Chapter 6

An ontogenetic perspective on the evolution of shell size and shell shape in the land snail genus *Plectostoma*

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Author Contributions

Conceived and designed the experiments: LTS. Performed the experiments: LTS. Analyzed the data: LTS. Contributed reagents/materials/analysis tools: LTS MS. Wrote the paper: LTS MS.

Supplementary Information

(https://drive.google.com/a/naturalis.nl/#folders/0BwCpl3C2XSo9Zi1mQ0swal9xelU)

Abstract

The rampant convergent and parallel evolution in shell form in the Gastropoda is well known. Many studies focus on the functional drivers which have been regarded as a major force in shell evolution. There is, however, a scarcity in studies that aim at understanding shell form evolution with respect to their ontogeny. Hence, we investigated the evolution of shell form in the micro-landsnail genus *Plectostoma* (Diplommatinidae) from the viewpoint of shell ontogeny. We examined the aperture ontogeny profiles that describe how aperture form and growth trajectory change along the shell ontogeny, and how the aperture ontogeny profiles relate to the observed shell forms. We also estimated the phylogeny of *Plectostoma* species, and examined patterns of character evolution for shell form. Our study revealed a general issue in the characterisation of shell shape and demonstrated how shell shape differences can be expressed as differences in the ontogeny of morphospace. It is clear that in *Plectostoma* the phylogenetic history does not prevent the course of shell ontogeny, and the resultant form. Finally, each species has a unique aperture ontogeny profile that determines its shell shape while retaining a conserved developmental program that maintains shell size.

Introduction

One of the central questions in the study of phenotypic evolution is why certain structures of a species evolve to obtain a certain form, whereas the same structure in other species does not. This disparity in phenotypic evolution generates the morphological variation that is the mainstay of biodiversity. Morphological diversity in organisms may be channelled by a combination of different evolutionary constraints, namely, phylogenetic, developmental, geometric and functional constraints (Seilacher, 1991; Arnold, 1992). However, it remains a challenge to unravel the evolutionary history of an organism's form because most organisms have very complex external forms consisting of many different structural modules.

The gastropod shell, however, is a single structure, which, across gastropod taxa, shares the same developmental process and similar functions since it first appeared during the Cambrian explosion. The shell is a product of accretionary growth where shell material is added at the existing aperture by the snail mantle edge (hereafter termed: aperture ontogeny). The aperture ontogeny consists of two major components: (1) the size and shape of the aperture and (2) the growth trajectories. Jointly, these components determine the shell form (**Chapter 3**). From a functional point of view, the shell is a solid exoskeleton in which the snail's soft body can fit to safeguard it against predators and, in the case of terrestrial snails, dehydration.

These characteristics produce similarity in the general form of the gastropod shell, despite a long evolutionary history and despite Gastropoda being an extremely speciose Molluscan class. Hence, shell form is prone to convergent evolution at various taxonomic levels (Wagner & Erwin, 2006). Phylogenetically closely related species, even within genus level, are known not to have similar shell size (Teshima et al., 2003; Parmakelis et al., 2003; Johnson et al., 2004; Ketmaier, Giusti & Caccone, 2006; Bichain et al., 2007; Kameda, Kawakita & Kato, 2007; Elejalde et al., 2008a; Fiorentino et al., 2008b; Puslednik et al.,

2009; Buckley et al., 2011; Stankowski, 2011; Criscione, Law & Koehler, 2012; Johnson et al., 2012; Koehler& Johnson, 2012; Lee, Lue & Wu, 2012; Criscione & koehler, 2013; Du et al., 2013, but see Martinez-Orti et al., 2008; Kotsakiozi et al., 2013). Similary, shell shape similarity does not usually translate to a close phylogenetic relationship between species (Boato, 1991; Emberton, 1995; Teshima et al., 2003; Tongkerd et al., 2004; Elejalde et al., 2005; Noshi & Sota, 2007; Elejalde et al., 2008a; Elejalde et al., 2008b; Stankowski, 2011; Johnson et al., 2012; Haase, Esch & Misof, 2013).

Studies of convergent evolution of shell form, as the works cited in the previous paragraph, quantify shell size and shape by treating the shell as a single entity. By convention, shell size is quantified by measuring the linear dimensions of the entire shell, such as shell height and width. Shell shape is obtained by taking ratios of shell dimensions or by geometric morphometrics, which are then used as criteria to assign the shell shape into shape categories, such as elongated, depressed, and flatted shells. While these entire-shell based characterisation approaches allow us to understand how the shell form could evolve under functional constraints, this approach does not allow us to understand the evolution of the aperture ontogeny that is fundamental in determining the shell form. Studies which take such an ontogenetic approach to understanding of shell form evolution are scarce (**Chapter 3**).

Here, we investigate the evolution of shell form in the micro-landsnail genus *Plectostoma* (Diplommatinidae) from the viewpoint of shell ontogeny. *Plectostoma* can be considered as one of the most diverse genera in terms of shell form (Vermeulen, 1994, **Chapter 2**). All species begin their shell ontogeny as a regular shell form, but some species change their coiling direction towards the end of the shell ontogeny. In this paper, we restrict our discussion to the evolutionary patterns in *Plectostoma* shell size and shape, and to what extent these patterns are related to the shell ontogeny. We will not discuss the possible functional drivers, most of which are still unknown, except anti-predation functions of the twisted tuba in a few species (Schilthuizen et al., 2006; **Chapter 5**).

First, we modified the conventional approaches so that both shell ontogeny and shape information could be characterised and analysed together with the phylogenetic data. For shell size, we quantified the inner volume of the entire shell, and obtained aperture size ontogeny profiles along the whorl accretionary length (i.e., the ontogeny axis). For shell shape, we adopt the approach of **Chapter 4** that divided the shell into five homologous developmental parts, for which each species was then characterised. In addition, we also quantified the shell shape in terms of its growth trajectories – curvature and torsion, and aperture shapes along the ontogeny axis. Next, we estimated the molecular phylogenetic relationships of selected *Plectostoma* species, covering most of the shell form diversity. Then, we examined the pattern of evolution for the shell size and shape based on the characterised shell traits for all six shell characters by phylogenetic signal tests and ancestral character estimates. Lastly, we explored the ontogeny of shell size based on the aperture size ontogeny profile and the ontogeny shell shapes based on the ontogenetic morphospace that was constructed from the three others aperture ontogeny profiles (curvature, torsion, and aperture shape).

Materials and Methods

Ethics Statement

The permissions for collecting specimens in Malaysia were given to LTS by the Economic Planning Unit, Prime Minister's Department (UPE: 40/200/19/2524), State Planning Unit, Chief Minister's Department, Sarawak ((47) UPN/S/G1/I/10.1 Vol.27), Forest Department Sarawak (Research Permit NPW.907.4.4(V)-19; Park Permit No. 07/2010; Export Permit No. 09003).

Ontogeny of shell size and shape

The relationship between the shell size, ontogeny axis length and aperture size

We examined whether there are associations between the shell size (internal volume in mm^3), ontogeny axis length (corresponding to total whorl length in mm), and aperture size changes along the shell ontogeny. We obtained these three shell variables from 11 representative *Plectostoma* species (Table 1) by using the 3D approach as described in **Chapter 4**. Here, we only briefly describe this methodology.

First, 3D models of *Plectostoma* shells were obtained with CT-scanning. Then, we used the 3D modelling software Blender ver. 2.63 (www.blender.org) to retopologise the aperture outlines from the scanned 3D models and created retopologised shell models based on these aperture outlines. Next, we used custom written Python scripts to extract: (1) ontogeny axis data, in terms of the length, and growth trajectories for curvature and torsion, and (2) aperture form data, in terms of perimeter and shape, from the retopologised shell models in Blender. Finally, the growth trajectories and aperture form variables were analysed as they developed along the ontogeny (hereafter termed: aperture ontogeny profiles).

