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RESEARCH ARTICLE

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Embryonic development of the pelvic girdle and hindlimb skeletal elements in *Anilius scytale* (Linnaeus, 1758) (Serpentes: Aniliidae)

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Abstract

Anilius scytale is the sister lineage of all other alethinophidian snakes. Morphology of the hind limb complex in adult A. scytale (Aniliidae) has been documented. We herein, for the first time, describe the embryology of the skeletal elements of its hind limb and pelvic girdle and contextualize the evolution of these structures. We identified pregnant females of *A. scytale* in the Herpetology Collection of the Museu Paraense Emílio Goeldi and separated 40 embryos. The embryos were sequentially staged using external and internal anatomy, collectively comprising a developmental series representing six stages. We cleared-stained one specimen of stages 31, 34, 36, and 37. Using the embryological information gleaned from *A. scytale*, we reinterpret evidence relating to the ossification of the pelvis and hindlimbs. In *A. scytale* hindlimb buds develop as transient structures that developed before Stage 30 and regresses in subsequent stages. There is no external or internal evidence of the forelimb or scapular girdle. From Stage 31 onwards the ischium, pubis, ilium, femur and zeugopodial cartilages are visible. Pubis and femur ossify towards the end of embryonic life, and cloacal spurs do not develop in the embryo. Skeletal elements of the hindlimb and pelvic girdle develop initially in the ventral zone of the cloaca-tail region. In subsequent stages the hindlimb and pelvic girdle elements migrate dorsally, with the pubis/ischium positioned medial to the ribs. A similar process may be associated with the achievement of the condition of the pelvic girdle in adults of scolecophidians, pythonids and boids.

KEYWORDS
Alethinophidia, ontogeny, Squamata

1 | INTRODUCTION

In the embryology of lizards with well-developed fore and hindlimbs, each limb develops in a discrete position along the body axis, within limb fields (Gilbert, 2010). The limb fields are formed by migration of mesenchymal cells from the somatic layer of the lateral plate mesoderm. Aggregation of mesenchymal cells in the limb fields develop into limb buds. A limb bud is an external protuberance consisting of mesenchymal cells covered with an ectodermal layer (Gilbert, 2010: p. 487). Interactions between mesenchymal and ectodermal cells establish three the main developmental axes of the fore and hindlimbs (proximo-distal axis, anteroposterior axis, dorsoventral axis). Along these axes, cells express a subset of Hox genes that determine the position and morphology of chondroblast and myoblast cells that eventually generate the skeletal and muscular components of the girdles (scapular/pelvic), stylopodia (femur/humerus), zeugopodia (tibia, fibula/radius, ulna) and autopodia (manus/pes). In the adult stage of lizards with four well-developed limbs, the skeleton of the hindlimb is composed of the pelvic girdle (ilium, ischium, pubis) which articulates with the vertebral column at the sacral region, and the stylopod (femur), zeugopod (tibia, fibula) and autopod (tarsus, metatarsus, phalanges) (Hoffstetter & Gasc, 1969; Russell & Bauer, 2008: p. 136).

In Serpentes, squamates with reduced limbs, forelimb buds do not develop, and hindlimb buds are reduced structures that may give rise to an incomplete autopodial region (Boughner et al., 2007; Leal & Cohn, 2016; Raynaud, 1971). In some fossil snakes (e.g., *Pachyrhachis problematicus*, *Haasiophis terrasanctus*, *Eupodophis descouensi*) and adult stages of extant snakes (e.g., Aniliidae, Boidae, Cylindrophiidae, Loxocemidae, Pythonidae, Scolecophidia, Trophidophiidae) (sensu Burbrink et al., 2020), stylopodial (femur) and zeugopodial (tibia, fibula) elements are reduced to vestiges and the pelvic girdle lacks articulation with the vertebral column and is located medial to the ribs (Apesteguía & Zaher, 2006; Georgalis & Smith, 2020; Leal & Cohn, 2016; Palci et al., 2020).

Studies of the reduction and loss of limbs began 150 years ago (for a complete review, see Camaiti et al., 2021). Some of these studies have focused on the structural body shape of snakes, including anatomical comparisons of extant and fossil species, phylogenetic analyses, and developmental studies (Camaiti et al., 2021). However, developmental studies describing the ontogeny of the hindlimb and pelvic girdle have been conducted only for species of *Python* (Pythonidae) in spite of the great diversity of extant taxa that retain vestiges of these structures (Boughner et al., 2007; Leal & Cohn, 2016; Palci et al., 2020; Raynaud, 1971).

Despite being important for our understanding of the evolution of the snake-like form, the pelvic girdle has not been the focus of developmental and comparative studies in Squamata and Serpentes, and little is known about the embryonic pattern of these parts (Malashichev et al., 2005). The inconsistent results of the investigations on the pelvic girdle may be demonstrated by a brief assessment of several works. Studies of the embryology of the pleuroapophyses in squamates (El-Toubi, 1947; Kamal, 1952), the homology of the pelvic elements in snakes (Raynaud, 1972; Raynaud et al., 1975), and the discussion of the morphogenetic control of the pelvic-axial skeleton articulation (Borkhvardt & Malashichev, 2000; Malashichev, 2001) have all been conducted. According to developmental studies on mammals and aves, the interactions formed in the limb bud shape the morphogenetic control of the hind limb elements (femur, tibia, and fibula), and the developmental control of the pelvic girdle is established at the limb field region after the formation of the limb bud. (Malashichev et al., 2005; Pomikal & Streicher, 2010). Since tetrapod developmental processes are relatively conservative, squamates could also have a comparable embryonic control.

The family Aniliidae includes a single species, *Anilius scytale* (Linnaeus, 1758). The position of the Aniliidae as the sister lineage of all other Alethinophidian snakes has been consistently retrieved in phylogenetic analyses using different sources of evidence (Rieppel, 1988; Vidal & Blair Hedges, 2002; Gauthier et al., 2012; Pyron et al., 2013; Burbrink et al., 2020). However, there are incongruences between the hypotheses proposed as to the phylogenetic position of the Aniliidae. The clade Aniliodea is composed of the Aniliidae and Cylyndrophiidae is retreived in some studies (Rieppel, 1988; Gauthier et al., 2012), while an alternative hypothesis recovers the clade Amerophidia (Aniliidae, Trophidophiidae) as the sister clade of all other Alethinophidians (Vidal & Blair Hedges, 2002; Siegel et al., 2011; Pyron et al., 2013; Burbrink et al., 2020).

Anilius scytale (Linnaeus, 1758) is distributed in the Amazon basin and in gallery forests of the Cerrado biome as well as in relict forests of the Caatinga (Natera-Mumaw et al., 2015; Nogueira et al., 2020). It has fossorial habits and a viviparous reproductive mode (Martins & Oliveira, 1998; McDowell, 1975).

The adult morphology of *Anilius scytale*'s hindlimb and pelvic girdle has been studied in a succession of works (Fürbringer, 1870; McDowell, 1975; Palci et al., 2020). Fürbringrer described the presence of three pelvic elements, two of them ossified, as well as a bony femur, a cartilaginous tibia and a cloacal spur in males (Fürbringer, 1870). McDowell described the presence of one pelvic bone, a bony femur and cloacal spurs in males (McDowell, 1975: p. 23). Palci et al. (2020) described the presence of three pelvic bones, an ossified femur and cloacal spurs. Despite these significant efforts to describe the adult anatomy of *Anilius scytale*'s hindlimb and pelvic girdle, there is no information available on their embryonic development.

Given the scarcity of embryological studies of the hindlimb/pelvic morphological complex in extant snakes, as well as the fact that *Anilius scytale* resides within sister lineage of all other Alethinophidian snakes, and that its adult form retains hindlimb and pelvic elements, our goal is to contribute to the long-standing debate on snake evolution by describing the embryological development of the pelvic girdle and hindlimb morphology of *Anilius scytale*.

2 | MATERIALS AND METHODS

2.1 | Accessing the embryos

The Herpetology Collection of the Museu Paraense Emílio Goeldi (MPEG), municipality of Belém, state of Pará, Brazil, houses a large collection of specimens of *Anilius scytale*. We determined the sex of the specimens by making a small incision on the ventral side of the tail in the region posterior to the cloaca and checked for the presence of male genitalia (hemipenes). To assemble our embryological series, we employed gravid females from the collections of the MPEG. We selected 89 females of *A. scytale* (Appendix A) and examined them by palpating their posterior region to detect the possible presence of embryos at some stage of development. Once we identified a pregnant specimen, we made a transverse incision in the ventral region to remove the embryos (Figure 1). We separated a subset of nine females with embryos and collected 40 embryos. Each set of embryos was separated, identified with the mother's number, and preserved in 70% ethanol.

