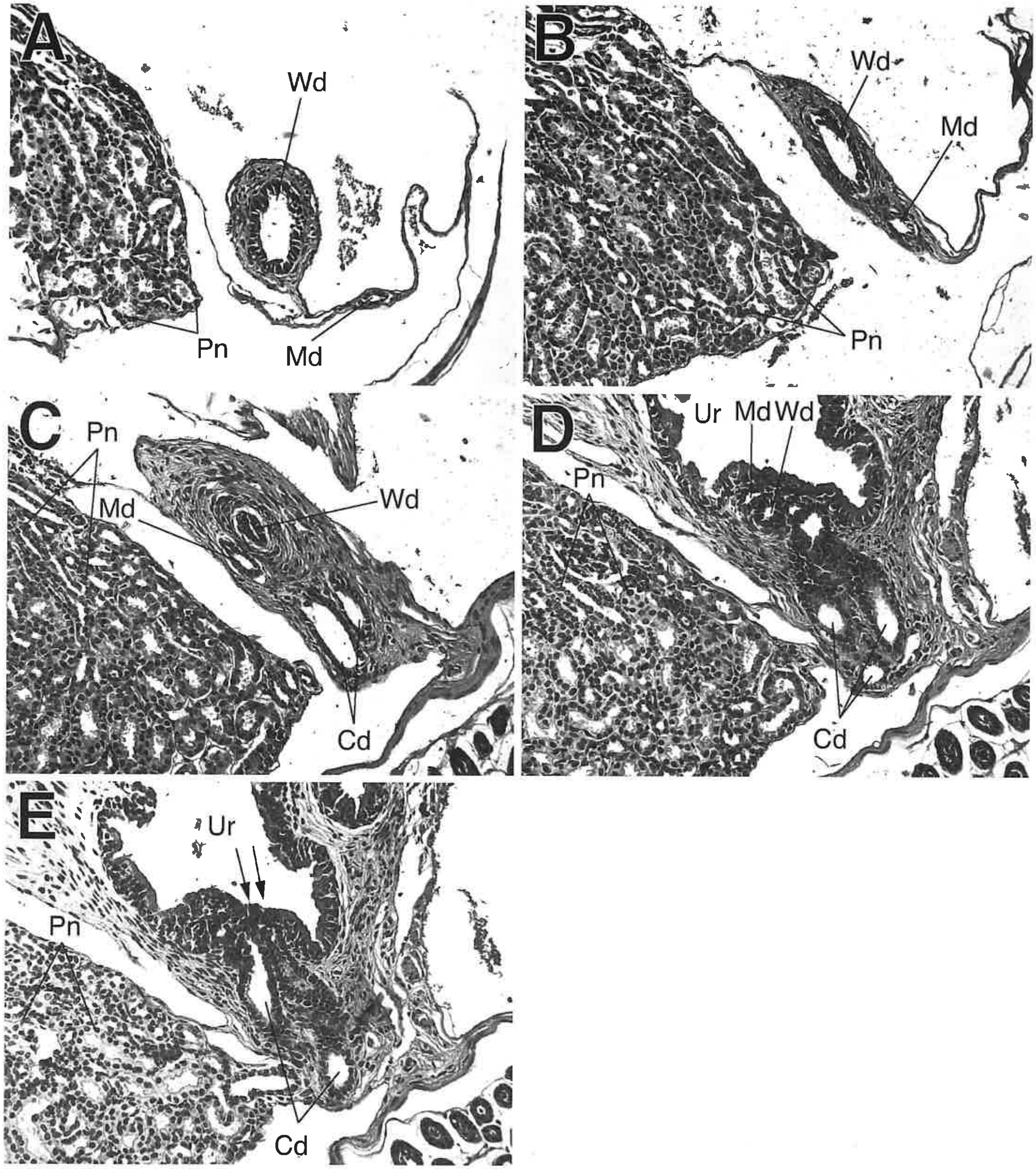

Fig. 3. Serial transverse histological sections from the left pelvic kidney of *Rhyacotriton olympicus*. Micrographs are arranged sequentially from cranial (A) to caudal (E). Cd, collecting ducts; Md, Müllerian duct; Pn, pelvic kidney nephron; Ur, urodaeum of cloaca; Wd, Wolffian duct; arrows indicate separate openings of collecting duct tubules.

Table 1. Data matrix of coded urogenital character states.

Taxa	Characters and states														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Ancestor	0	0	0	?	?	0	0	?	0	0	0	0	0	0	0
Sirenidae	0	0	0	0	?	0	0	?	0	0	0	0	0	0	0
Proteidae	0	0	0	1	?	1	?	0	0	1	1	1	1	1	1
Ambystomatidae	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1
Dicamptodontidae	1	?	?	?	?	?	?	?	1	1	1	1	1	1	1
Plethodontidae	1	1	1	?	0	0	1	0	0	1	1	1	1	1	1
Salamandridae	1	1	0,1	1	0	1	0	1	0	1	1	1	1	0	1
Amphiumidae	1	1	0	?	?	?	?	?	0	1	0	1	1	0	1
Hynobiidae	1	0	1	?	?	1	0	?	0	1	0	0	0	0	0
Rhyacotritonidae	1	0	1	1	0	0	0	1	1	1	1	1	1	1	1
Cryptobranchidae	1	0	1	1	?	1	0	?	0	1	0	0	0	0	0

(state 1). We concluded that state 0 was the state possessed by the ancestor to salamanders, as collecting ducts that communicate with the cloaca have not been identified in Gymnophiona (Wake, 1970) or Anura (Bhaduri, 1953; Bhaduri and Basu, 1957).