After that, we examined the pattern of aperture size changes along the shell ontogeny of each species. Then, we used Pearson correlation to test the correlation between the log-transformed shell volume and the log-transformed ontogeny axis length. In view of the strong correlation that we found (see Results), we also examined the pattern of aperture size changes of all 11 shells after their respective ontogeny axis length (mm) was rescaled by standardisation, which was done by dividing the ontogeny axis position of the apertures of each shell by axis length. All data analysis and exploration were done in R version 3.0.1 (R Core Team, 2013) (R scripts in Supplementary File 1).

Table 1. Specimen data for phylogenetic analysis.

Species	Collection number	18S	28S	16S	COI	Locality	Collection Date	Latitude, Longitude
Plectostoma austeni (Smith, 1894)	BOR 5546	#####	#####	#####	#####	Malaysia; Sarawak; Serian; Gunung Rimau near Kampung Benuk	19-Aug-2010	1.319, 110.291
Plectostoma christae (Maassen, 2001)	BOR 5572	KC420367	KC420316	KC420413	KC420271	Malaysia; Kelantan; Limestone in FELDA Ciku 5	17-Feb-2010	5.004, 102.2
Plectostoma concinnum (Fulton, 1901)	n.a.	#####	#####	#####	#####	Malaysia; Sabah; Sandakan; limestone hill `Keruak`	16-Dec-2010	5.518, 118.291
Plectostoma crassipupa (van Benthem Jutting, 1952)	BOR 5512	KC420400	KC420353	KC420451	KC420304	Malaysia; Kelantan; ; Limestone hill near Kampung Paloh, on the right hand side of the road no 8 to	16-Jun-2011	4.992, 102.228

						Gua Musang		
Plectostoma davisoni Liew, Vermeulen, Marzuki & Schilthuizen 2014	BOR 5508	KC250938	KC250963	KC250913	KC250872	Malaysia; Kelantan; Limestone hill on the right hand side of the road D29, km 17 from Jelawang to Gua Musang	28-May-2011	4.985, 101.965
Plectostoma grandispinosum (Godwin Austen, 1889)	BOR 5590	KC250946	KC250971	KC250921	KC250879	Malaysia; Sarawak; Miri; along the trail to the Bukit Kasut, Niah National Park	12-Jun-2010	3.804, 113.78
Plectostoma ikanensis Liew, Vermeulen, Marzuki & Schilthuizen 2014	BOR 5504	KC250929	KC250954	KC250903	KC250862	Malaysia; Kelantan; ; Limestone hills `Ciku 2`. In the FELDA plantation Ciku 2	28-May-2011	4.924, 102.177
Plectostoma kubuensis Liew, Vermeulen, Marzuki & Schilthuizen 2014	BOR 5519	KC420366	KC420315	KC420412	KC420270	Malaysia; Perlis; Bukit Kubu. Loc 3	21-May-2011	6.404, 100.144
Plectostoma tenggekensis Liew, Vermeulen, Marzuki & Schilthuizen 2014	BOR 5596	KC420380	KC420332	KC420431	n.a.	Malaysia; Pahang; loc. 14 Bukit Tenggek (c. 45 km NW of Kuantan)	27-Jun-1997	4.014, 103.159
Plectostoma laidlawi (Sykes, 1902)	BOR 5510	KC420372	KC420323	KC420421	KC420279	Malaysia; Kelantan; ; Limestone hill in Kampung Bayu. About 337 km from Kuala Lumpur by road no. 8	28-May-2011	5.09, 102.22
Plectostoma pulchellum (Godwin Austen, 1890)	BOR 5563	KC250924	KC250949	KC250898	KC250857	Malaysia; Sarawak; Mulu National Park, Moon Cave	24-Sep-2010	4.044, 114.815
Plectostoma pumilio (Smith, 1894)	BOR 5550	#####	#####	#####	#####	Malaysia; Sarawak; Serian; Unnamed limestone hill near Kg. Sematan, along the new road to Bau	19-Aug-2010	1.296, 110.274
Plectostoma relauensis Liew, Vermeulen, Marzuki & Schilthuizen 2014	BOR 5511	KC420370	KC420321	KC420419	KC420277	Malaysia; Kelantan; Taman Negara, Sungai Relau Station. Gua Gajah	15-Jun-2011	4.642, 102.063
Plectostoma retrovertens (Tomlin, 1938)	BOR 5559	KC420392	KC420345	KC420443	KC420297	Malaysia; Pahang; Karak; Bukit Chintamanis	29-Aug-2010	3.446, 102.014
Plectostoma salpidomon (van Benthem Jutting, 1952)	BOR 5569	KC250934	KC250959	KC250909	KC250868	Malaysia; Pahang; Kuala Lipis; Gua Bama	16-Feb-2010	4.194, 101.967
Plectostoma senex (van Benthem Jutting, 1952)	BOR 5575	KC250926	KC250951	KC250900	KC250859	Malaysia; Pahang; Kuantan; Gua Charas	20-Feb-2010	3.908, 103.147
Plectostoma sinyumensis (Maassen, 2001)	BOR 5537	KC250936	KC250961	KC250911	KC250870	Malaysia; Pahang; Gunung Jebak Puyuh, near Gunung Senyum	16-Jul-2010	3.7, 102.453
Plectostoma siphonostomum (van Benthem Jutting, 1952)	BOR 5557	KC250932	KC250957	KC250906	KC250865	Malaysia; Pahang; Chegar Perah; Limestone hill on the left hand side of the road no. 8 toward Kuala Lipis. Near Kampung Chegar Perah I and II FELDA	27-Aug-2010	4.487, 101.976
Plectostoma stellasubis (Vermeulen, 1994)	BOR 5588	KC250925	KC250950	KC250899	KC250858	Malaysia; Sarawak; Miri; Location near the Great Cave. Niah National Park	11-Jun-2010	3.804, 113.78
Plectostoma umbilicatum (van Benthem Jutting, 1952)	BOR 5503	KC420374	KC420325	KC420423	KC420281	Malaysia; Pahang; Gua Tongkat	29-May-2011	3.891, 102.473
Plectostoma wallacei busauense (Smith, 1893)	BOR 5545	KC250941	KC250966	KC250916	KC250875	Malaysia; Sarawak; Serian; Gunung Barau near Kampung Benuk	19-Aug-2010	1.323, 110.3

Shell shape ontogeny in aperture ontogenetic morphospace

In addition to the shell shape and size, we examined the remaining aperture ontogeny profiles for curvature, torsion and aperture shape (for the latter, we used the first principal component, which explained 46 % of the variation) of the 11 shells along the standardised ontogeny axis. To remove the size component from the morphospace, we standardised the curvature and torsion profiles by multiplying them with the aperture size profile, because the raw aperture curvature and torsion estimates may be related to the aperture size (Okamoto, 1988).

Then, we visualised the aperture ontogenetic morphospace by plotting the three aperture ontogeny profiles, namely: (1) aperture shape scores; (2) standardised torsion, and (3) standardised curvature. Finally, each of the apertures in the morphospace was labelled with its species identity and its standardised ontogeny position (%) in two separate panels.

To explore this ontogenetic morphospace, we first identified the outlier aperture ontogeny in the morphospace, defined as the apertures with ontogeny profiles larger than quartile 3, and smaller than quartile 1, by at least 1.5 times the interquartile range. This range was selected for the sake of convenience so that the outliers can be identified within and between species ontogenetic morphospace. After the region of ontogenetic morphospace with outliers was identified, the remaining occupied ontogenetic morphospace was examined. We investigated whether the occupancy of ontogeny morphospace was associated with the shell shape categories (see below) and whether it was specific to species or to a particular ontogeny stage. All data explorations and analyses were done in R (R scripts in Supplementary File 1).

