

Speciation, phenotypic plasticity, or ontogeny, the case of the genus *Galkinius* (Pyrgomatidae, Cirripedia, Crustacea)

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Barnacles of the genus *Galkinius* occupy a large spectrum of host corals, making it one of the least host-specific genera within the Pyrgomatidae. Molecular analyses show that within the genus *Galkinius* there are highly supported clades, suggesting that the genus *Galkinius* is a complex of evolutionarily significant units (ESUs). The morphology of the opercular valves has been used as the basis for the separation of species of *Galkinius*. In this study, morphological variability was found both between specimens within ESUs extracted from different host species and between specimens extracted from the same colony. Identifications based on the opercular valves cannot therefore be assigned to different species despite being genetically distinguishable. It is proposed that in many cases the differences between valve morphology of different species of *Galkinius* are the outcome of ontogeny. Allometric growth of the valves has resulted in differences in the proportions of the parts of the valve.

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INTRODUCTION

The genus *Creusia* was erected by Leach (1817) to accommodate coral-inhabiting barnacles (Pyrgomatidea) with shells composed of four compartments and fused scuta and terga. Darwin assigned all coral barnacles to one genus *Pyrgoma*, stating ‘I feel no hesitation in including the several genera in one genus. . . for the fact that the scutum and tergum being calcified together. . . it is certainly unimportant’ (Darwin, 1854: 355). With some reservations he recognized within *Pyrgoma* the subgenus *Creusia* and assigned all

pyrgomatids with four shell plates to one species, *Creusia spinulosa* Leach, with 11 varieties. Varieties 9, 10 and 11 (Figs 1D, 6B), which are distinguished by ‘The scutum and tergum. . . being calcified together without any trace of a suture’ (Darwin, 1854: 380), are the subject of the present study. Another prominent character of these varieties is that the ‘adductor ridge is enormously developed, so as to depend far beneath the true basal margin’ (Darwin, 1854: 53). *Creusia* was later recognized by various authors as a distinct genus, which included these three varieties [Annandale, 1924; Broch, 1931; Hiro, 1935; 1938; Nilsson-Cantell, 1938; Utinomi (= Hiro), 1962; Ross & Newman, 1973]. Annandale (1924) was the first to recognize Darwin’s varieties as distinct species and he

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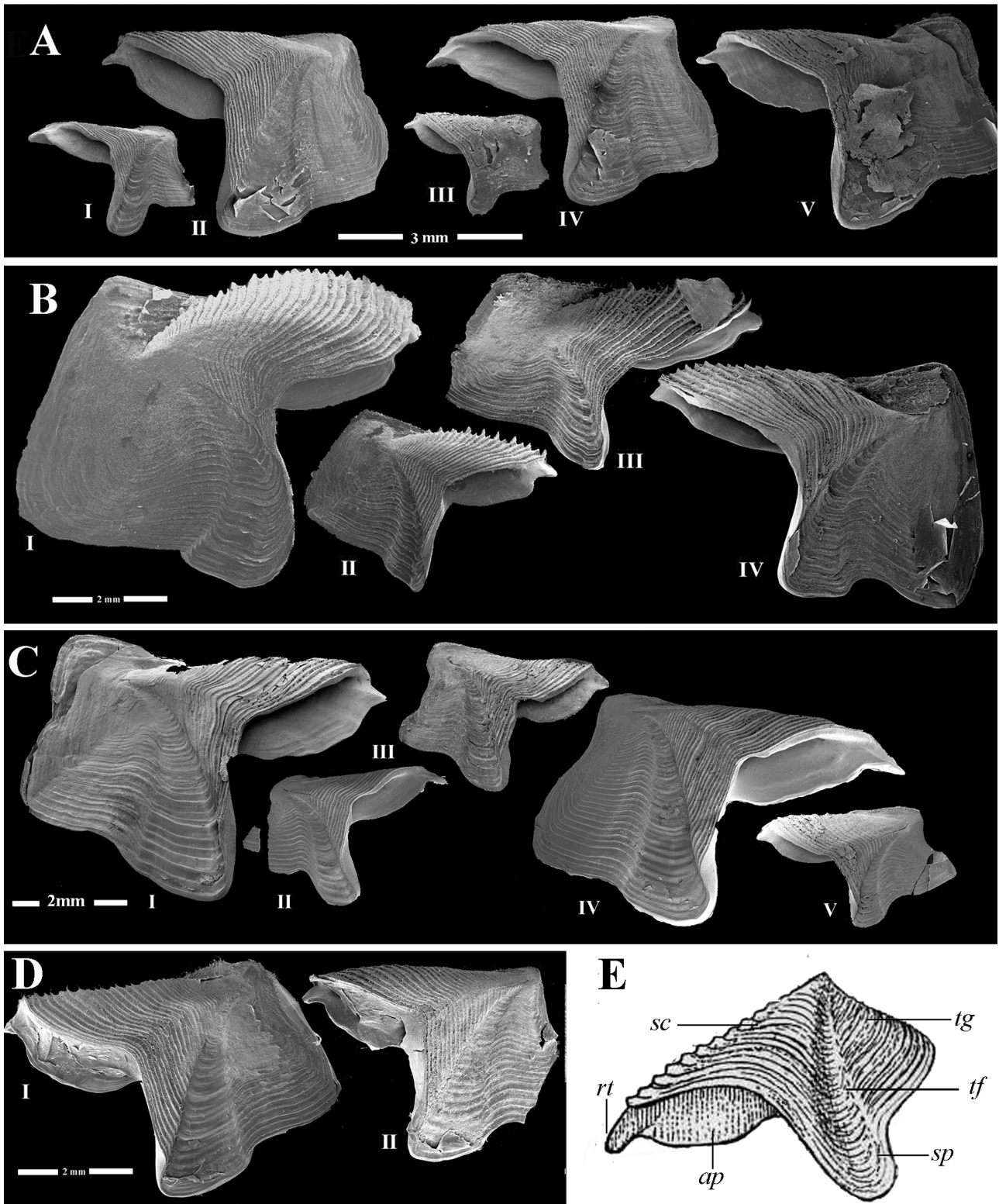