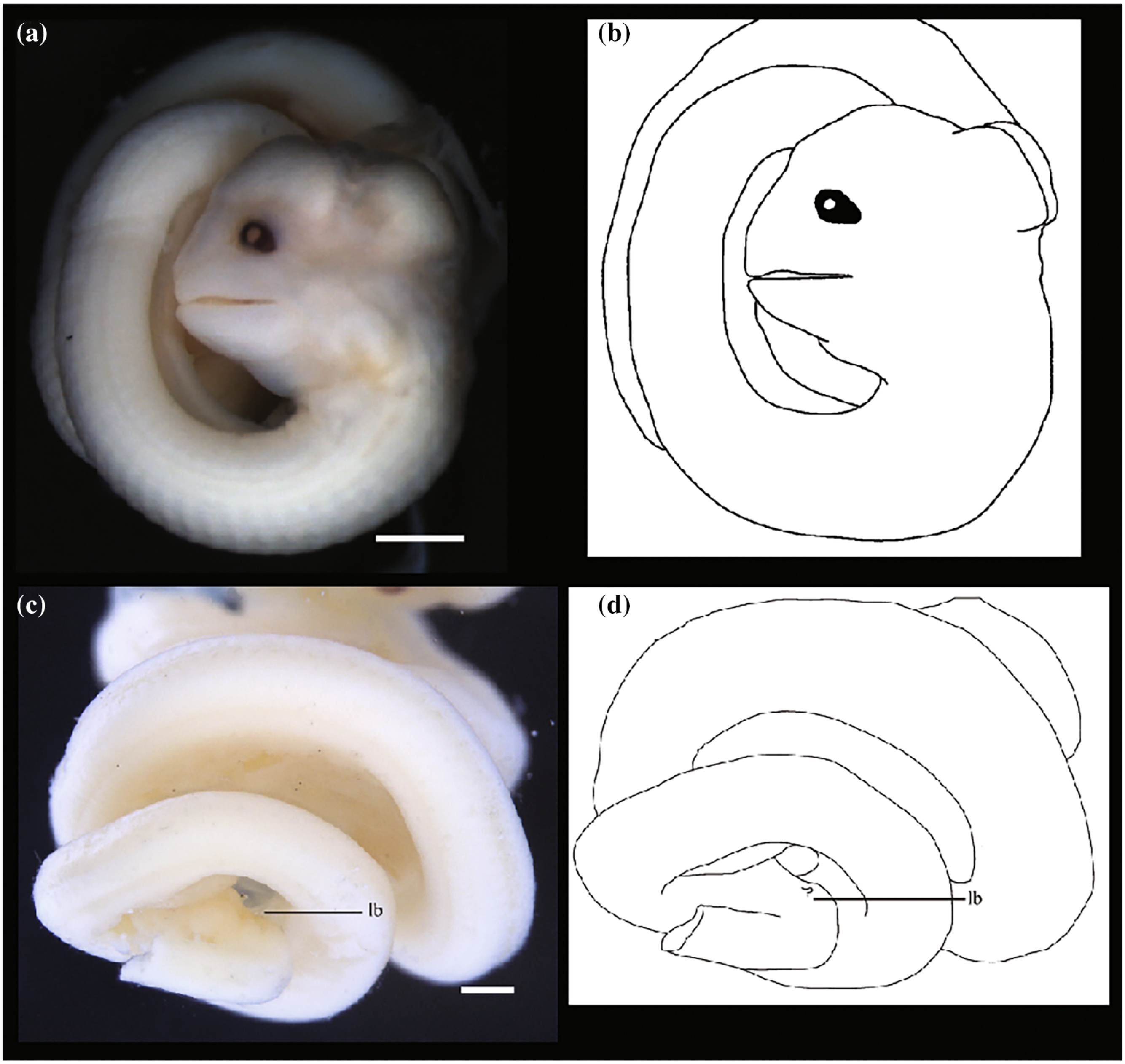

FIGURE 1 Embryonic Stage 30 of *Anilius scytale* (female MPEG 6184). (a) Lateral view; (b) detail of the cloacal region. Scale bar: (a–c) 0.5 mm; (b) 1 mm. Abbreviation: lb, limb bud.

2.2 | Analyzing and staging the embryos

We subdivided embryo specimens into a discrete sequential series of six putative stages to describe the embryonic development of *Anilius scytale*. We did not sexed the embryos.

The putative embryological stages accord Zehr's with six stages in developmental table for *Thamnophis sirtalis sirtalis*, based on transformations of the external morphology: endolymphatic sac, ocular morphology and adnexa, pharyngeal arches and slits, scales morphology, scale pigmentation, hindlimb bud, and hemipenis (Zehr, 1962). To account for the observed transformations of internal morphology of the hindlimbs and pelvic girdle cartilages in our specimens, we further subdivided Zehr's Stage 37, which is based solely on external morphology (Table 1).

We measured snout-vent length (SVL) and head length (HL) of each embryo. Specimens were photographed using a Leica M205 modular stereomicroscope (Leica Microsystems GmbH, Wetzlar Germany).

2.3 | Analyzing and staging the adults

To confirm the presence of a cloacal spur in the adult stage of *Anilius scytale*, we selected a sample of 10 males and 10 females. We defined the adult stage following Maschio, Prudente, Lima, & Feitosa (Maschio et al., 2007). The cloacal spur is an integumentary derivative that externally caps the distal end of the vestigial limb. It is found in the cloacal region in a lateroventral position.

2.4 | Analyzing the internal anatomy

We elected at least one specimen of the putative stages 31, 34, 36, and 37 for clearing and staining, employing Potthoff's (Potthoff, 1984) protocol.

2.5 | Skeletal homology

Our hypotheses about homology of the skeletal elements of the hindlimb and pelvic girdle are based on topology and topographic relationships at each embryonic stage, transformations of their topology and topographic relationships through the developmental sequence, and on comparisons of their developmental pattern with the topology and topographic relationships in adult of *Iguana iguana* (Linnaeus, 1758), which represents the plesiomorphic state for squamates (see Russell & Bauer, 2008: p. 05).

TABLE 1 Embryonic stages sensu Zehr (1962), female collection numbers, and body measurements of the analyzed embryos of Anilius scytale.

Embryonic stages	Female numbers	SVL (mm)	HL (mm)
22	MPEG 26451	32.69	
	MPEG 26451	33.12	
	MPEG 26451	34.19	
	MPEG 26451	34.39	
30	MPEG 6184	38.86	–
	MPEG 22909	74.00	3.46
	MPEG 22909	78.00	3.10
	MPEG 22909	80.00	2.92
31	MPEG 22909	85.00	3.23
	MPEG 22909	86.00	3.81
	MPEG 22909	89.00	4.15
	MPEG 22909	90.00	3.90
34	MPEG 7452	85.00	3.10
	MPEG 7452	91.00	3.76
	MPEG 22450	132.00	5.54
36	MPEG 22450	134.00	7.32
	MPEG 22450	136.00	7.15
37a	MPEG 23902	189.00	7.64
	MPEG 18600	159.00	7.15
	MPEG 18600	162.00	7.65
37b	MPEG 18600	169.00	7.61
	MPEG 18600	174.00	7.67
	MPEG 18600	177.00	7.75
	MPEG 18600	179.00	8.57
	MPEG 15300	199.00	9.36
	MPEG 15300	204.00	9.77
	MPEG 15300	204.00	8.69
	MPEG 15300	210.00	10.15
	MPEG 15300	212.00	9.72
	MPEG 15300	217.00	10.36
	MPEG 22910	127.00	7.42
	MPEG 22910	128.00	7.97
	MPEG 22910	129.00	7.61
	MPEG 22910	134.00	6.77
37c	MPEG 22910	145.00	8.06
	MPEG 22910	146.00	7.33
	MPEG 22910	152.00	7.44
	MPEG 22910	153.00	7.51
	MPEG 22910	156.00	7.12
	MPEG 22910	157.00	8.58

Abbreviations: HL, head length; SVL, snouth-vent length.