Evolution of shell size and shape

Molecular Phylogenetics

We included 21 *Plectostoma* species in our molecular phylogenetic analysis, seven endemic to Borneo and 14 to Peninsular Malaysia. These species form a fair representation for *Plectostoma* shell diversity (Vermeulen, 1994; **Chapter 2**). In addition to these ingroup taxa, four outgroup taxa were included in the phylogenetic analysis. Sequence data for these outgroup taxa, which include three genera of the Diplommatinidae and a species of the Cochlostomatinae, were obtained from Webster et al. (2012). The details of these specimens and the Genbank accession numbers are listed in Table 1.

We extracted DNA from one specimen (entire animal and its shell) for each species by using the E.Z.N.A. Mollusc DNA kit (OMEGA bio-tek) and the manufacturer's extraction protocol. After extraction, PCR was carried out to amplify four regions, namely, 16S (mitochondrial, Palumbi 1996), COI (mitochondrial, Folmer et al. 1994), 28S (nuclear, Park and Foighil 2000), and 18S (nuclear, Stothard et al. 2000). We followed the PCR protocols of Webster et al. (2012). After that, positive PCR products were sequenced by Macrogen sequencing service (Macrogen Inc., Europe).

Alignment of sequences was done with Bioedit ver 7.1.3 (Hall 1999) and adjusted manually. The final aligned data matrix consists of 2,234 positions (Supplementary File 2).We divided the dataset into six partitions which represent the three separate codon positions of COI and

the remaining three sequenced genetic regions. We inferred a phylogeny using both Bayesian and maximum likelihood analyses.

For Bayesian analysis, we used jModelTest 2.1.4 (Darriba et al., 2012) to select the most appropriate model, based on the Akaike Information Criterion (AICc) for each of the six partitions. The best fits were: the HKY+I+G model for 16S; GTR+I+G for 28S, COI(1^{st} codon); GTR+I for COI(2^{nd} codon); HKY + G for COI(3^{rd} codon); and JC for 18S. Bayesian inference was run in MrBayes ver. 3.2.1 (Huelsenbeck and Ronquist 2001) with the following setting: mcmc ngen=1,000,000; nchains=4; samplefreq=100; average deviation of split frequencies < 0.01; and a burn-in value of 25%. We retained the consensus tree for further analysis. Maximum likelihood analysis was done in RAxML v8.0.0 (Stamatakis, 2014) via the CIPRESS portal v3.3 (Miller, Pfeiffer and Schwartz, 2010). We set the GTRGAMMA model for the concatenated six partitions and 1,000 bootstrap replicates.

Ancestral state reconstructions

We scored shell shape as five discrete characters representing five subsequent phases in shell ontogeny, namely, apex shape, apical spire shapes, basal spire shape, tuba coiling type, and aperture opening orientation. The detailed description of these shell parts from the developmental and morphological points of view can be found in **Chapter 2**, with the addition of one extra category for apical spire shape, namely equal lateral, when the ratio of apical spire height and width is equal to one (Supplementary File 3).. For this reason, five species that had previously been categorised as oblong were moved to this new category. In addition, four of the shells that were categorised into moderately convex/slightly convex apex were now categorised as moderately convex; and one *Plectostoma laidlawi* was now in the distinctly convex apex category (cf. Table 3 in **Chapter 2**).

Then, we reconstructed ancestral states of the five discrete shell shape characters and the continuous shell size variable on the Bayesian estimated consensus tree. The ancestral state reconstructions were done with both maximum likelihood using the 'ace' function in R package 'ape' (Paradis, Claude & Strimmer, 2004), and maximum parsimony using MESQUITE 2.75 (Maddison & Maddison, 2011).

Phylogenetic signal

We investigated whether closely related species are more likely to have similar shell traits than expected by chance by examining the phylogenetic signal with two approaches, namely, maximum likelihood in terms of lambda (λ) (Pagel, 1999), and maximum parsimony in terms of randomisation tests. As required by lambda analysis, we transformed the Bayesian consensus tree into an ultrametric tree by using Sanderson's semi-parametric penalized likelihood approach (Sanderson, 2002) as implemented in the R package 'ape' (i.e., 'chronopl' function). All data analysis and exploration was done in R version 3.0.1 (R Core Team, 2013) (R scripts in Supplementary File 1).

We estimated λ and its model likelihood score of each shell trait (i.e. alternative model) on the basis of the *Plectostoma* phylogenetic tree by using the "*fitContinuous*" function for shell

size and the "*fitDiscrete*" function for the five shell shape characters, in the "Geiger" package (Harmon et al., 2008) via R (R Core Team, 2013). After that, we repeated the analysis for a null model, for which the *Plectostoma* phylogenetic tree was transformed to a total basal polytomy tree (i.e. $\lambda = 0$, no phylogenetic signal) by using the "*transform*" function. Lastly, we examined whether there was a significant phylogenetic signal in each of the shell traits by running a likelihood ratio test for both alternative and null model likelihood scores.

In addition to the likelihood method above, we performed a randomisation test for the five discrete shell shape characters based on the parsimony method implemented in Mesquite (Maddison & Maddison, 2011). First, we created a null model that consists of 999 random trees for each shell trait by reshuffling terminal taxa. The null model is a distribution of steps in character for all random trees, and has percentile boundary of 0.05. Then, we obtained the steps value for each shell trait and compared the value with the respective null model. Shell traits were considered to have significant phylogenetic signal if the steps value fell outside the percentile boundary. In addition, we tested the phylogenetic signal in shell size with K of Blomberg et al. (2003) by using the 'phylosig' function in R package 'phytools' (Revell, 2012).

Results

Ontogeny of shell size and shape

The relationship between the shell size, ontogeny axis length and aperture size

There are associations among shell volume, ontogeny axis length and aperture size profile (Figure 1A). Figure 1C shows a strong correlation between the log-transformed shell volume and the log-transformed ontogeny axis length ($r = 0.91$, $t = 6.6805$, $df = 9$, $p = 0.000$). In addition, the larger shells always have larger aperture sizes than smaller shells at the same point of their standardised ontogeny axis (Figure 1B).

All species, with the exception of *Plectostoma grandispinosum*, have similar patterns in aperture size changes along the standardised shell ontogeny axis (Figure 1D). Initially, aperture size increases constantly before it reaches the first plateau at about 70 – 75 % of the shell ontogeny. Then, aperture size decreases toward the shell's constriction around 80 – 90 % of the shell ontogeny. After the constriction phase, the aperture size increases until the end of the shell ontogeny. *P. grandispinosum*, on the other hand, has its first aperture size plateau at 50 % and its constriction at about 60 % of the shell ontogeny. We found that *P. grandispinosum* has a *ca*. 30 % longer ontogeny axis during the tuba phase as compared to the rest of the species, if the standardised ontogeny axis of *P. grandispinosum* was rescaled until its constriction phase – a developmental homology matched with the other species.

Shell shape ontogeny in aperture ontogenetic morphospace

Figure 2 shows the aperture ontogeny profiles for the 11 *Plectostoma* species on the standardised ontogeny axis (raw data: Supplementary File 4). The modest changes in curvature and torsion profile of the shells are generally in accord with their regularly coiled conical (*i.e.,* logarithmically spiralling) shell before the constriction phase and tuba phase of

the ontogeny (raw curvature and torsion in Figures 2A and 2B; standardised curvature and torsion in Supplementary File 5: Figures S1-S5).