- *Character 4: Müllerian duct:* The Müllerian duct is absent (state 0). The Müllerian duct is present (state 1). The state of this character in the ancestor to salamanders could not be determined from current literature. In both Gymnophiona and Anura the presence and absence of a Müllerian duct is polymorphic. Thus, the ancestral condition for character polarization was coded as '?'.
- *Character 5: Ciliated collecting ducts:* The collecting ducts are not ciliated (state 0). The collecting ducts are ciliated (state 1). Little data exist on the microanatomy of the collecting ducts in Gymnophiona and Anura. Møbjerg et al. (2004) indicated that the collecting ducts are not ciliated in one species of caecilian. In Anura, the epithelial cells of the collecting ducts may contain one central cilium (Møbjerg et al., 1998), or possibly lack cilia altogether (Uchiyama et al., 1990). We are reluctant to utilize the absence of ciliated cells in this region as the pleisiomorphic state of amphibians due to the lack of consistent data. Thus, this character was coded as '?' for the ancestor to salamanders.

Genital kidney characters and states

- *Character 6: Bidder's duct:* A Bidder's duct is absent (state 0). A Bidder's duct is present (state 1). A Bidder's duct was not observed in Gymnophiona (Wake, 1970), and we know of no description that indicates its presence in Anura. Thus, we concluded that the ancestor to salamanders lacked this duct.
- *Character 7: Renal corpuscle within the epididymal complex:* A renal corpuscle is present (state 0). A renal corpuscle is absent (state 1). A renal corpuscle has previously been identified in the epididymal complex in Gymnophiona (Wake, 1970) and Anura (Spengel, 1876), and thus, this was considered the state of the ancestor to salamanders.
- *Character 8: Ciliated cells lining the Wolffian duct:* The composition of the epithelial lining of the Wolffian ducts does not include ciliated cells (state 0). The composition of the epithelial lining of the Wolffian ducts does include ciliated cells (state 1). Little data exist on the cellular composition of the Wolffian duct in amphibians. Wake (1970) and Møbjerg et al. (2004) indicated that the Wolffian ducts in Gymnophiona possess no ciliated cells. In frogs, the epithelium of some portions of the Wolffian ducts possesses cilia (Ecker and Haslam, 1889). We are uncomfortable delineating a state for the ancestor of salamanders for this character based on the poor diversity in

sampling. Thus, we coded the condition for the ancestor to salamanders as '?'.

Male cloacal gland characters and states (from Sever, 1991a)

- *Character 9: Primary and secondary folds in the male cloacal tube:* Folds in the primary cloacal tube are absent (state 0). Folds in the primary cloacal tube are present (state 1).
- *Character 10: Anterior ventral glands:* Anterior ventral glands are absent (state 0). Anterior ventral glands are present (state 1).
- *Character 11: Posterior ventral glands:* Posterior ventral glands are absent (state 0). Posterior ventral glands are present (state 1).
- *Character 12: Kingsbury's glands:* Kingsbury's glands are absent (state 0). Kingsbury's glands are present (state 1).
- *Character 13: Dorsal pelvic glands:* Dorsal pelvic glands are absent (state 0). Dorsal pelvic glands are present (state 1).
- *Character 14: Lateral pelvic glands:* Lateral pelvic glands are absent (state 0). Lateral pelvic glands are present (state 1).
- *Character 15: Dorsal or vent glands:* Dorsal or vent glands are absent (state 0). Dorsal or vent glands are present (state 1).