The ilium of *Iguana iguana* is a postero-dorsally oriented cartilage that grows dorsolaterally towards the lymphapophyses that are located at the lateral region of the vertebrae in the region of the cloaca (for a discussion of the differentiation of vertebrae in legless squamates, see Hoffstetter & Gasc, 1969: p. 240). In adult forms of legged lizards the ilium articulates with the vertebral column at the sacrum (Hoffstetter & Gasc, 1969: p. 238). The ischial cartilage is oriented ventrally, and the pubic cartilage is oriented anteriorly. In adult forms of normal-limbed squamates the ischial and pubic cartilages grow towards the sagittal midline of the body and fuse, forming the ischiadic and pubic symphyses (Russell & Bauer, 2008: p. 136). The femoral cartilage is a posterolaterally oriented element articulating at the confluence point of the three pelvic cartilages. In adult forms of normal-limbed squamates, the cartilages of the ilium, ischium and pubis have a sutural region at the acetabulum (Russell & Bauer, 2008: p. 136). In adult forms, the acetabular region is positioned latero-dorsal to the ischium (Russell & Bauer, 2008, Figure 1. p. 14).

2.6 | Phylogenetic analyses

To reconstruct the evolutionary history of these elements, we use the phylogenetic framework presented by Palci et al. (Palci et al., 2020: Supporting information Data S3). We reinterpret their characters 114, 115 and 117 in the phylogeny of snakes, using embryological evidence from *Anilius scytale* to establish the most parsimonious reconstruction of these characters (Palci et al., 2020). Using this embryological information to complement descriptions of adult specimens, we reinterpret coded states of *Anilius scytale* as follows: character 114—Ossification of the ilium: (0) present, (1) absent; character 115—Ossification of the ischium: (0) present, (1) absent; character 117—Hindlimb: (0) hindlimb present, with distinct femur, tibia and fibula, (1) hindlimb present, but with only one bone (femur), sometimes with a keratinous distal spur, (2) hindlimb absent.

We coded *Anilius scytale* as having a cartilaginous ilium and ischium, and having a hindlimb present, with a distinct femur, tibia and fibula. Our recodification of character 117 homologizes the two zeuopodial cartilages at the distal end of the femur, registered in the embryological development of *A. scytale*, with the presence of a tibia and fibula in at least one semaphoront stage, while affirming the absence of cloacal spurs in the ontogeny of *A. scytale*. The reconstructions of character history were performed with states unordered (characters 114, 115) and ordered states (character 117), with all transformations equally weighted, using Mesquite v3.7 (Maddison & Maddison, 2021).

3 | RESULTS

3.1 | Description of the embryos

Stage 22.

Embryos = 4 (Table 1).

Hemipenis- the hemipenial buds not visible. *Limbs*– the limb buds not visible.

Stage 30.

Embryos = 1 (Table 1).

Hemipenis—Hemipenial buds visible, short pair oval-shaped without ornamentation. *Limbs*—Limb bud present laterodorsal (Figure 1b) to the genital ridge and hemipenial buds.

Stage 31.

Embryos = 7 (Table 1).

Cartilaginous vertebrae (cervical, caudal) and ribs present. No vertebral or rib ossification centers visible. Centrum chondrification surrounding the notochord. Verterbral articulations (via condyle-cotyle) are incomplete. Prezygapophyseal and postzygapophyseal wings present. Ribs composed of slender cartilage tapering distally with an acute ending. No sacral region visible. Four vertebrae with lymphapophyses. *Hemipenis*—Increased length, with bilobed shape, no ornamentation or sulcus. *Limbs*—Pelvic spurs not visible. Cartilaginous ilium, pubis, ischium and femur are visible in the latero-ventral region of the cloacal zone (Figures 2a,b). Although each cartilaginous element is identifiable, the clearing-staining technique does not allow us to establish whether the elements form a continuous cartilaginous piece. Ilium slender with a free, postero-dorsally oriented extremity that is with flexed so as to lie parallel to the vertebrae. The pubis is shorter, antero-ventrally oriented and tapers distally. The ischium is short and ventrally oriented. The femur is the largest cartilage of the hindlimb and has a posterior orientation. Its proximal end articulates with the pelvic girdle. One zeugopodial cartilage is present at the distal end of the femur (Figures 2a,b).

Stage 34.

Embryos = 2 (Table 1).

The anterior third of the vertebral column has developed vertebral and rib ossifications, neural spines and condyle-cotyle articulations. Each neural arch has an ossification center. The neural arch is expanded, and contacts those of each adjacent vertebra, and a low neural spine is developed. The notochord is obliterated by chondrifications of the centra. Vertebral articulations via condyles-cotyles are complete. Each centrum has developed a short and low hypapophysis. Caudal vertebrae with transverse processes. The first transverse process is fused with the vertebrae and the remainder articulates with the vertebrae. *Hemipenis*—One embryo

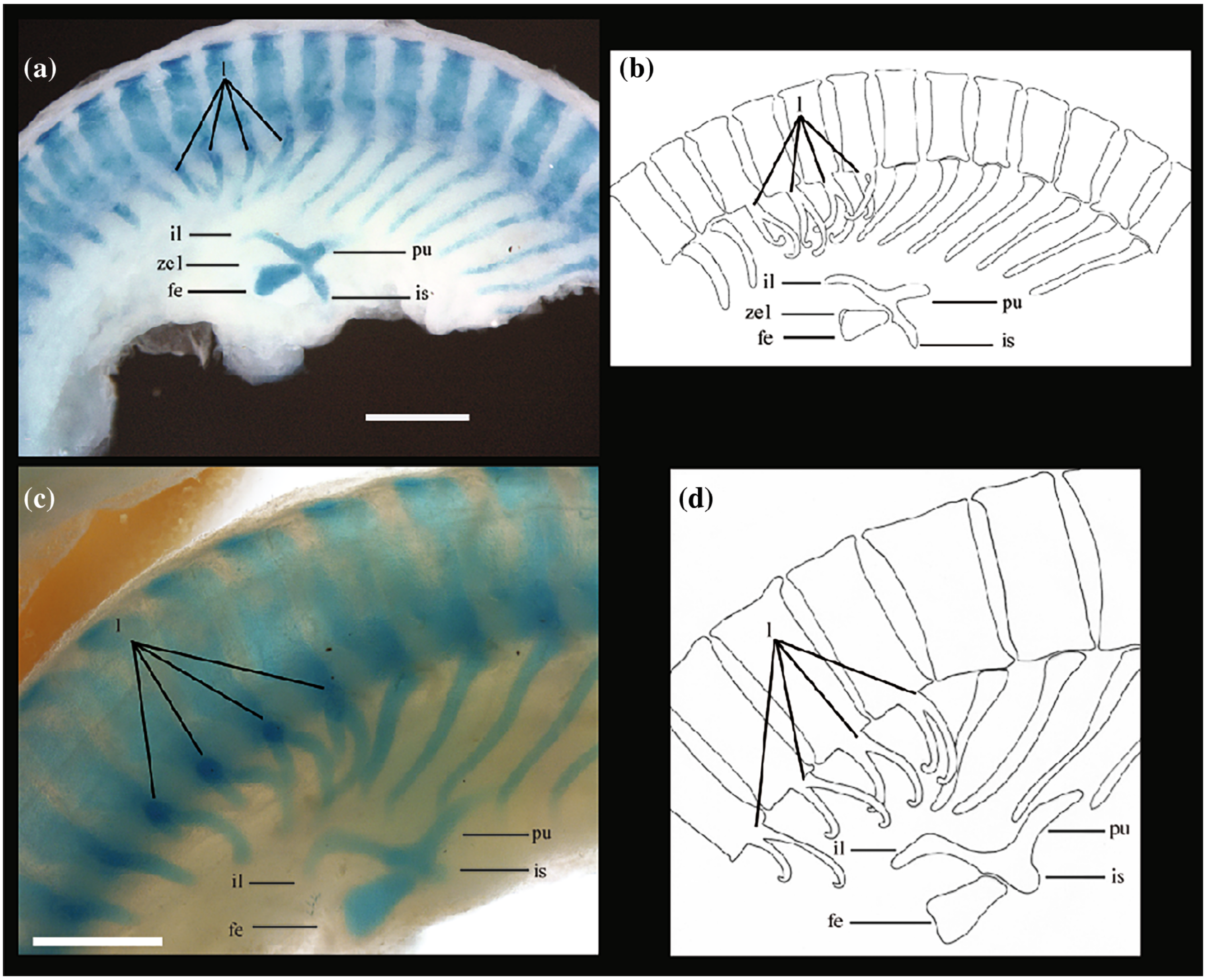

FIGURE 2 Lateral view of the skeletal elements of the hindlimb and pelvic girdle of embryonic stages 31 and 34 of *Anilius scytale*. (a) Stage 31 (female MPEG 22909); (b) diagram of the Stage 31 embryo (female MPEG 22909); (c) Stage 34 (female MPEG 7452); (d) diagram of the Stage 34 embryo (female MPEG 7452). fe, femoral cartilage; il, ilial cartilage; is, ischial cartilage; l, lymphapophyseal cartilage; pu, pubic cartilage; l, lymphapophyseal cartilage; ze 1, 1 zeugopodial cartilage. Scale bar: 0.5 mm.

with bilobed hemipenis with larger morphology, the other embryo with reduced hemipenis. *Limbs*–The hindlimb and pelvic girdle elements lie medial to the ribs (Figures 2c,d). The cartilages of the hindlimb and pelvic girdle are at the same stage of development as in Stage 31.