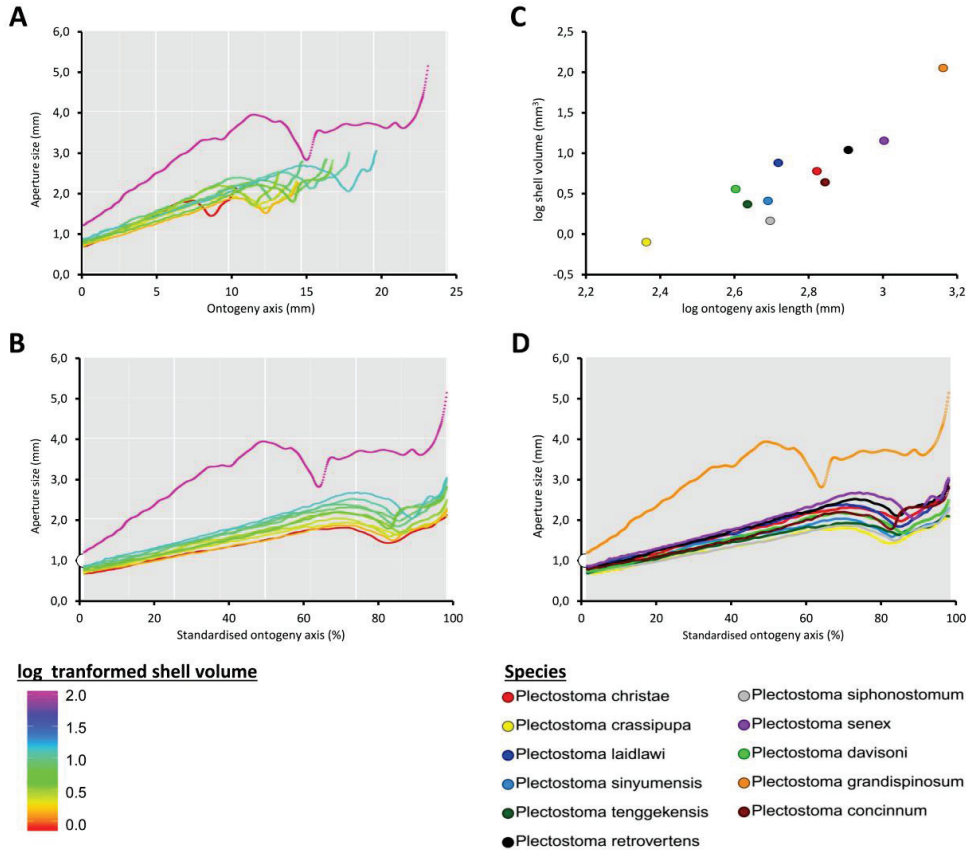

Figure 1. The ontogeny analysis of shell size of the 11 *Plectostoma* species. (A) Plot of aperture size ontogeny profile vs. ontogeny axis, and each profile annotated by its size. (B) Plot of aperture size ontogeny profile vs. standardised ontogeny axis, and each species profile annotated by its size. (C) Correlation between log-transformed shell volume and log-transformed ontogeny axis length. (D) Same as C, but each profile annotated by the species identity.

Figure 3 shows the ontogenetic morphospace of the 11 species. The outliers of the aperture shape changes along the ontogeny always are located either at the very beginning of the shell ontogeny (before 10%) or at the later phase of the ontogeny (after 60%) (Figure 3B). Nine of the 11 species occupied the outlier aperture shape space, either at the beginning or at the later stage of shell ontogeny, but never both (Figure 3E).

The outliers of the aperture standardised torsion always are located at the end of the ontogeny (after 80%) and some of these outliers are also outliers in the standardised curvature (Figure 3A). This space is occupied by the species with a twisted tuba, namely, *P. laidlawi*, *P. tenggekensis*, *P. retrovertens*, *P. davisoni*, *P. grandispinosum*, and *P. concinnum* (Figure 3D).

When the non-outlier ontogenetic morphospace in Figure 4 was examined closely, the species that share similar shell shapes as far as the five shell characters are concerned, do not necessarily share the same ontogenetic morphospace (see also Supplementary File 5: Figure S6-S8). Most species occupy a species-specific region in the aperture shape and standardised curvature morphospace in the first half of shell ontogeny (0 % – *ca*. 50 %) (Figure 4B and Figure 4E).

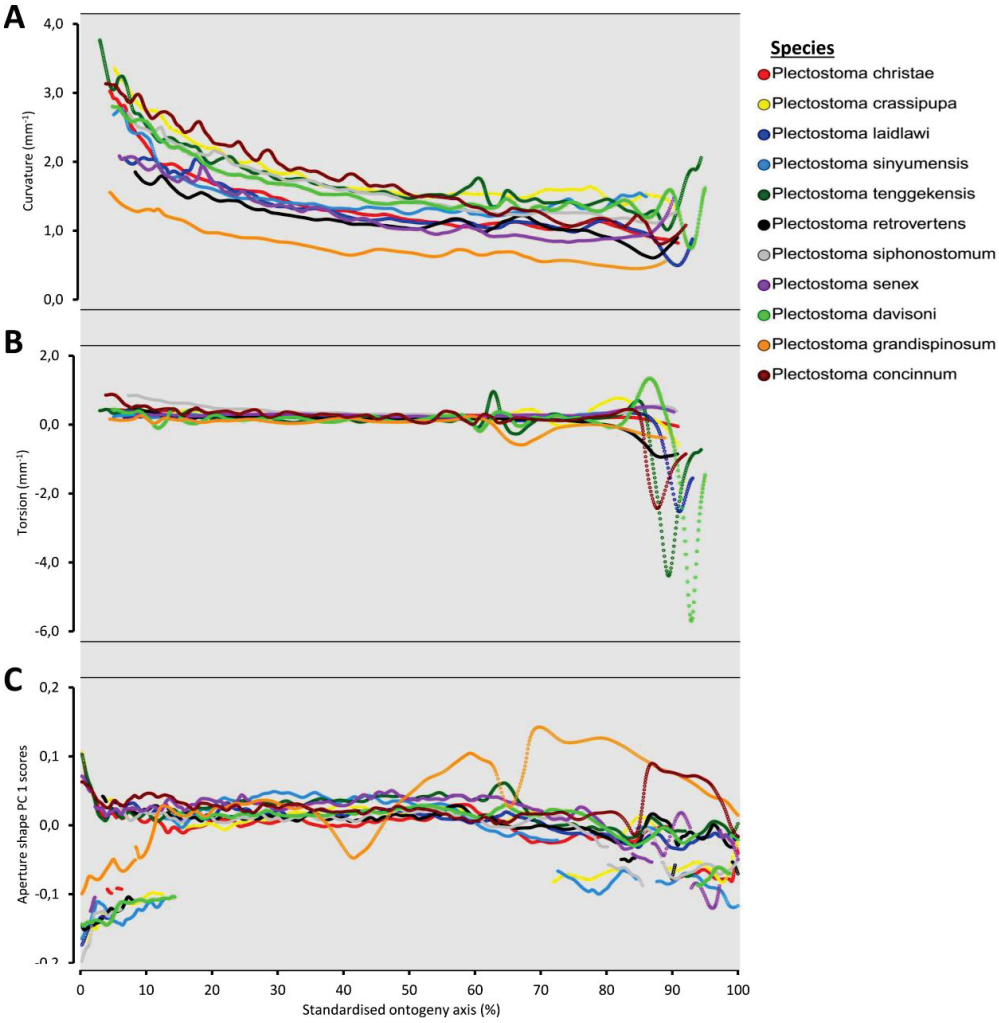

Figure 2. Aperture ontogeny profiles of the 11 *Plectostoma* species. (A) Plot of curvature vs. standardised ontogeny axis. (B) Plot of torsion vs. standardised ontogeny axis. (C) Plot of aperture shape scores vs. standardised ontogeny axis.