Optimization of the characters and associated states listed above on the Wiens et al. (2005) topology resulted in a tree with a length of 25 (CI = 0.60, RI = 0.69; Fig. 4). Collecting ducts that do not communicate along the length of the Wolffian ducts adjacent to the pelvic kidneys (character 1, state 1) evolved either once on the branch leading to all salamanders, excluding the Sirenidae (with secondary loss in the Proteidae; character 1, state 0), or evolved independently on the branches leading to the Cryptobranchidae + Hynobiidae and the Salamandroidea excluding the Proteidae. The pleisiomorphic condition for salamanders was recovered as collecting ducts that communicate with the Wolffian duct adjacent to the pelvic kidney along its entire length (character 1, state 0). Collecting ducts that do not anastomose before interacting with the cloaca or Wolffian ducts (character 2, state 0) was recovered as the pleisiomorphic condition for salamanders. Collecting ducts that anastomose before interacting with the cloaca or Wolffian ducts (character 2, state 1) either evolved twice on the branches leading to the Ambystomatidae + Dicamptodontidae + Salamandridae and Amphiumidae + Plethodontidae, or once on the branch leading to the Ambystomatidae + Dicamptodontidae + Salamandridae + Rhyacotritonidae + Amphiumidae + Plethodontidae with a reversal on the branch leading to the Rhyacotritonidae. Collecting ducts that do not directly interact with the cloaca (character 3, state 0) was recovered as the pleisiomorphic condition for salamanders. Collecting duct interactions with the cloaca (character 3, state 1) evolved independently on the branches leading to the Cryptobranchidae + Hynobiidae, and possibly the branches leading to the Rhyacotritonidae and Plethodontidae. However, an equally parsimonious solution results with a single transition to the derived morphology on the branch leading to the Amphiumidae + Plethodontidae + Rhyacotritonidae, with a reversal on the branch leading to the Amphiumidae. The presence of a Müllerian duct (character 4, state 1) appears to be a synapomorphy for all salamanders excluding the Sirenidae, which does not possess a Müllerian duct (character 4, state 0); however, the fact that this trait is polymorphic in the ancestor to salamanders results in the pleisiomorphic condition of this character for salamanders recovered as equivocal. Non-ciliated collecting ducts (character 5, state 0) were recovered as the pleisiomorphic condition for salamanders, with ciliated collecting ducts (character 5, state 1) recovered as an autapomorphic structure for the Ambystomatidae.

In the genital kidney, the presence of a Bidder's duct (character 6, state 1) was recovered as the ancestral condition for salamanders excluding Sirenidae, which lacks a Bidder's duct (character 6, state 0). A Bidder's duct is lost on the branch leading to the Amphiumidae + Plethodontidae + Rhyacotritonidae. The presence of a renal corpuscle within the genital kidney (character 7, state 0) was recovered as the pleisiomorphic condition for salamanders. The lack of a renal corpuscle (character 7, state 1) was recovered as autapomorphic for the Plethodontidae. Due to lack of data, the evolutionary history of