Figure 1. Opercular valves of *Galkinius* extracted from *Hydnophora*; A, NHM-2013 798–802; host: *Hydnophora exesa*. B, WAM-22091; host: *H. exesa*. C, NHM-2013 793–797; host: *H. exesa*. D, TAU AR29315; host: *H. exesa*. E, opercular valve of *Galkinius indica*, after Darwin (1854: plate XIV, 6.u; *Creusia spinulosa* var. 11). Abbreviations: ap, adductor plate; rt, rostral tooth; sc, scutum; sp, spur; tf, tergal furrow; tg, tergum.

raised variety 11 and named it *Creusia indica* Annandale, in which he recognized three forms or phases, *typica* from *Favia*, *merulinae* from *Merulina laxa* Dana, and *symphylliae* from *Symphyllia agaricia* Milne Edwards & Haime. This approach was followed by Ross & Newman (1973) who 'blanketly endorse all known taxa, subspecies and varieties to specific rank'; variety 11 remained *C. indica*, variety 10 was named *Creusia decima* Ross & Newman, and variety 9 became the nominotypical *Creusia spinulosa*. Galkin (1986) proposed the genus name *Utinomia* to accommodate *C. indica* and *C. decima*. Ross & Newman (1995) indicated that the name *Utinomia* is pre-occupied and proposed *Galkinia* as a replacement. However, Perreault (2014) discovered that *Galkinia* was itself pre-occupied and replaced it with *Galkinius*.

Ross & Newman (2002), in an updated nominal list of coral-inhabiting barnacles, based on earlier studies, allocated the pyrgomatids with four shell plates and fused scutum and tergum to two genera, the monospecific *Creusia* and *Galkinius* with four nominal species. The original name, *C. spinulosa*, was assigned to Darwin's variety 9. Variety 10 was named *Galkinius decima* (Ross & Newman), whereas the other three nominal species of *Galkinius* are *Galkinius indica* (Annandale), *Galkinius angustiradiata* (Broch), and *Galkinius supraspinulosa* Ogawa.

Recently, Chan, Chen & Achituv (2013a) and Chan, Chen & Lin (2013b) studied the genus *Galkinius* from Taiwan, and using morphological and molecular approaches they recognized nine species of which six are new. The previously reported species *G. angustiradiata* and *G. supraspinulosa* were not included in this study. Chan *et al.* (2013a, b) also demonstrated that host specificity varied amongst various *Galkinius* species; thus, *G. indica* (Annandale, 1924) is specific to *Hydnophora* (Pallas), and *Galkinius adamanteus* Chan *et al.* and *Galkinius equus* Chan *et al.* are specific to *Favites abdita* (Ellis and Solander). By contrast, *Galkinius depressa* Chan *et al.* and *Galkinius altiapiculus* Chan *et al.* are epibiotic on a wider range of coral hosts including *Goniastrea*, *Favites*, *Montipora*, *Platygyra*, and *Merulina*. A key for separation of the species based on the morphology of the opercular valves was presented. In the present study we included material from more hosts and localities, in an attempt to examine variations of host specificity of *Galkinius* in a greater number of coral genera from a wider latitudinal range.