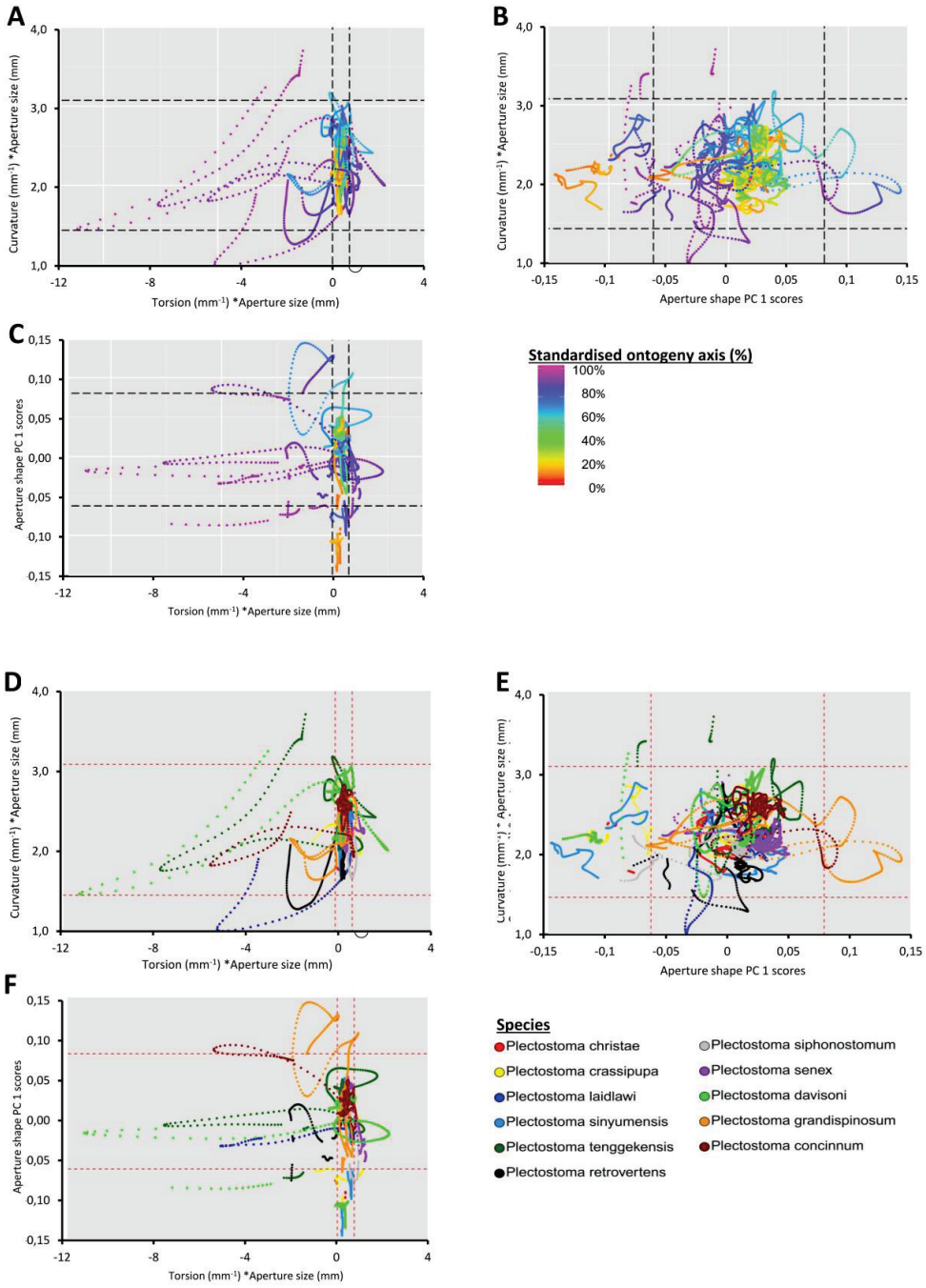

Figure 3. Shell ontogenetic morphospace of the 11 *Plectostoma* species. (A) – (C) Three panels that show each dimension of the ontogenetic morphospace, and each aperture annotated by its position along the standardised ontogeny axis. (D) – (E) Three panels that show each dimension of the ontogenetic morphospace and each aperture annotated by its species identity. The dashed line marks the outlier values for each ontogenetic morphospace axis.

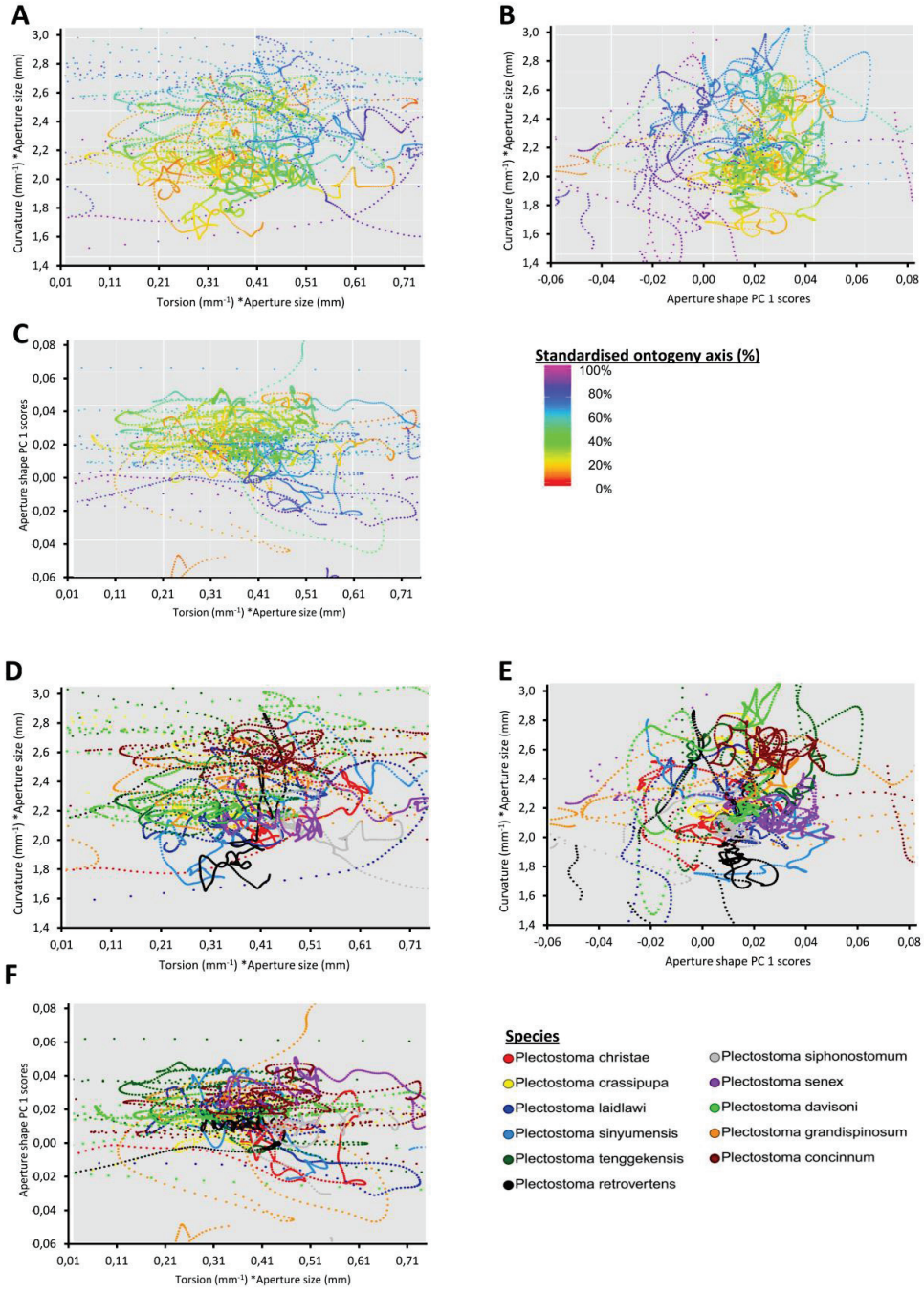

Figure 4. Shell ontogenetic morphospace of the 11 *Plectostoma* species after exclusion of the outlier region (see Figure 3). (A) – (C) Three panels that show each dimension of the ontogenetic morphospace, and each aperture annotated by its position along the standardised ontogeny axis. (D) – (E) Three panels that show each dimension of the ontogenetic morphospace and each aperture annotated by its species identity.