Stage 36.

Embryos = 3 (Table 1).

All vertebrae and ribs are in the process of ossification. The vertebrae still cartilaginous at their synapophyses and in their condylar regions. Neural arch ossification includes the zygosphene-zygantrum system. Rib capitulum and distal section still cartilaginous. Rib distal ends curve medially (Figures 3a–d). Five vertebrae with lymphapophyses (Figures 3c,d). Lymphapophyses ossified except for their distal ends. First and second lymphapophyses are articulated, the other three are fused to their vertebrae. *Hemipenis*—Hemipenis larger, bilobed, sulcated and ornamented with flounces. *Limbs*—The pubis has become longer. The ischium has become more slender and longer, with its position has changed to a medial orientation (Figures 3a,b). The femur develops an ovoid shape. The acetabular articulation is clearly visible, marking the connection between ilium, pubis and ischium. Two zeugopodial cartilages are located at the dorsal and ventral regions of the distal end of the femur (Figures 3c,d). The dorsal zeugopodial cartilage shorter and rounded (Figures 3c,d: ze1); the ventral one is longer and more slender (Figures 3c,d: ze2).

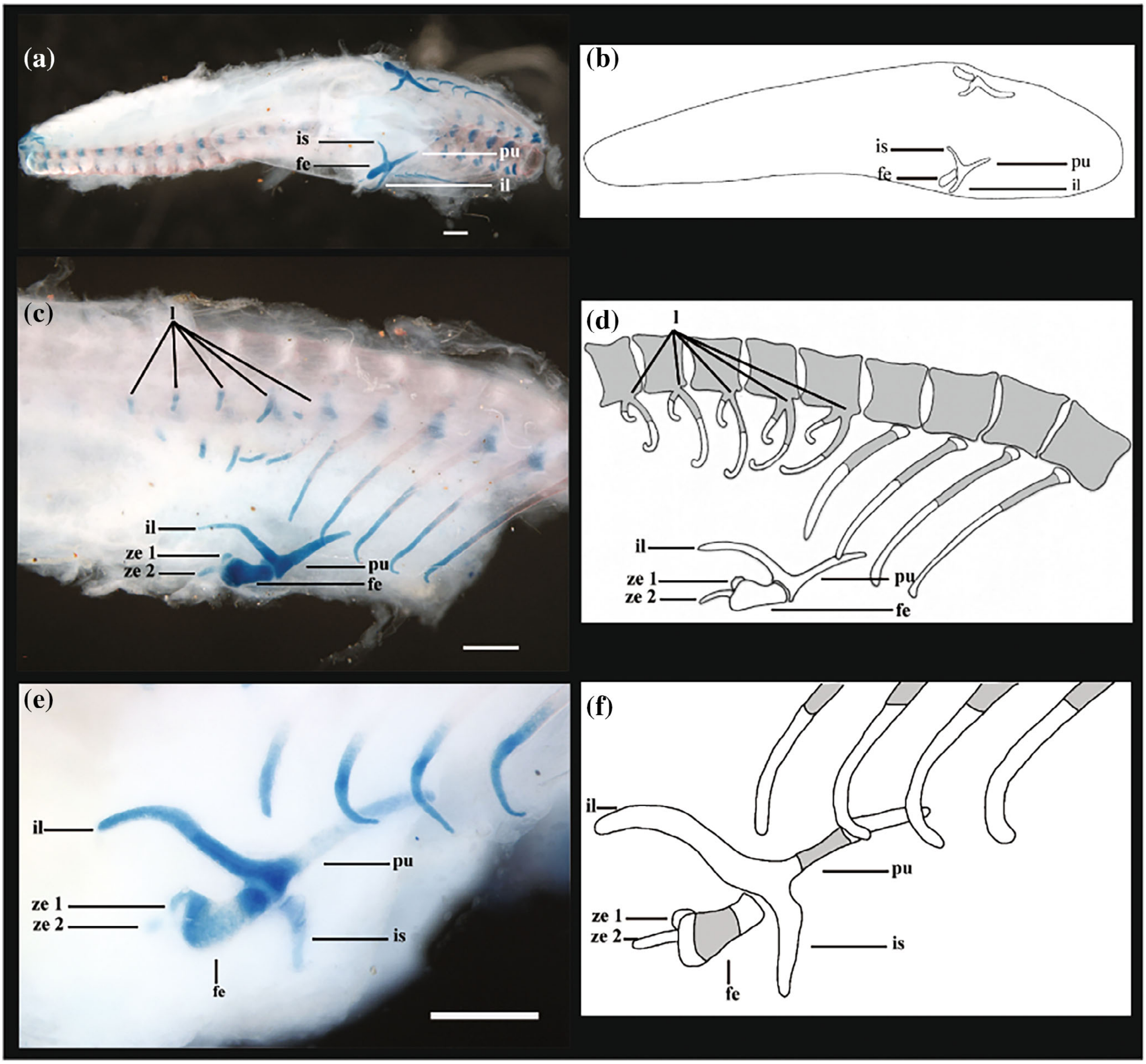

FIGURE 3 Skeletal elements of the hindlimb and pelvic girdle of embryonic stages 36 and 37a of *Anilius scytale*. (a) ventral view of the Stage 36 embryo (female MPEG 22450); (b) diagram of the ventral view of the Stage 36 embryo (female MPEG 22450; (c) lateral view of the of the Stage 36 embryo (female MPEG 22450); (d) diagram of the lateral view of the of the Stage 36 embryo (female MPEG 22450); (e) lateral view of the Stage 37a (female MPEG 23902); (f) diagram of the lateral view of the Stage 37a embryo (female MPEG 23902). fe, partially ossified femur (red section); il, ilial cartilage; is, ischial cartilage; l, partially ossified lymphapophyses; pu, partially ossified pubis (red section), ze 1, zeugopodial cartilage 1; ze 2, zeugopodial cartilage 2. Scale bar: 0.5 mm.

Stage 37a.

Embryos = 1 (Table 1).

Vertebra and rib ossification process is advanced, but a small section of the (synapophyseal condyle-cotyle, rib capitula and rib's distal end) remain cartilaginous (Figures 3e,f). Five lymphapophyses are evident, with their distal ends cartilaginous. The first lymphapophysis is articulated and the following three are fused to the vertebrae. *Hemipenis*—Hemipenis is not visible externally. *Limbs*—The pubis has developed an ossification center in its proximal region (Figures 3e,f). The femur has developed an ossification center in its proximal region. The two zeugopodial cartilages remain visible at the dorsal and ventral aspects of the distal region of the femur (Figures 3e,f). The dorsal zeugopodial cartilage is the shorter of the two and is rounded in shape (Figures 3e,f: ze1); the ventral zeugopodial cartilage longer and more slender (Figures 3e,f: ze2).

Stage 37b.

Embryos = 6 (Table 1).

Limbs—The ossification centers of the ilium and pubis stain more intensely, suggesting increased calcification (Figures 4a,b). The ischium is reduced in length. The two zeugopodial cartilages at distal region of the femur remain visible (Figures 4a,b).

Stage 37c.

Embryos = 16 (Table 1).

Limbs—The degree of ossification of the pubis and femur is augmented (Figure 4c,d). The distal end of the femur is altered in shape, developing a straight border. The ventral zeugopodial cartilage at the distal region of the femur is visible (Figure 4c: ze 2).