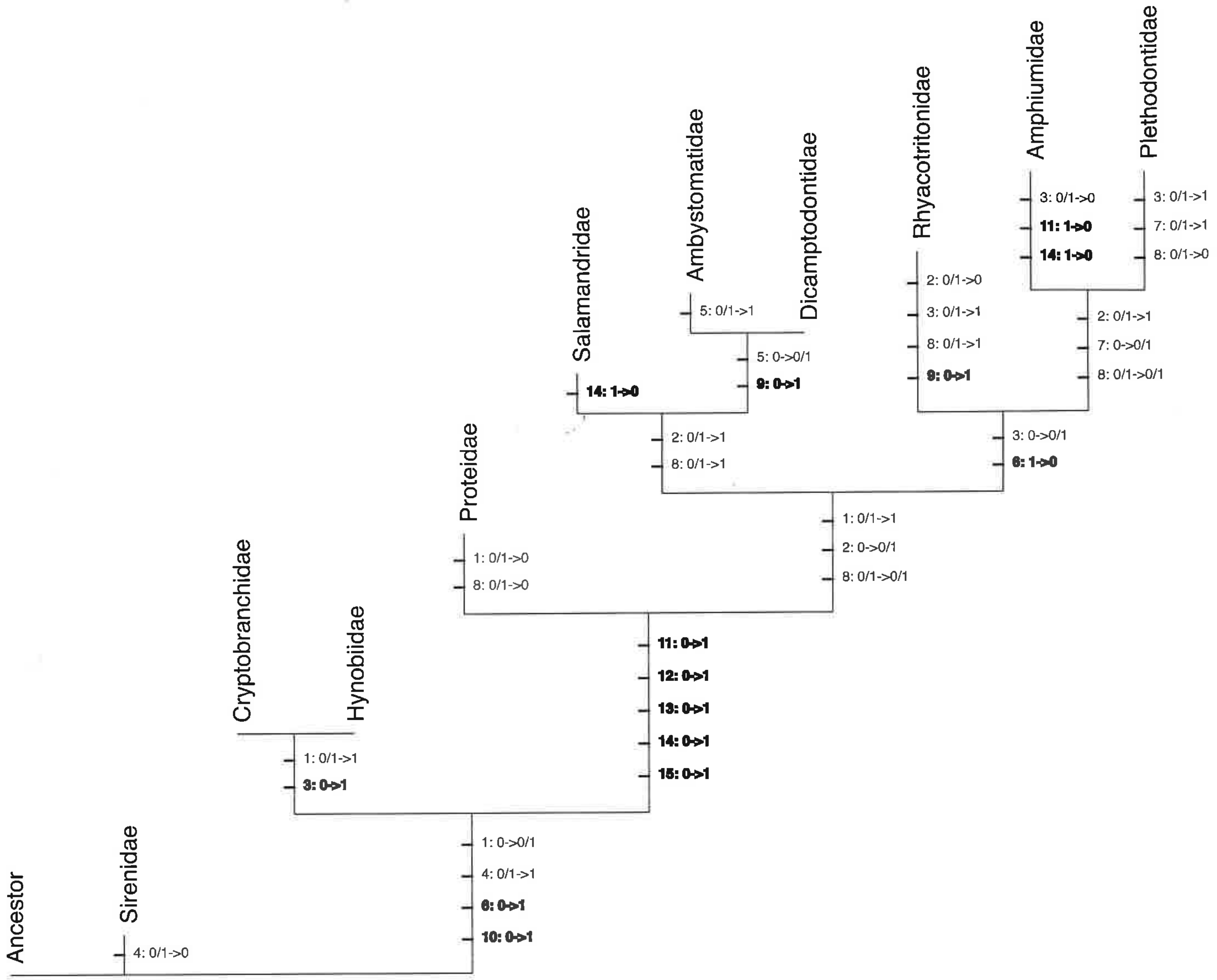

Fig. 4. Optimization of male salamander urogenital duct character state to the phylogeny reconstructed by Wiens et al. (2005; most parsimonious tree reconstructed from morphology with non-adult characteristics of paedomorphs recorded as unknown, RAG-1, and rRNA). Numbers on branches indicate character state transitions. Bold text signifies unambiguous transitions. Branch lengths were altered to better visualize possible evolutionary transitions. No transitions occurred on the branches leading to Sirenidae and Salamandridae, and these branches were subsequently collapsed. In the original analysis by Wiens et al. (2005) Sirenidae was recovered as the sister lineage to all extant salamanders.

ciliated Wolffian ducts could not be recovered. From the limited data currently possessed, Wolffian ducts without ciliated cells (character 8, state 0) could be the pleisiomorphic condition for salamanders, with a Wolffian duct possessing ciliated cells (character 8, state 1) evolving independently on the branches leading to the Ambystomatidae + Dicamptodontidae + Salamandridae and Rhyacotritonidae. However, an equally parsimonious solution is obtained with a ciliated Wolffian duct as the pleisiomorphic condition of salamanders, with independent transitions to a non-ciliated Wolffian duct in the Plethodontidae and Proteidae.

In the male cloaca, the lack of folds in the cloacal tube (character 9, state 0) was recovered as the ancestral condition for salamanders. Cloacal tube folds (character 9, state 1) evolved independently on the branches leading to the Ambystomatidae + Dicamptodontidae and the Rhyacotritonidae. In terms of cloacal glands, the pleisiomorphic characteristic for salamanders was recovered as lacking cloacal glands for every