Evolution of shell size and shape

Molecular Phylogenetics

The phylogenetic relationships among *Plectostoma* species can be seen in Figure 5. The monophyly of the *Plectostoma* clade and the majority of its internal nodes are well supported by Bayesian posterior probabilities (> 0.95). Similarly, the monophyly of *Plectostoma* and the four major clades are also well supported in the maximum likelihood analysis (bootstrap > 85%) (Supplementary File 6). Each of the major clades consists of species that are diverse in shell form.

Ancestral state reconstructions

Figure 6 shows the results from maximum likelihood ancestral state reconstruction for the shell shapes and shell size. The results are consistent with the reconstruction based on maximum parsimony (Supplementary File7). The ancestral shell size is estimated to be about 2.5 mm^3 – an intermediate size for *Plectostoma* species (95% CI: 1.7 – 3.2) (Figure 6F). The ancestral shapes of the three shell spire parts are present in almost all deep nodes (i.e. backbone nodes for the four clades) in the phylogeny. The different apex and spire shapes have been derived from their respective ancestral states multiple times in all four major clades during the radiation of *Plectostoma* (Figure 6A, 6B, and 6C).

Figure 6D shows that a twisted tuba is an ancestral trait for *Plectostoma*, backbone nodes, and its clades 1, 2 and 3. The transition from twisted tuba to the other two tuba types does not occur in clade 1. There, a regularly coiled tuba has been derived from the twisted tuba independently from those in clades 3 and 4; and a distorted tuba has been derived from a twisted tuba independently in clades 2 and 3. There is a single case of secondary gain of a twisted tuba in clade 4 after it was lost. The ancestral *Plectostoma* shell had a leftward aperture (Figure 6E). This ancestral apertural state has been retained in the ancestral shell of clades 1 and 2, but there are several transitions to other aperture inclinations in the remaining backbone nodes and particularly in clade 3.

Phylogenetic signal

Shell size, the shapes of all shell spire parts (apex, apical spire, and basal spire), tuba coiling type, and aperture opening orientation show no significant phylogenetic signal, based on likelihood and parsimony methods (Table 2).

Table 2. Phylogenetic signal test results obtained from likelihood method (λ) and randomisation method (Steps in character).

Shell Traits	Lambda (λ)	Likelihood score (alternative model)	Likelihood score (null model, λ=0)	*p*-value		Steps in Character	95% confidence interval of steps
Size [1]	0.84	-35.86	-37.15	0.109		-	-
Apex	0.92	-13.80	-14.56	0.219		5	4 – 10
Apical spire	0.88	-16.34	-16.72	0.387		4	3 – 6
Basal spire	0.53	-20.73	-22.16	0.091		7	5 – 11
Tuba	1	-16.03	-16.72	0.239		4	4 – 6
Apertural view	0.00	-26.62	-26.62	1.000		8	8 – 11

[1] randomisation method cannot be done on size, thus Blomberg et al.'s K was done (K = 0.92, p = 0.062).

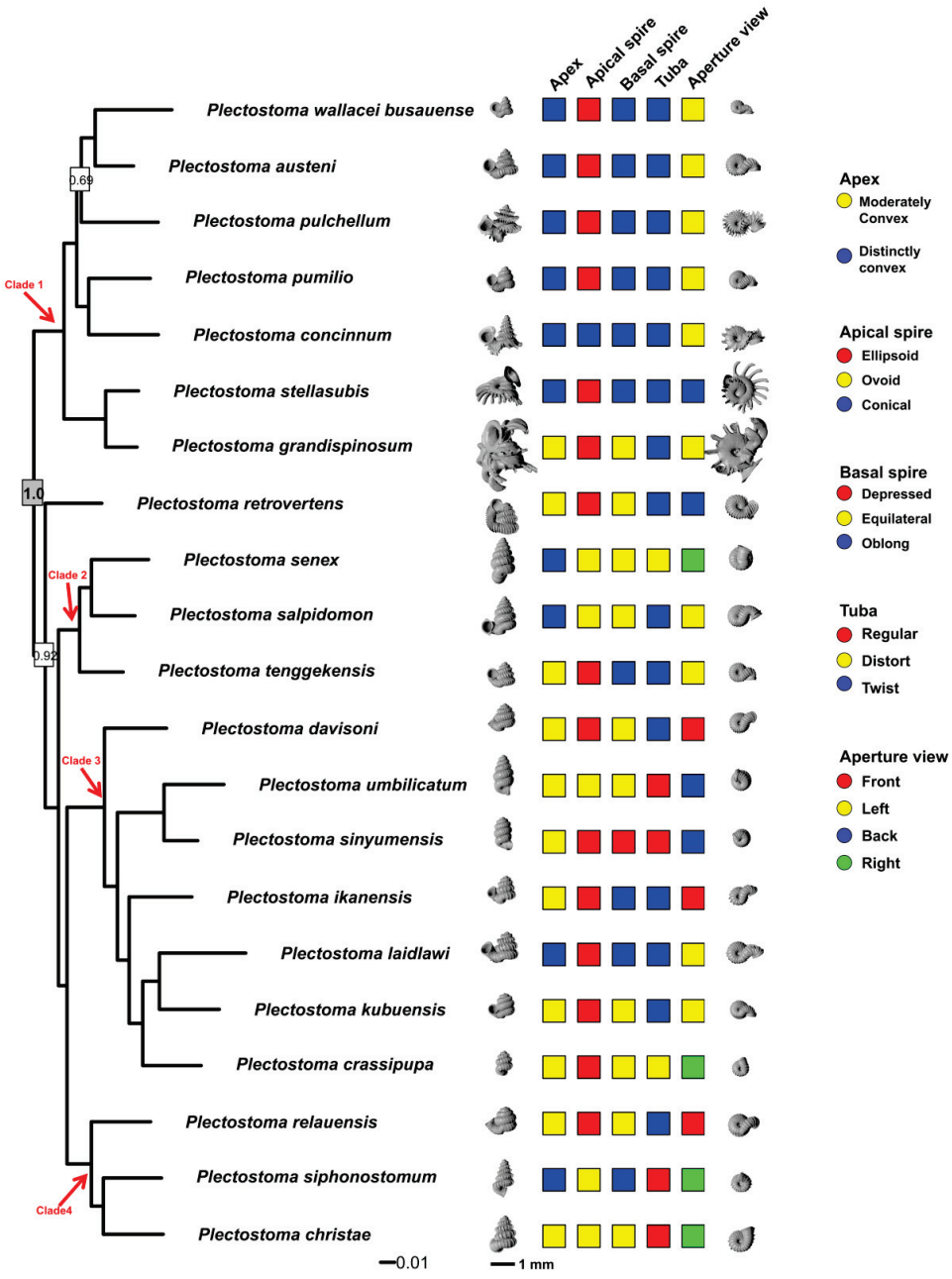

Figure 5. Phylogenetic tree and character states for each shell part for 21 *Plectostoma* species. The Bayesian estimated consensus phylogenetic tree, in which the monophyly of *Plectostoma* was well supported (grey box, posterior probability >95%) consists of four major clades. All nodes were well supported, except the two nodes that are annotated in the white box. The character states of five shell parts were annotated by different colours, and the left lateral and bottom views of the shell are shown.