Adult stage.

10 males; 10 females.

The cloacal spur is absent in the adult stage.

3.2 | Phylogenetic analyses

Our reconstructions increased the number of steps when compared to that of Palci et al. (2020) (Figures 5–7). A cartilaginous ilium in *Anilius scytale* rendered an uncertain reconstruction for the ancestral state of Serpentes (Figure 5). Its ossification evolved independently as a reversion in lineages of the Alethinophidia, *Xenopeltis unicolor*, *Cylindrophis ruffus*, *Python* and (*Boa*, *Eunectes*, *Epicrates*) (Figure 5). A cartilaginous ischium in *Anilius scytale* recovered this state as being present in the ancestor of the Alethinophidia (Figure 6), although the ancestral condition of the Serpentes remains uncertain (Figure 6). The presence of a hindlimb with tibia/fibula in *Anilius scytale* appears as a reversion, since the state of a hindlimb with a femur and cloacal spurs evolved in the Serpentes clade (Figure 7).

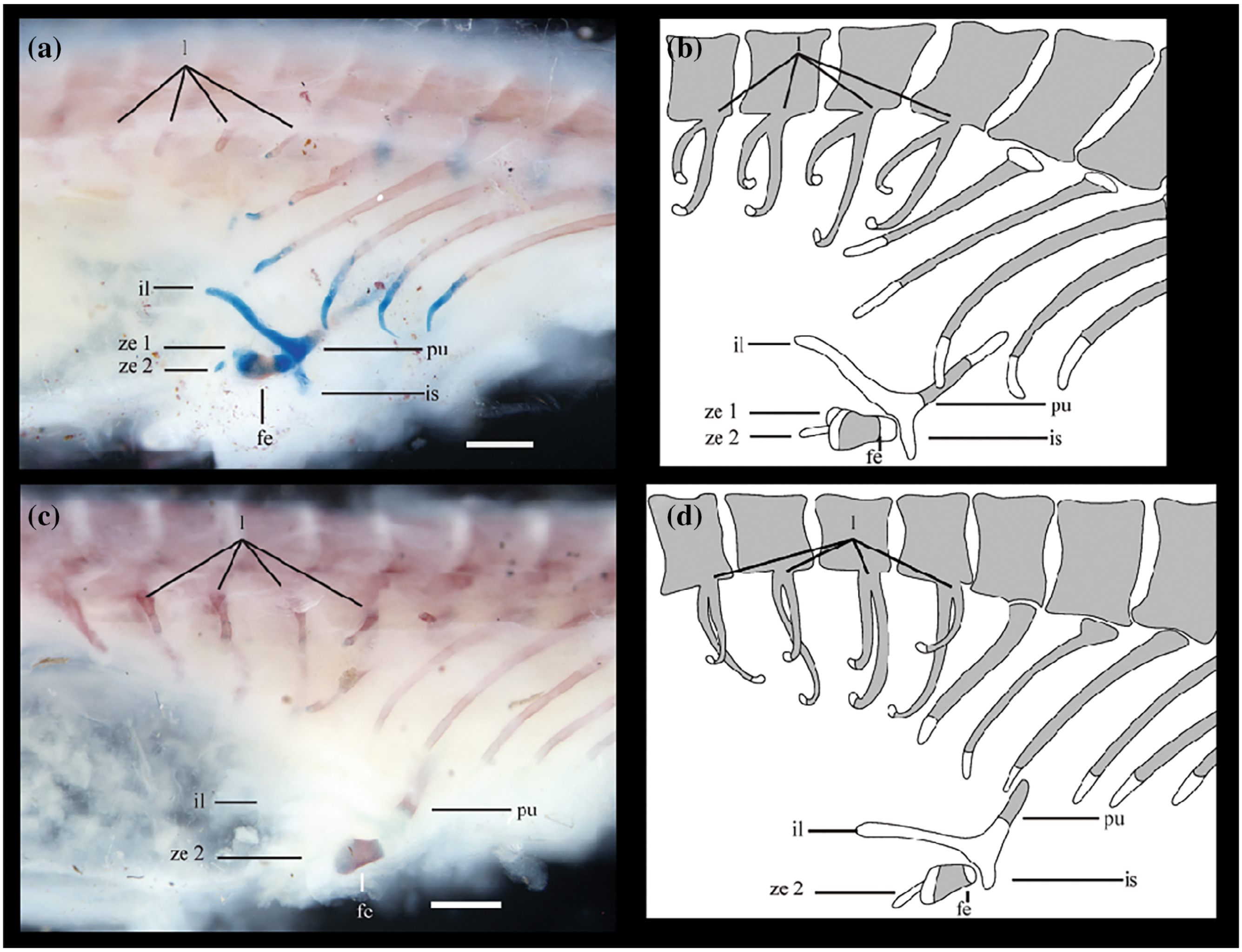

FIGURE 4 Skeletal elements of the hindlimb and pelvic girdle of embryonic Stage 37 b and c of *Anilius scytale*. (a) lateral view of the Stage 37b embryo (MPEG 18600); (b) diagram of the lateral view of the Stage 37b embryo (MPEG 18600); (c) lateral view of the Stage 37c (MPEG 22910); (d) diagram of the lateral view of the Stage 37c embryo (MPEG 22910). fe, partially ossified femur (red section); il, ilial cartilage; is, ischial cartilage; l, partially ossified lymphapophyses; pu, partially ossified pubis (red section), ze 1, zeugopodial cartilage 1; ze 2, zeugopodial cartilage 2. Scale bar: 0.5 mm.