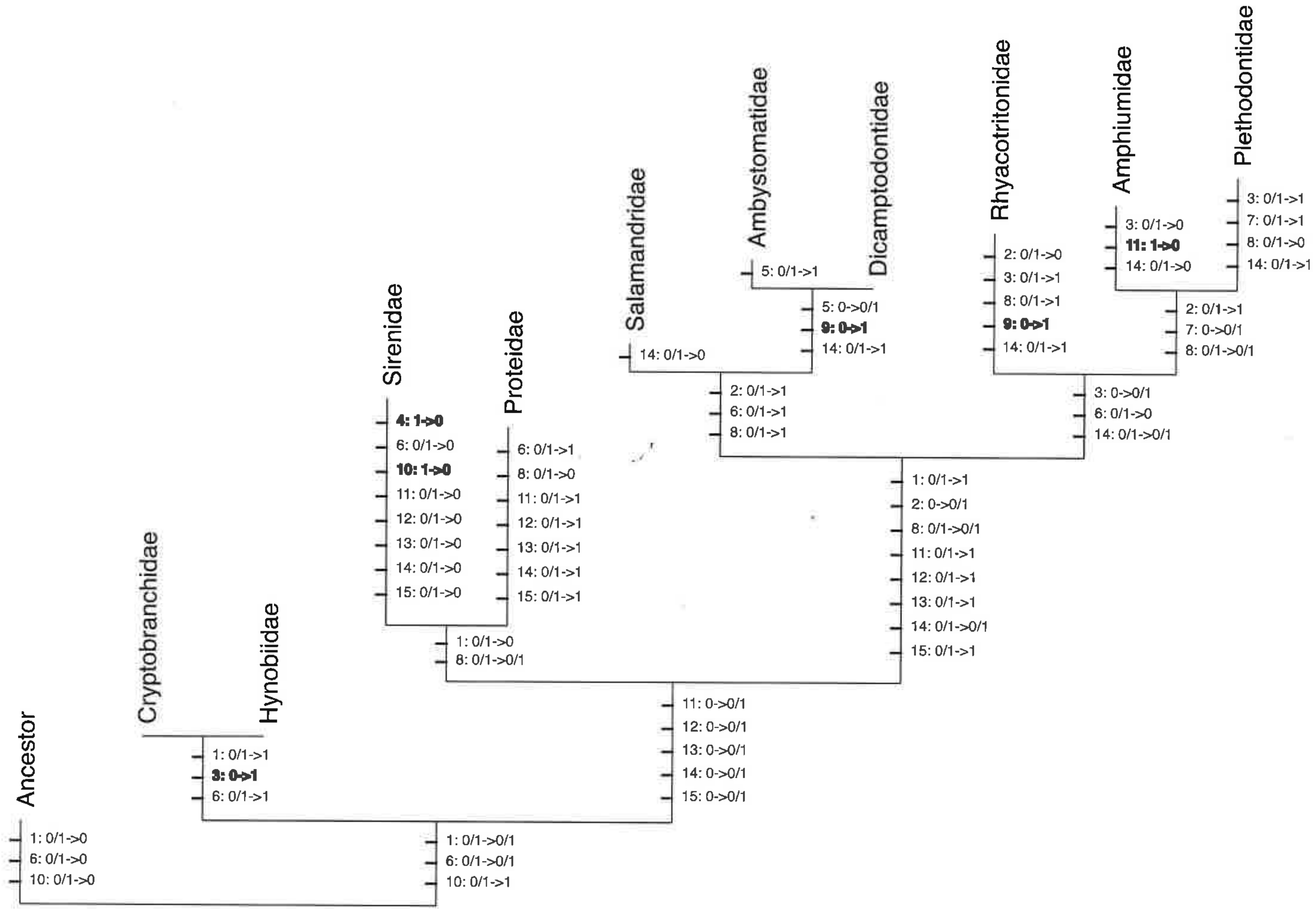

Fig. 5. Optimization of male salamander urogenital duct character states to the phylogeny reconstructed by Struck (2007; most parsimonious tree reconstructed from morphology, RAG-1, rRNA, and mtDNA). Numbers on branches indicate character state transitions. Bold text signifies unambiguous transitions.

gland complex (characters 10, 11, 12, 13, 14, and 14, states 0). The presence of anterior ventral glands (character 10, state 1) may represent a non-ambiguous synapomorphy for all salamanders to the exclusion of the Sirenidae, while the presence of posterior ventral glands (character 11, state 1) was recovered as the ancestral state for the Salamandroidea with a subsequent loss (character 11, state 0) on the branch leading to the Amphiumidae. The presence of Kingsbury's (character 12, state 1) and dorsal pelvic (character 13, state 1) glands may represent a non-ambiguous synapomorphy for the Salamandroidea, whereas the presence of lateral pelvic glands (character 14, state 1) evolved on the branch leading to the Salamandroidea, with subsequent independent losses in the Amphiumidae and Salamandridae. Dorsal or vent glands (character 15) followed the same evolutionary trajectory as Kingsbury's and dorsal pelvic glands.

Optimization of the characters and associated states listed above on the Struck (2007) topology resulted in a tree with a length of 32 (CI = 0.47, RI = 0.47; Fig. 5). The pleisiomorphic condition of the collecting duct morphology was recovered as equivocal for salamanders. However, collecting ducts that do not communicate with the Wolffian ducts along their lengths traveling adjacent to the pelvic kidney (character 1, state 1) were recovered as the ancestral state for the Cryptobranchidae + Hynobiidae and all other salamander excluding the Proteidae and Sirenidae. Collecting ducts that communicate individually with the Wolffian ducts along the length of the Wolffian duct traveling adjacent to the pelvic kidney (character 1, state 0; the condition also found in the ancestor to salamanders) were recovered as the pleisiomorphic condition for the Sirenidae + Proteidae clade. Non-anastomizing collecting ducts (character 2, state 0) were recovered as the ancestral state for salamanders, and collecting ducts that do anastomose (character 2, state 1) may have evolved independently on the branches leading to the Ambystomatidae + Dicamptodontidae + Salamandridae and Amphiumidae + Plethodontidae clades. However, the retention of the pleisiomorphic condition in the Rhyacotritonidae resulted in the recovery of the ancestral state of the Ambystomatidae + Dicamptodontidae + Salamandridae + Rhyacotritonidae + Amphiumidae + Plethodontidae clade as equivocal. Thus, collecting ducts that anastomose may have evolved a single time, with a subsequent reversal in the Rhyacotritonidae. Collecting ducts that communicate with the Wolffian duct (character 3, state 0) were recovered as the pleisiomorphic condition for salamanders. Collecting ducts that bend laterally and communicate with the cloaca (character 3, state 1) evolved independently on the branch leading to the Cryptobranchidae + Hynobiidae and at least once within the Amphiumidae + Plethodontidae + Rhyacotritonidae lineages. However, the ancestral state for this lineage could not be recovered. Thus, either the derived condition evolved once on the branch leading to the Amphiumidae + Plethodontidae + Rhyacotritonidae or independently on the branches leading to the Plethodontidae and Rhyacotritonidae. The former transition would result in a reversal on the branch leading to the Amphiumidae. Existing morphological descriptions indicate that all salamanders excluding the Sirenidae possess a Müllerian duct (character 4, state 1), and that the absence of a Müllerian duct (character 4, state 0) may be autapomorphic for the Sirenidae. Similarly, existing data on collecting ducts indicate that the ancestral state for all salamander families is collecting ducts with a non-ciliated epithelium (character 5, state 0), and that presence of partially ciliated collecting ducts (character 5, state 1) is autapomorphic for the Ambystomatidae.