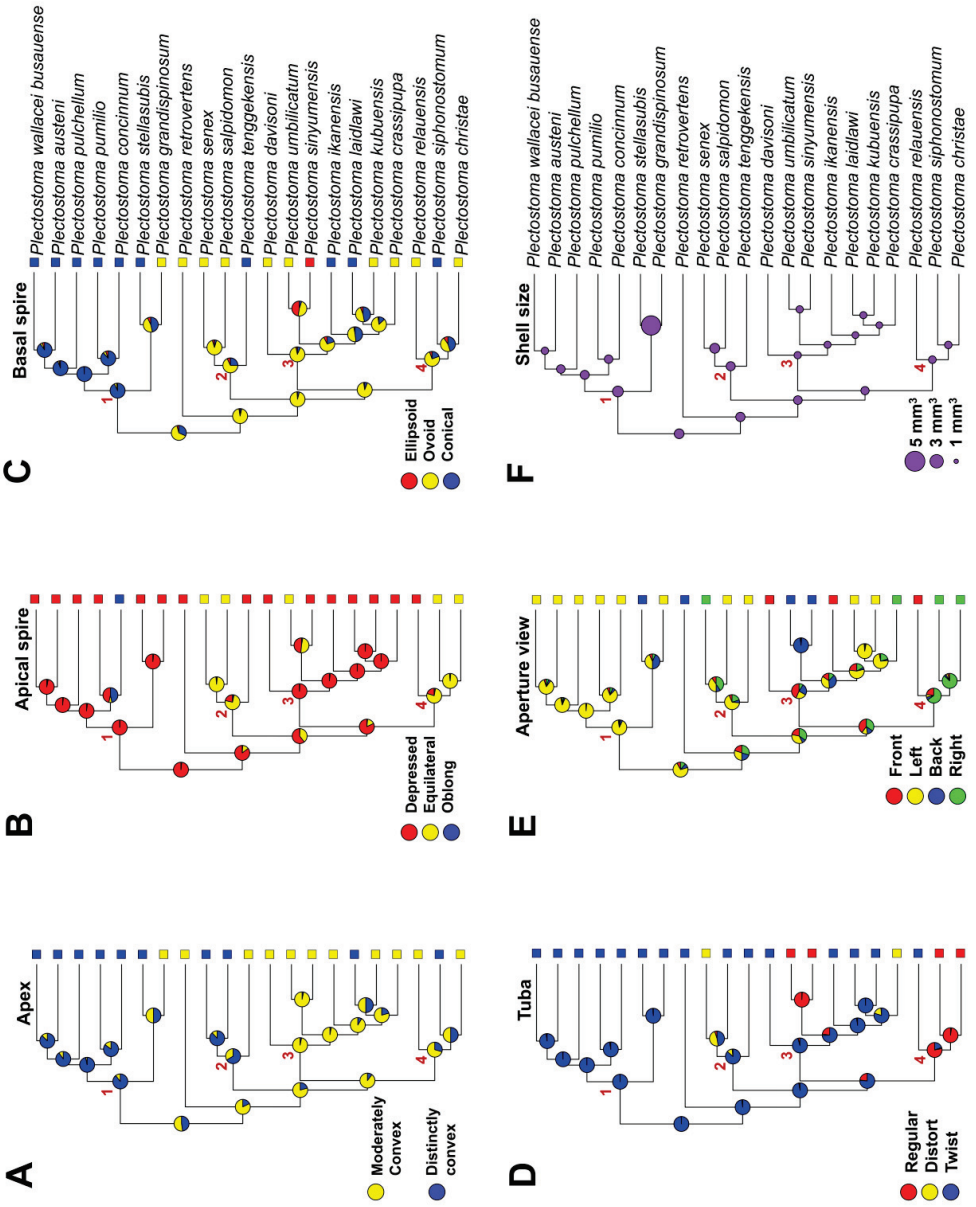

Figure 6. Ancestral state reconstructions for shell shape and size, using the maximum likelihood method. (A) Shell apex shapes. (B) Shell apical spire shapes. (C) Shell basal spire shapes. (D) Tuba coiling types. (E) Direction of aperture view. (F) Shell size.

Discussion

Shell size ontogeny and evolution

From a developmental point of view, we may expect a snail to grow a shell into which its entire soft body fits when it withdraws into the shell. From functional and developmental points of view, the shell volume is a more accurate measurement of shell size than linear dimensions such as shell height and width (see also Gould, 1984). Conventional linear measurements are extremely effective for size comparisons between shells of similar shape. However, they have limitations when comparison is made between shells that are of different shape. For example, shell height comparison between a discoidal shell and a fusiform shell tells very little about the size differences because the dimensional measurements are tied to shell shapes that result from different coiling strategies. Similarly, the whorl count that is often used in conjunction with the shell dimensional measurements has the same problem when dealing with shells that are very different in shape (Cain, 1980). These two issues are particularly relevant to *Plectostoma* shells, where comparison between diverse shell forms cannot be easily carried out with such conventional shell size measurements.

Shell size is controlled by the shell growth rate, which, in turn has both genetic and environmental components (see review by Goodfriend, 1986; and others: Baur & Raboud, 1988; Baur, Baur & Froberg, 1994; d'Avila & Bessa, 2005; Miereles et al., 2008; Martin & Bergey, 2013) that are hard to disentangle. In the case of *Plectostoma*, we do not know to what extent environmental factors may impact the, presumably genetically determined, aperture size ontogeny profiles in a species. Nevertheless, all *Plectostoma* species have identifiable shell-developmental homologies – the constriction before the tuba and differentiated peristome and aperture, which allows for reference points in the ontogeny and to define the end of shell ontogeny.

The evolution of *Plectostoma* shell size (shell volume) is not constrained by phylogeny. This finding confirms the results of most previous studies in which shell size (estimated by other metrics) tends to be as dissimilar between closely related species within a genus, as between more distantly related species (Teshima et al., 2003; Parmakelis et al., 2003; Johnson et al., 2004; Ketmaier, Giusti & Caccone, 2006; Bichain et al., 2007; Kameda, Kawakita & Kato, 2007; Elejalde et al., 2008b; Fiorentino et al., 2008; Puslednik et al., 2009; Buckley et al., 2011; Stankowski, 2011; Criscione, Law & Koehler, 2012; Johnson et al., 2012; Koehler& Johnson, 2012; Lee, Lue & Wu, 2012; Criscione & koehler, 2013; Du et al., 2013; but see Martinez-Orti et al., 2008; Kotsakiozi et al., 2013).

A general developmental program may exist that governs the length of the ontogeny axis and size changes of the aperture profile in the final determination of shell size. In general, the larger shells of *Plectostoma* is result from shell growth in which the aperture size is larger and the ontogeny axis (more or less equal to total whorl length) is longer than in the smaller *Plectostoma* shells (Figures 1B and 1C). A few previous studies have suggested that larger shell size tends to correspond with larger whorl size (an estimation for aperture size), but smaller whorl number (Cameron, 1981; Goodfriend 1983 cited in Goodfriend, 1986; Gould,

1984, Gould, 1989). However, all there studies used different measurements for shell size (shell weight in Cameron, 1981; shell diameter in Goodfriend, 1983; and linear measurement of shell dimensions in Gould, 1989). Thus, how the shell size is exactly determined by aperture size changes and total number of whorls added along the ontogeny remains unresolved.