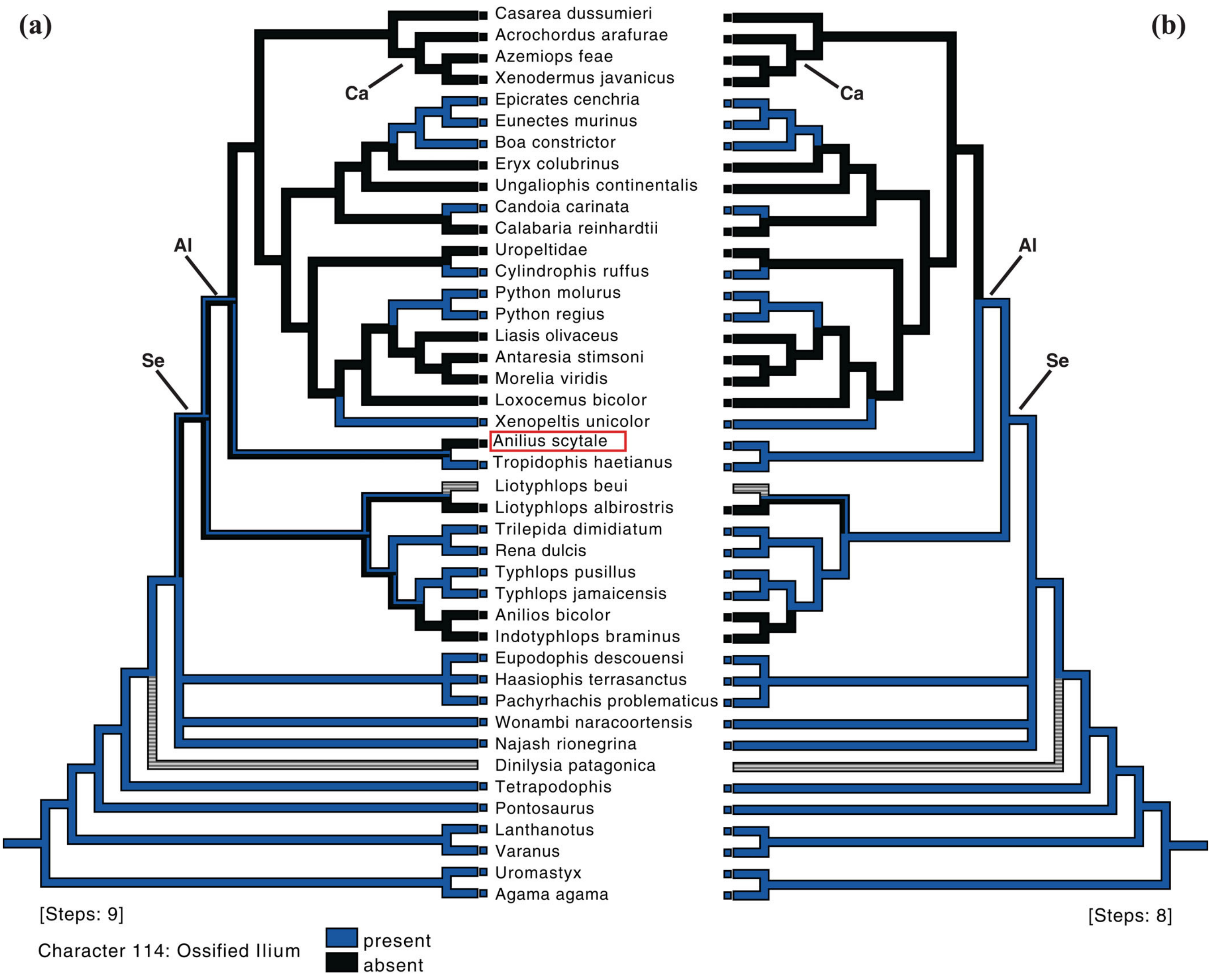

FIGURE 5 Comparisons between reconstruction of the ancestral state of the character ossified ilium with maximum parsimony optimization (character 114 sensu Palci et al., 2020). (a) recoded with *Anilius scytale* embryological data; (b) coded based on adult stage data (Palci et al., 2020). Al, Alethinophidia; Ca, Caenophidia; Se, Serpentes.

4 | DISCUSSION

In the embryology of *Anilius scytale* the hindlimb buds develop as transient structures that appeared between the Stage 22 and the Stage 30, and regress subsequently. The skeletal elements of the pelvic girdle (ilium, ischium, pubis) and the hindlimb element of the stylopod (femur) are visible from Stage 31 onwards. From Stage 31 onwards we registered the presence of zeugopodial cartilages, its number is seemingly variable in the embryo stages probably due to some variables that affect the results of the clearing and double staining technique. The pubis and femur begin to ossify towards the end of embryonic life. There is no external or internal evidence of forelimbs or the scapular girdle.

The skeletal elements of the hindlimb and pelvic girdle develop initially as cartilaginous elements in the ventral region in the vicinity of the cloaca/tail zone of the vertebral column. In subsequent stages this complex migrates dorsally positioning the pubis and ischium medial to the ribs. In adult forms of scolecophidians, pythonids and boids the pelvic elements also lie medial to the ribs (Fürbringer, 1870: 122; Leal & Cohn, 2016: Figure 4g).

4.1 | Homology hypothesis for the skeletal elements of the hindlimb and pelvic girdle in *Anilius scytale*

In the embryology of *Anilius scytale*, the hindlimb and pelvic girdle elements, here putatively named the ilium, ischium, pubis and femur, develop topological and topographical relationships similar to those seen in adult

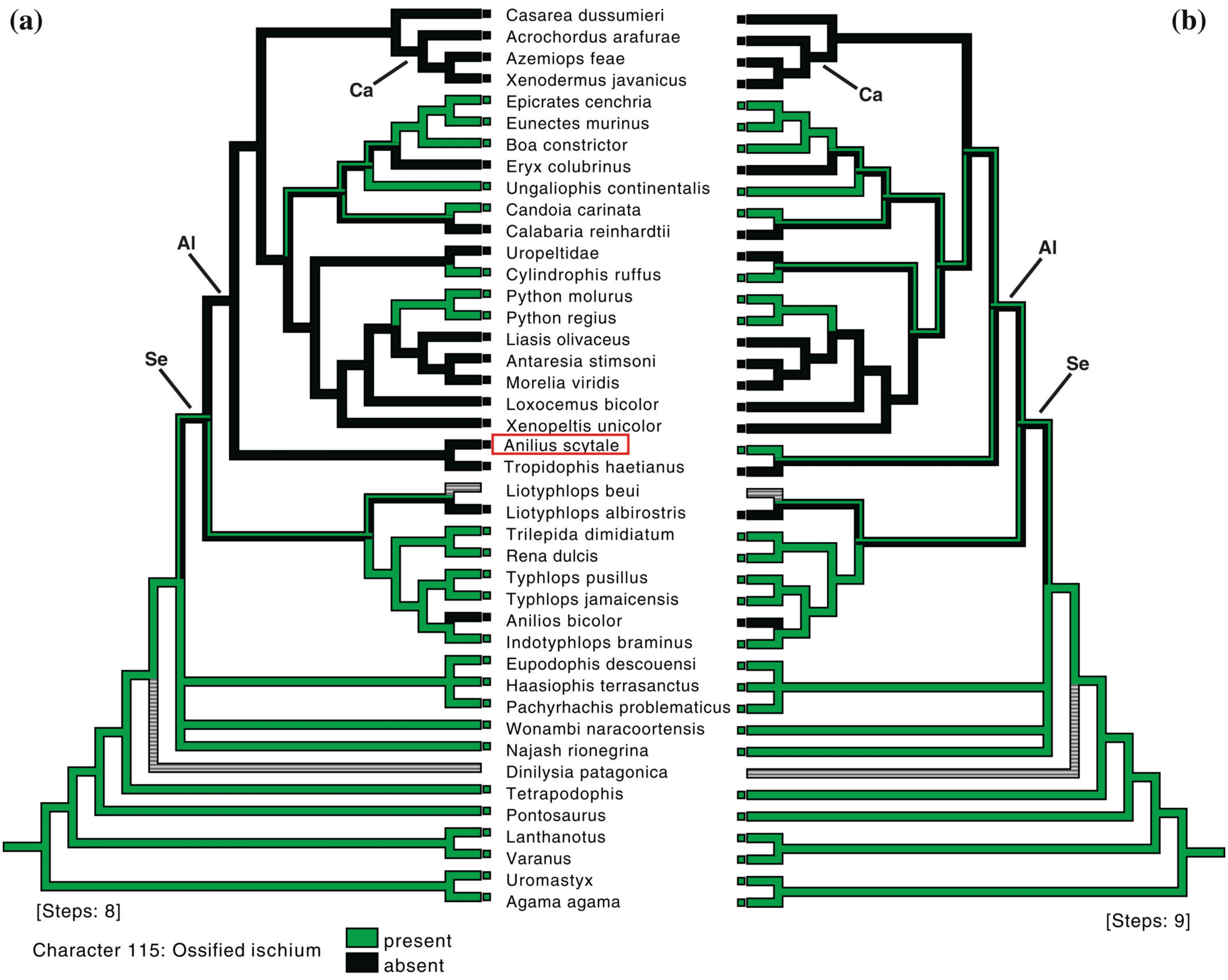

FIGURE 6 Comparisons between reconstruction of the ancestral state of the character ossified ischium with maximum parsimony optimization (character 115 sensu Palci et al., 2020). (a) recoded with *Anilius scytale* embryological data; (b) coded based on adult stage data (Palci et al., 2020). Al, Alethinophidia; Ca, Caenophidia; Se, Serpentes.

forms of normal-limbed squamates such as *Iguana iguana* (see Russell & Bauer, 2008: Figure 1. p. 14). However, in the fully expressed topology and topography of the pelvic elements seen in Stag 37 do not meet at the ventral midline of the body.

In *Anilius scytal*e the ilium does not articulate with the vertebral column. In adult forms of *Iguana iguana*, and other normal-limbed squamates, the ilium articulates with the pleurapohyses of the sacral vertebrae in the cloacal region (Hoffstetter & Gasc, 1969: p. 238). Similar topographic and topological relationships are observed in adult forms of some extant and fossil non-caenophidian snakes.

In taxa representing some basal lineages of extant [e.g., *Trilepida salgueroi* (Amaral, 1955)—Leptotyphlopidae] and fossil (e.g., *Najash rionegrina* Apesteguía & Zaher, 2006) snakes, the ilium is an elongated element with its distal region curved dorsally (Zaher et al., 2009: Figure 13A; Pinto et al., 2015: Figure 10). In *N. rionegrina*, however, the ilium articulates with the vertebral column. In other fossil snakes (e.g., *Pachyrhachis problematicus* Haas, 1979) as well as in extant species [e.g., *Python regius* (Shaw, 1802)—Pythonidae], the ilium is straight and shorter and remains posterodorsally oriented but does not articulate with the vertebral column (Zaher et al., 2009: Figure 13B; Leal & Cohn, 2018: Figure 2).