In terms of the genital kidney, the ancestral state for the backbone of the salamander phylogeny was equivocal between the absence (character 6, state 0) and presence (character 6, state 1) of a Bidder's duct. However, the data indicate that at least one of these states evolved or was lost independently in different salamander families. The Cryptobranchidae + Hynobiidae, Ambystomatidae + Dicamptodontidae + Salamandridae, and Proteidae lineages possess a Bidder's duct, while this characteristic is lacking on the branches leading to the Sirenidae and Amphiumidae + Plethodontidae + Rhyacotritonidae. Only the Plethodontidae lack a renal corpuscle (character 7, state 1) separating the vasa efferentia from the more distal epididymal ducts. Too little data are available to recover ancestral state for salamanders in terms of Wolffian ducts without ciliated cells (character 8, state 0) or Wolffian ducts with ciliated cells (character 8, state 1). The branches leading to the Ambystomatidae + Dicamptodontidae + Salamandridae and Rhyacotritonidae possess Wolffian ducts with ciliated epithelial cells, whereas the branches leading to the Plethodontidae and Proteidae do not possess ciliated Wolffian ducts.

A cloacal tube lacking folds (character 9, state 0) was recovered as the ancestral state for salamanders, while cloacal tube folds (character 9, state 1) evolved independently on the branches leading to the Rhyacotritonidae and the Ambystomatidae + Dicamptodontidae.

All salamanders possess anterior ventral glands (character 10, state 1) except the Sirenidae, indicating an independent loss of these glands in the Sirenidae (character 10, state 0). The lack of posterior ventral glands (character 11, state 0) was recovered as the ancestral state of salamanders and these glands (character 11, state 1) may have evolved independently on the branches leading to the Proteidae and Ambystomatidae + Dicamptodontidae + Salamandridae + Rhyacotritonidae + Amphiumidae + Plethodontidae. A reversal to the pleisiomorphic condition occurs in the latter clade, as the Amphiumidae lack posterior ventral glands. The ancestral state for all salamanders excluding the Cryptobranchidae + Hynobiidae could not be recovered for this character, and thus, it cannot be unequivocally confirmed that the Proteidae evolved posterior ventral glands independently or if the Sirenidae lost these glands. Identical results were obtained for Kingsbury's (character 12) and dorsal pelvic (character 13) glands, although a loss was not observed in the Amphiumidae. Lateral glands (character 14) follow a similar trajectory but were again lost in the Amphiumidae, and also the branch leading to the Salamandridae. Dorsal or vent glands (character 15) followed the same evolutionary trajectory as Kingsbury's and dorsal pelvic glands.

Discussion

Comparative Morphology of the Urogenital Ducts in Salamanders—Morphological aspects of the genital kidney have been recently reviewed by Aranzábal (2003; 2009); however, the most in-depth review of this structure was by Williams et al. (1984). In general, vasa efferentia transport sperm from the testes into modified nephrons. In all salamanders, except for the Plethodontidae (Strickland, 1966; Williams et al., 1984), Rhyacotritonidae, and Sirenidae (Willett, 1965), a longitudinal duct (Bidder's duct or marginal longitudinal duct) connects the lumina of the multiple vasa efferentia and also serves to communicate the vasa efferentia leading immediately from the testes to the afferent epididymal ducts (Williams et al., 1984). This duct differs from the testicular longitudinal canal that has also been termed the longitudinal duct in some studies (e.g., Baker and Taylor, 1964).

The afferent epididymal ducts empty into a renal corpuscle and, thus, are delineated grossly from the efferent epididymal ducts by a renal corpuscle in all salamanders except the Eurycinae (Williams et al., 1984), Rhyacotritonidae, and Sirenidae. The renal corpuscle and efferent epididymal ducts represent the remnant mesonephros that is modified for sperm transport. The efferent epididymal ducts subsequently empty into the Wolffian ducts. In the Eurycinae, no renal corpuscle or Bidder's duct is observed and, thus, distinguishing between the vasa efferentia and the afferent and efferent epididymal ducts is difficult. The seemingly homogeneous ducts that communicate the testes with the Wolffian ducts in the Eurycinae were termed transverse tubules by Williams et al. (1984).

The Rhyacotritonidae and Sirenidae (Willett, 1965) have identical genital kidney duct morphologies that are different from all other salamanders but similar to that of caecilians (Wake, 1970). Whereas a renal corpuscle delineates the vasa efferentia from the efferent epididymal ducts, no longitudinal duct connects the vasa efferentia and no discernible afferent epididymal duct exists between the renal corpuscle and the vasa efferentia. Furthermore, histological analysis of the efferent epididymal ducts indicated that the efferent epididymal ducts are nothing more than nephrons; e.g., a neck region, proximal portion, intermediate segment, distal portion, and collecting ducts (Willett, 1965), with the neck region and intermediate segments reduced or absent in the Rhyacotritonidae. The absence of a longitudinal duct, but presence of renal corpuscles, was also observed in caecilians (Wake, 1970). Furthermore, via gross examination Wake (1970) noted that a thickened fold of mesentery created the appearance of a duct communicating the different tubules of the vasa efferentia in caecilians. However, upon careful histological examination of serial testicular duct sections, Wake (1970) observed no such duct. Considering that the efferent epididymal ducts are highly convoluted, the presence of a longitudinal duct could be a byproduct of gross examination and poor histological description. Obviously more detailed accounts of the testicular ducts are necessary to illuminate the uncertainty of this structure. Earlier reports (e.g., Baker and Taylor, 1964) only mention gross morphology derived from histology, but did not describe histological features. The absence of histological descriptions makes comparison of the duct network difficult, if not impossible.