In addition to the strong relationships among shell size, ontogeny axis length, and aperture size along the shell ontogeny, there is a consistent pattern of size changes along the standardised ontogeny axis; for example, the constriction occurs at approximately the same point in the standardised shell ontogeny in all species. Both the shell size relationships and the aperture size ontogenetic pattern are quite conserved among *Plectostoma* species, regardless of shell shape. However, a few species with an extremely long tuba, such as *P. grandispinosum*, deviate slightly from these rules by having a 30% longer ontogeny axis in the tuba phase of the ontogeny.

In brief, we showed that comparing shell size in terms of aperture size ontogeny and the ontogeny axis length may help to gain a better understanding of development and evolution of gastropod shell size. In *Plectostoma*, the size of the shell is determined by a conserved aperture size ontogeny and total shell ontogeny length. It is likely that ontogeny axis length and aperture size are strongly tied in the shell ontogeny. Hence, the parallel evolution of shell size in *Plectostoma* is a reflection of parallel evolution of ontogeny length and aperture size along the shell ontogeny, and does not involve significant changes in the pattern of the aperture size ontogeny profile.

Shell shape ontogeny and evolution

In contrast to the shell size, which can be characterised in a standard metric, shell shape analysis is more challenging because shape is much more difficult to characterise. Thus, shell shape has often been characterised semi-quantitatively, as it was in our study. Besides, shell form is usually treated as a single, functionally significant entity (see Introduction). Hence, the parallel evolution of shell shape in different lineages would imply the parallel evolution of the shell's adaptive function in these lineages; however, this does not need to imply a parallel evolution of the shell ontogeny.

As the shell is essentially a petrified ontogeny of the organ that secretes this exoskeleton (i.e., the mantle and the aperture ontogeny), the evolvability and heritability of aperture ontogeny can be examined on the basis of shell shape. The unidirectional accretionary growth of the shell may suggest that seemingly large shape differences between two shells may actually be caused by small differences in the aperture ontogeny; and also that shell whorls produced early in the ontogeny could have an influence on the subsequent aperture ontogeny and hence the subsequent shell form (Gould, 1984; Hutchinson, 1989). Hence, it is important to understand the evolution of shell form in view of the aperture ontogeny, growth trajectories, and aperture shape, which could provide further insight into the evolutionary lability of shell form.

We show the spire shape of *Plectostoma* shells not to be constrained by phylogeny. This result is mirrored in other studies that examined the relationship between the phylogeny and shell shapes among species within a genus (Boato, 1991; Emberton, 1995; Teshima et al., 2003; Tongkerd et al., 2004; Noshi & Sota, 2007; Elejalde et al., 2008a; Elejalde et al., 2008b; Elejalde et al., 2009; Stankowski, 2011; Johnson et al., 2012, Haase, Esch & Misof, 2013). Tuba coiling type in *Plectostoma* also is not constrained by phylogeny; similar results were obtained in studies of convergent evolution of the irregular coiling of the last whorl in other micro-snail taxa in Southeast Asia (Tongkerd et al., 2004). Homoplasy of shell traits at such a low taxonomic level, across different taxa, raises the question whether shell shapes that evolve in parallel could have the same shell ontogeny; or, in other words, occupy the same ontogenetic morphospace. To answer this question, we discuss the evolution of shell spire and tuba shape, respectively, based on the occupancy of ontogenetic morphospace.

Spire

The shell spire of all *Plectostoma* species has a regular shape, coiled around an imaginary axis. The shape differences between shell spires can be detected from a geometric perspective, for example height and width ratio and diameter differences between shell whorls. Although small spire shape differences between species are detectable by our qualitative approach, all *Plectostoma* species have a conical spire and live in a vertical limestone habitat. Hence, the slight differences in spire shape may not change the shell's adaptation to the inclination of the habitat (for similar results in other land snails, see review in Goodfriend, 1986; Okajima & Chiba, 2009; Okajima & Chiba, 2011; Noshita, Asami & Ubukata, 2012; Okajima & Chiba, 2012; Stankowski, 2013). The lack of adaptive differences could be one of the explanations for the lack of phylogenetic signal in *Plectostoma* spire shape.

For the ontogenetic point of view, similarly-shaped shell spires do not have the same aperture ontogeny profiles or occupy the same region in ontogenetic morphospace. In fact, the ontogenetic morphospace dimensions of the standardised curvature and aperture shape during the intermediate phase of shell ontogeny (*ca*. 20 – 60 %) are species-specific (Figure 4E). Neighbouring species in this part of ontogenetic morphospace do not necessarily have similar apical spire shapes (Figures 4B, 4E, and Supplementary File 5: Figures S6-S8). This suggests that two species may obtain similar spire shape with unique but different aperture ontogeny profiles. This also highlights the fact that our semi-quantitative spire shape categories which are similar to the conventional approach in the determination of shell shape (based on dimensional ratios) cannot effectively capture the ontogenetic differences between species (see also Haase, Esch & Misof, 2013).

Tuba

In contrast to the majority of gastropod, in which the last shell whorl is usually coiled in the same way as the preceding whorls, the shells of many species in Diplommatinidae, Streptaxidae, and Vertiginidae deviate from this generality. Although this character state is obviously derived, the opposite appears to be the case within the genus *Plectostoma*: a

twisted tuba is the ancestral state, whereas a distorted and a regularly coiled tuba are derived character states. It is clear that the magnitude of change in the aperture ontogeny profile in *Plectostoma* is related to the degree of distortion in tuba coiling (Figures 3A, 3D, 4A and 4D).

The twisted tuba occupies a larger ontogenetic morphospace than a regular or distorted tuba. The aperture ontogeny profiles for standardised torsion and curvature of the shells change drastically when forming the twisted tuba at the end of the *Plectostoma* shell ontogeny (after *ca*. 80 %) (Figure 3A). In addition, the aperture shape changes drastically as well for the species with a long tuba, such as *P. grandispinosum* and *P. retrovertens*. It is clear that the aperture ontogeny needs to undergo drastic changes to accomplish the transition from the regular spire to the twisted tuba, and therefore occupy a larger region in ontogenetic morphospace, as compared to species with a regular or slightly distorted tuba.

Conclusions

Our study has revealed a methodological issue in shell shape characterisation, and has shown an alternative to describing measurable differences between shell shapes in view of geometry and ontogeny. We support the concern of Haase et al. (2013) that using shell dimensional ratio as a proxy for shell shape may be oversimplified and inaccurate in the determination of similarity between shells, especially when the differences are small. We have also revealed that each species has a unique aperture ontogeny profile that is responsible for its shape while retaining a conserved shell size developmental program to gain its size. It is clear that the phylogeny does not limit changes in shell ontogeny. Further studies are needed to assess how other evolutionary processes and constraints, geometrical as well as functional, could have driven the parallel evolution of *Plectostoma* shell forms.

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Supplementary Information

Supplementary File 1. R scripts and data.
Supplementary File 2. Sequence data for 21 *Plectostoma* species and 4 outgroup taxa in nexus format for MrBayes analysis.
Supplementary File 3. Character matrix.
Supplementary File 4. Aperture ontogeny profiles for 11 *Plectostoma* species raw data.

Supplementary File 5. Aperture ontogeny profiles for 11 *Plectostoma* species, in which each was labelled by respective shell shape.
Supplementary File 6. Phylogenetic tree inferred by maximum likelihood analysis.
Supplementary File 7. Ancestral state reconstructions for shell shape and size, and rib form, as derived by the maximum parsimony method.