In *Anilius scytale*, the cartilage of the femur contacts the pelvic girdle cartilages at a specific location. At Stage 36, this contact region transforms into an articular region, here proposed to be the acetabulum. The acetabular region lies laterodorsal to the ischium. In the pelvic girdle of the adult stage of *Iguana iguana*, the acetabulum is

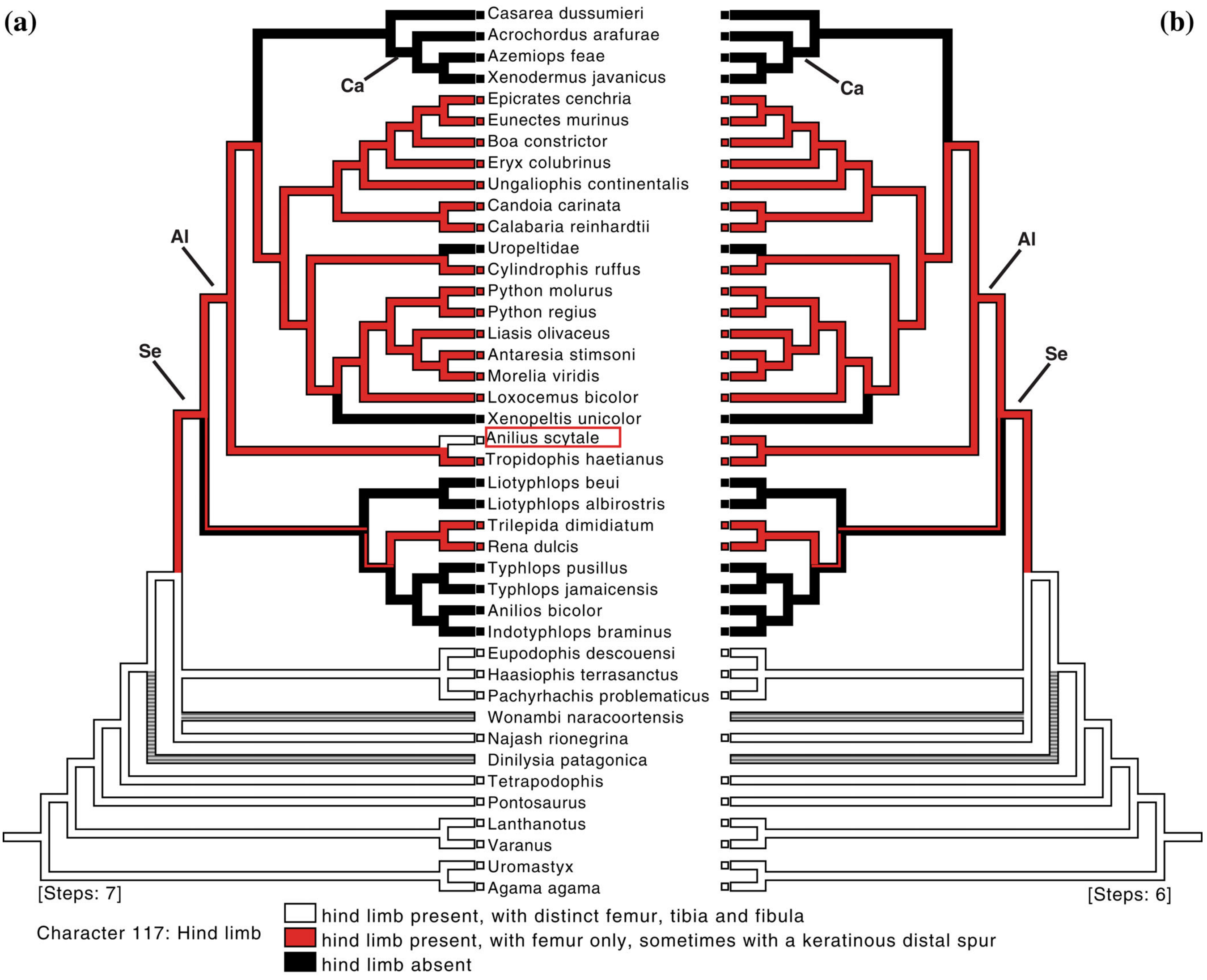

FIGURE 7 Comparisons between reconstruction of the ancestral state of the character Hindlimb with maximum parsimony optimization (character 117 sensu Palci et al., 2020). (a) recoded with *Anilius scytale* embryological data; (b) coded based on adult stage data (Palci et al., 2020). Al, Alethinophidia; Ca, Caenophidia; Se, Serpentes.

situated at the contact region between the ilium, ischium and pubis, and constitutes the articular area for the femur (Russell & Bauer, 2008: Figure 1. p. 14).

In the embryology of *Anilius scytale* we registered the presence of zeugopodial cartilages at the distal end of the femur. The dorsal cartilage (ze1) is visible at Stage 31. In the subsequent Stage 34 there is no zeugopodial cartilage visible. In Stages 36, 37a and 37b there are two cartilages that articulate with the distal end of the femur. The dorsal cartilage (ze1) is the smaller and has a globular shape. The ventral cartilage (ze2) is longer and more slender. At Stage 37c only the ventral cartilage (ze2) is visible. The inconsistency of the presence of the two zeugopodial cartilages in the embryology of *Anilius scytale* may be due to the variables that affect the clearing and double-staining technique. The alcian blue stain has affinities for mucopolysaccharides molecules of the cartilage tissues and the alizarin red for the calcium forms present in some tissues (Dingerkus & Uhler, 1977). The clearing and double-staining technique can never be predicted with certainty, but it is known that some fixing procedures affect its results (Wassersug, 1976; Potthoff, 1984). The use of buffered formalin with sodium phosphate over 24 h lead to tissues decalcification and altered the stain uptake of the cartilages (Potthoff, 1984). All female specimens used in our research were fixed following the procedures of field works expeditions of the MPEG using a solution a 10% formalin non-buffered for more than 24 h.

The presence of a single cartilage distal to the femur of *Anilius scytale* was reported by Fürbringer (1870: p. 85), who identified it as the tibia. There is indirect embryological evidence to support Fürbringer's homology hypothesis of this cartilage as an element of the zeugopodium. Its presence in some pythons has been interpreted to be evidence for a vestigial element of the zeugopodial chondrifications. The latest embryonic stage of *Malayopython reticulatus* (Pythonidae) develops a cartilage at the distal end of the femur, interpreted to be a fused tibia and fibula (Raynaud, 1985: p. 121). Leal and Cohn (2016: Figure 4) observed, in the initial stages of limb development of *Python regius* (Pythonidae), the transitory presence of chondrogenic condensations of the three limb regions, stylopod (femur), zeugopod (tibia, fibula), and autopod (digital plate). The autopod and zeugopod condensations disappeared in subsequent stages. We believe that this additional evidence supports Fürbringer‘s homology hypothesis about the cartilage lying distal to the femur in *A. scytale* being a zeugopodial cartilage (Fürbringer, 1870).

Our data illuminate Fürbringer's homology hypothesis, corroborating the presence of two zeugopodial cartilages. We confirm the homology hypothesis of tibia with one of these cartilages and propose the homology hypothesis of the fibula for the other. The position and morphology of both cartilages do not currently allow us to establish precisely the identity of each of them.

4.2 | Comparative anatomy of the skeletal elements of the hindlimb and pelvic girdle in adults of *Anilius scytale*

We observed a discrepancy when comparing the morphology of the final developmental stage of the series (Stage 37), with descriptions of adults of *Anilius scytale* (Fürbringer, 1870; McDowell, 1975; Palci et al., 2020). Embryos at Stage 37a exhibit all three pelvic elements, a femur, and a zeugopodial cartilage. The pubis and the femur are almost completely ossified, with only their distal ends remaining cartilaginous.

Based on one adult specimen Fürbringer (1870: p. 85) described the presence of three pelvic elements, the ilium, ischium and pubis, a developed femur and a tibia. The tibia was small but bore a large claw (Fürbringer, 1870, Plate III, Figure 45). The pubis was the longest of the pelvic girdle elements and was ossified. A short and straight ilium and a short, stout pubis were also observed.

McDowell (1975:p. 23) described the presence of a single pelvic bone, an ossified femur and the presence of spurs in the males. However, McDowell (1975) did not mention which pelvic element he was describing.