Salamanders can be grouped into three categories depending on their male pelvic kidney morphology: 1) the simple condition (or larval condition), 2) the complex non-anastomosing condition, and 3) the complex anastomosing condition. The simple condition is found only in the Proteidae (Rosenquist and Baker, 1967) and Sirenidae (Willett, 1965) and can also be considered the larval condition, as this condition is found in larval salamanders (Rodgers and Risley, 1938). The simple condition is defined by collecting ducts that exit the kidneys laterally and communicate individually with the Wolffian ducts along their entire length adjacent to the pelvic kidneys. This condition is also observed in caecilians (Wake, 1970) and some anurans that have not completely lost a pelvic portion of the kidney (Bhaduri and Basu, 1957). The functional implication of this morphology is that Wolffian ducts carry both urinary and reproductive material along their entire length adjacent to the pelvic kidneys.

The complex non-anastomosing condition has been described in the Cryptobranchidae (Ratcliff, 1965), Hynobiidae (Yamagiwa, 1924), and Rhyacotritonidae (this study). This kidney condition is defined by collecting ducts that exit the pelvic kidney laterally, bend caudally, and communicate with the urodaeum (cranial region of the cloaca) individually. The functional implications of this pelvic kidney morphology are that Wolffian ducts carry only reproductive materials, as urinary material passes directly from the pelvic kidney to the cloaca.

The complex anastomosing condition has been described in the Ambystomatidae (Baker and Taylor, 1964; Siegel et al., 2010), Amphiumidae (Baker, 1945), Plethodontidae (Strickland, 1966), and Salamandridae (Francis, 1934; Baker, 1965). Collecting ducts that exit the pelvic kidney laterally, bend caudally, and anastomose define this condition. Two subtypes of this morphology have been described as follows: a) collecting ducts that empty into the Wolffian ducts via common collecting ducts and b) collecting ducts that empty into the urodaeum via common collecting ducts. Subtype ‘a’ was described in the Ambystomatidae (Baker and Taylor, 1964; Siegel et al., 2010) and Amphiumidae (Baker, 1945). Subtype ‘b’ was described in the Plethodontidae (Strickland, 1966). Both ‘a’ and ‘b’ subtypes have been described for the Salamandridae, indicating that this trait is polymorphic within newts (Francis, 1934; Baker, 1965). As with the complex non-anastomosing condition, the functional implication of this morphology is that Wolffian ducts transport only reproductive material. However, the joining of the common collecting duct with the most caudal portion of the Wolffian ducts in the Ambystomatidae, Amphiumidae, and some Salamandridae indicate some mixing of urinary and reproductive material within the most distal extremity of the Wolffian duct.

The isolation of the urinary and reproductive tracts of salamanders with the complex conditions may have paved the way for secondary sexual function in the collecting ducts of salamanders (Siegel et al., 2010). In the Ambystomatidae, Plethodontidae, and Salamandridae the collecting ducts hypertrophy during the mating season and synthesize abundant secretions of unknown function (Aron, 1924; Adams, 1940; Sakai and Kawahara, 1983; Norris, 1987; Siegel et al., 2010; Siegel et al., in press). It has been hypothesized that these secretions aid in sperm viability, capacitation, activation, or function as a constituent of the spermatophore (Siegel et al., 2010). Current data indicate that this secondary modification of the pelvic kidney collecting ducts is restricted to salamanders with the complex anastomosing pelvic kidney morphology; however, it must be recognized that kidneys from seasonal collections of salamanders exhibiting the non-anastomizing kidney morphology have not yet been investigated. The unknown function of collecting duct secretions originally described in the 1920s (Aron, 1924) exemplifies our poor understanding of salamander reproductive biology, and we look forward to subsequent inquiry into these structures.

This investigation provides no novel data on the comparative morphology of the male cloaca than previously provided by the definitive works of Sever (1991a, b; 1992a–d). We refer readers to these articles for an in-depth discussion of salamander cloacal morphology.

Urogenital Duct Morphology and the Evolutionary History of Salamanders—Urogenital duct characters support a more traditional hypothesis of salamander phylogeny with a monophyletic Salamandroidea (e.g., Duellman and Trueb, 1986; Larson and Dimmick, 1993; Wiens et al., 2005) over that of a paraphyletic Salamandroidea (e.g., Gao and Shubin, 2001; Larson, 1991; Larson et al., 2003; Frost et al., 2006). Descriptive statistics (e.g., tree length, CI and RI) indicated more synapomorphic value and less homoplasy when urogenital duct characters were optimized to a phylogeny with a monophyletic Salamandroidea. The presence of cloacal glands and, thus, the capability of internal fertilization, have long been thought to unite salamanders of the Salamandroidea (e.g., Sever, 1991a). However, recent phylogenetic analyses concluded that these complex structures might have evolved independently due to the recovery of a paraphyletic Salamandroidea with respect to the placement of the Sirenidae within the traditional Salamandroidea (Larson, 1991; Gao and Shubin, 2001; Larson et al., 2003; Frost et al., 2006). We conjecture that this alternative hypothesis supports internal fertilization and the presence of cloacal glands as the pleisiomorphic condition in salamanders and that these features were subsequently lost in some paedomorphic lineages (i.e., cryptobranchids, hynobiids, and sirenids). When cloacal characters are removed from the data matrix, optimization on the Wiens et al. (2005) and Struck (2007) topologies results in a tree length of 14 (CI = 0.57, RI = 0.50) and 15 (CI = 0.53, RI = 0.42), respectively. Thus, the difference between descriptive statistics decreases drastically when cloacal characters are excluded, indicating the possible evolutionary disagreement in different regions of the urogenital duct system.

Willett (1965) believed that insights from the urogenital anatomy of salamanders would lead to more accurate reconstruction of higher-level salamander relationships than external and osteological features. This conclusion was based on the observation that incidences of paedomorphism cause the comparison of external and osteological features of salamanders to be difficult, as confirmed by Wiens et al. (2005) and Struck (2007). Willett (1965) believed that no matter what the trajectory of the majority of somatic tissues (retention of larval features or a transition to an adult form), the urogenital tract of salamanders would always proceed to an easily comparable adult form. Thus, Willett (1965) predicted an affinity of the Sirenidae and Proteidae based on identical morphology of the pelvic kidney collecting ducts. However, we feel that her theories on paedomorphic character ambiguity may also cause inaccurate assessment of salamander relationships when investigating kidney anatomy alone. For example, the collecting duct form found in male proteids and sirenids is the form also found in larvae of all other salamander families (Rodgers and Risley, 1938). Thus, it is possible that the retention of the larval collecting duct morphology in adults is due to these taxa reaching an adult form at an earlier developmental stage than other paedomorphs that possess the derived collecting duct morphology. We recognize that the result of identical kidney anatomy in proteids and sirenids could be caused by a common paedomorphic event, which would seemingly support similarities found within sirenid and proteid karyological morphologies (Morescalchi, 1975) and recent topologies reconstructed from combined molecular and morphological data sets (see Gao and Shubin, 2001; Frost et al., 2006; Struck, 2007). However, we also feel that the previously proposed distant relationships between these paedomorphs (see Duellman and Trueb, 1986; Larson and Dimmick, 1993; Wiens et al., 2005; Zhang and Wake, 2009) could indicate that this identical morphology was achieved by chance (e.g., convergence), as all salamanders possess this kidney morphology at least some time during their development. Obviously, ontological studies are necessary to test these alternative hypotheses.

In terms of the Rhyacotritonidae, no urogenital duct character states were identified as unambiguous synapmorphies uniting rhyacotritonids with other families or groups, besides cloacal characters (Sever, 1991a), when utilizing the topologies of Wiens et al. (2005) and Struck (2007) for optimization. Interestingly, the male genital kidney anatomy of the Rhyacotritonidae is identical to that of the Sirenidae (Willett, 1965), and the male pelvic kidney anatomy of the Rhyacotritonidae is identical to that of the Cryptobranchidae (Ratcliff, 1965) and Hynobiidae (Yamagiwa, 1924). Thus, investigation of the genital and pelvic kidney collecting ducts of rhyacotritonids brings us full circle to a prediction by the original describer of *Rhyacotriton* who placed this family within the Hynobiidae (Gaige, 1917); i.e., that the Rhyacotritonidae are a more basal taxon of salamander, a hypothesis supported by nuclear encoding rRNA

(Larson, 1991) and nuclear encoding rRNA and mitochondrial DNA (Larson et al., 2003). However, in terms of cloacal glands, the Rhyacotritonidae possess advanced features that seemingly unite this taxon with the Salamandroidea (Sever, 1991a), as also recovered when phylogenies have been reconstructed with a suite of molecular (mitochondrial and nuclear) and morphological character sets (Duellman and Trueb, 1986: Larson and Dimmick, 1993; Hay et al., 1995; Wiens et al., 2005; Struck, 2007; Zhang and Wake, 2009).

No matter what alternative hypothesis of phylogeny is adhered to, it is evident that morphological features of the urogenital system need further investigation. Of interest is that cloacal characters support a monophyletic Salamandroidea (Sever, 1991a); however, the similar kidney structure of the Proteidae and Sirenidae may be interpreted as support for alternative hypotheses of relationship that recovered a paraphyletic Salamandroidea (e.g., Gao and Shubin, 2001; Frost et al., 2006). Much remains unknown on the micro-anatomy of the urogenital system in salamanders, in particular the epididymal complex, and we feel insights from salamander reproductive anatomy will continue to play a leading roll in our understanding of higher-level salamander evolution.

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