Palci et al. (2020) analyzed one dry skeleton, one radiographically imaged and one examined via micro CT. They described the presence of a femur (ossified) and an ossified anterior pelvic element (Palci et al., 2020: p. 637). Even though Palci and colleagues described two bones, a femur and an unnamed pelvic element (named in their figures as the pubis, Figure 2q), in their character matrix (Supporting Information Data S3), they codify *Anilius scytale* as having an ossified pubis, ilium and ischium. We did not observe the adult hindlimb and pelvic girdle skeletons of *Anilius scytale*. However, Stage 37b is close to the end of the embryonic sequence and its external morphology presents a full-grown appearance and adult coloration (Zehr, 1962: p. 327). In the postembryonic life of squamates, the major morphological changes are ossification of epiphyses and sesamoids, and calcification of cartilaginous tissues (Maisano, 2002). There are no registered observations of the disappearance of existing cartilages or bones.

Based on our developmental evidence we agree with Fürbringer's (1870) description of the skeletal elements of the hindlimb and pelvic girdle in adult forms of *Anilyus scytale*. However, we do not have evidence of adult forms of *A. scytale* that would enable us to confirm the tissue condition (cartilage/bone/calcification) of the ilium and ischium.

Fürbringer (1870), McDowell (1975) and Palci et al. (2020) recorded the presence of cloacal/pelvic spurs in *Anilus scytale*. The cloacal/pelvic spur is an external projection of the hindlimb's distal end. Its presence is commonly reported for the non-caenophidian Alethinophidia (*Cylindrophis*, *Anilius*, Pythonidae, Boidae). McDowell (1975: p. 10) reported the presence of well-developed cloacal/pelvic spurs in males of the families Pythonidae and Boidae. The males of some species of these families use their spurs during combat and courtship (Gillingham & Chambers, 1982). We did not find evidence of a cloacal/pelvic spur in the developmental series or in the adult stage of *A. scytale*.

Palci et al. (2020: p. 649) suggested that the hindlimb and pelvic elements would be retained in non-caenophidian alethinophidians due to their role in sexual stimulation via movements of the cloacal spur during courtship and copulation. This new function of the hindlimbs would be associated with the retention of ossified elements (illium, ischium, pubis, femur) and the presence of a cloacal spur. The morphology of *Anilius scytale* does not corroborate this hypothesis (Figures 5–7). *Anilius scytale* lacks cloacal spurs but retains all pelvic elements and some hindlimb elements, including a bony pubis and femur. The presence of skeletal structures in the hindlimbs of *A. scytale* does not seem to be related to sexual selection.

4.3 | The transient condition of the limb bud

In *Anilius scytale*, the hindlimb bud is transient in nature. It is visible in Stage 30, but in subsequent stages it is not visible externally, and there is no external trace of it in adult forms. In normal-limbed tetrapods, the limb buds developed at discrete locations along the body long axis, within limb fields. Each limb field is formed by the proliferation of mesenchymal cells that form the somatic layer of the lateral plate mesoderm and eventually differentiate into the girdle and limb skeletal and muscle precursor cells (Gilbert, 2010: Figure 13.2). The limb buds are intermediary stages in the development of the "internal" and external elements of the limb and occur as buds or bulges of the ectoderm resulting from migrations of mesenchymal cells into that region (Gilbert, 2010: p. 487).

A transient condition of the hindlimb bud evolved independently in squamate lineages. In the embryonic life of limbless lizards, such as *Anguis fragilis* Linnaeus, 1758 (Anguidae) and *Scelotes inornatus* (Smith, 1849) (Scincidae), and the snake *Python regius* (Pythonidae), the limb buds develop and then regress, with internal elements of the pelvic girdle and limb developing (Leal & Cohn, 2016; Raynaud, 1985). Adults of *Anguis fragilis* develop vestiges of the stylopodium (the proximal section of the femur) and all the elements of the pelvic girdle (ilium, ischium, pubis) (Raynaud, 1985: 100; Skawiński et al., 2021). Adults of *Scelotes inornatus* retain internally the proximal part of the ossified femur that ends in a cartilaginous projection (Raynaud, 1985: p. 100). In *Python regius* (Pythonidae), the hindlimb bud exhibits a similar transient nature. The resulting morphology of the skeletal elements of the hindlimb and pelvic girdle of *P. regius* consists of all the pelvic elements, a femur and a zeugopodial element, which bears a pelvic spur (Leal & Cohn, 2016: Figure 1a).

Although the embryological data for *Anilius* suggest new interpretations of the development of hindlimbs and the pelvic girdle, we recognize gaps in the analyzed embryological series, which should be rectified in future studies.

5 | CONCLUSIONS

In the embryology of *Anilius scytale* the hindlimb buds develop as transient structures that appeared between the Stage 22 and the Stage 30, and regress subsequently. The skeletal elements of the hindlimb and pelvic girdle are evident from Stage 31 onwards as follows: the pelvic girdle (ilium, ischium, pubis); the limbs stylopodium (femur) and zeugopodium (two cartilages). The pubis and femur are ossified towards the end of embryonic life. The pelvic or cloacal spur does not develop. There is no external or internal evidence of forelimbs or the scapular girdle. The skeletal elements of the hindlimb and pelvic girdle initially develop ventral to the vertebral column in the cloacal-tail region. In subsequent stages this complex migrates dorsally, positioning its anterior elements (pubis and ischium) medial to the ribs. A similar process may be associated with the condition observed in adult forms of scolecophidian, pythonid and boid snakes. The pelvic girdle has not been the focus of developmental and comparative studies in Squamata and Serpentes, and little is known about the embryonic pattern of these parts.

This study highlights the difficulty in obtaining an informative embryological series with a sufficient number of individuals representing the initial developmental stages of the hindlimb. Although our embryological series for *Anilius scytale* lacks some stages, we nonetheless were able to obtain valuable information about the development of its hindlimbs and pelvic girdle by accessing material deposited in scientific collections.

AUTHOR CONTRIBUTIONS

Ricardo Arturo Guerra-Fuentes: Conceptualization; writing – original draft; methodology; validation; visualization; writing – review and editing; formal analysis; project administration; data curation; supervision; resources. **Romário Gemaque de Sousa:** Investigation; methodology; data curation; formal analysis. **Ana Lúcia da Costa Prudente:** Conceptualization; investigation; funding acquisition; writing – original draft; methodology; validation; visualization; writing – review and editing; formal analysis; project administration; data curation; supervision; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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APPENDIX A

List of analyzed material from nonpregnant females. All females are from the Herpetology collection of the Museu Paraense Emilio Goeldi and all bear the institutional identifier MPEG.

BRAZIL. Amazonas. Coari: - 18228, - 22197, - 22198; Manicoré: - 20843; Maraã: - 16782; Presidente Figueiredo: - 17428, - 17433, - 17456, - 17505, - 17518, - 17547; Unicará: - 23508. Maranhão. Babaçulândia: - 23628; Colônia Nova: - 10302; Nova Vida: - 11090, - 11901, - 12674, - 12676, - 16039; Santa Luzia do Paruá: - 11188, - 12024, - 12025, - 12026, - 12027, - 12834, - 13658, - 14363. Mato Grosso. Tanguro: - 21736. Pará. Abaetetuba: - 18818; Almeirim: - 21348, - 21349, - 21351; Ananindeua: - 16630, - 16632, - 16635, - 16636; Babaçulândia: - 23628; Barcarena: - 18293; Belém: - 2050, - 10905, - 16432, - 16823, - 24065, - 24066, - 24067, - 24455; Capitão Poço: - 15402; Dom Eliseu: - 14275; Gurupá: - 15443; Igarapé-Açu: - 876; Maracanã: - 1599, - 1889, - 5802; Monte Dourado: - 15442; Ourém: - 1642, - 1660, - 1662, - 2169, - 4247, - 4251, - 7006, - 7027, - 7029; PA 70/Km 72: - 9494; Paragominas: - 18846; Peixe Boi: - 281, - 283, - 4604; Santa Isabel do Pará: - 19664; Santo Antônio do Tauá: - 2644, - 3966, - 6955, - 6961; Viseu: - 1290, - 7353, - 7418, - 7667, - 11468, - 11493, - 15434.

List of analyzed material from pregnant females (number of embryos).

BRAZIL. Pará. Ananindeua: - 15300 (embryos= 6); Belém: - 18600 (embryos= 6); Canaã dos Carajás: - 22450 (embryos= 3), - 26451 (embryos= 4); Marabá: - 7452 (embryos= 2); Ourém: - 6184 (embryos= 1); Parauapebas: - 22909 (embryos= 7), - 22910 (embryos= 10). Without locality: - 23902 (embryos= 1).