MATERIAL AND METHODS

Specimens extracted from dry corals in the collections of the Western Australia Museum (WAM), the coral collection of Naturalis (the Rijksmuseum van Natuurlijke Historie Leiden; RMNH), The Natural

History Museum, London (NHM), and Tel Aviv University Zoological Collection (TAU) were used for morphological study. For both the morphological and the molecular studies, specimens collected in the field or from aquaria were used. Material for the molecular study was obtained from the crustacean collection of WAM and collections made by the WAM team at the Ningaloo Reef region of Western Australia. Additional material was obtained from a tropical fish shop supplied by a farm in Bali, Indonesia. Barnacles were extracted from the coral, and fixed in ethanol. For DNA extraction, adductor and depressor muscles and mantle tissue were removed from ethanol-preserved specimens, and the cirri and mouthparts were stored separately for further study. Full details of the material examined are presented in Supporting Information Table S1.

For the morphological study of hard parts, the wall plates and opercular valves were immersed for about 2 h in household bleach, rinsed in distilled water, and then dried on a small hot plate at 90 °C. The specimens were examined under a dissecting microscope and adhering chitin was removed using needles and fine forceps. Dried samples of wall plates and opercular valves were mounted on brass stubs, coated with gold, and examined with a Qaunta Feg 250 scanning electron microscope; images were copied and stored using AUTOBEAM software, integrated in LINK ISIS.

DNA was extracted using a Macherey–Nagal genomic DNA isolation kit. For amplification of cytochrome *c* oxidase subunit I (*COI*) the universal primers HCOI2198 and LCOI1490 (Folmer *et al.*, 1994) were used. REDTaq ReadyMix R2523 (Sigma-Aldrich, St. Louis, MO) was used for sequence amplification by PCR (Saiki *et al.*, 1988). Amplification was carried out in a personal combi-thermocycler (Biometra, Germany). PCR products were purified by centrifugation using a High Pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany) or by Mclab laboratories (San Francisco, California). PCR products were sequenced on both strands using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems by Mclab laboratories).

Sequences were deposited in GenBank under accession numbers KP702762–KP702820. In addition to the newly generated sequences, randomly selected sequences of the new species described by Chan *et al.* (2013b) were retrieved from GenBank and included in the molecular analyses. The list of samples used for analyses and their GenBank accession numbers are presented in Supporting Information Table S2.

Sequences were aligned using MUSCLE (Edgar, 2004) embedded in MEGA6. Distances were calculated using the Kimura two-parameter model (Kimura, 1980) embedded in MEGA6 (Tamura *et al.*, 2013).

Table 1. List of hosts of *Creusia* and *Galkinius* arranged by family. Details on sources and collection sites are given in Supporting Information Table S1

Host	Family
Acroporidae:	<i>Acropora</i> sp.; <i>Montipora peltiformis</i> ; <i>Montipora aequituberculata</i> .
Diploastreidae:	<i>Diploastrea heliopora</i> .
Merulinidae:	<i>Hydnophora exesa</i> ; <i>Hydnophora microconos</i> . <i>Merulina ampliata</i> ; <i>Oulophyllia crispa</i> ; <i>Platygyra lamellina</i> ; <i>Mycedium elephantotus</i> .
Faviidae:	<i>Caulastrea echinulata</i> ; <i>Favia amicum</i> , <i>Favia favius</i> , <i>Favia laxa</i> , <i>Favia lizardensis</i> , <i>Favia rosaria</i> , <i>Favia speciosa</i> ; <i>Favia valenciennes</i> , <i>Favites abdita</i> ; <i>Favites chinensis</i> ; <i>Goniastrea aspera</i> ; <i>Goniastrea palauensis</i> ; <i>Goniastrea pectinata</i> ; <i>Leptastrea inaequalis</i> , <i>Leptastrea pruinosa</i> ; <i>Leptastrea purpurea</i> ; <i>Leptastrea transversa</i> ; <i>Montastrea curta</i> , <i>Montastrea magnistellata</i> , <i>Montastrea valenciennesi</i> .
Fungiidae:	<i>Cycloseris (Fungia) hexagonalis</i> ; <i>Fungia (Verrillfungia) scabra</i> ; <i>Lithophyllon undulatum</i> ; <i>Lithophyllon mokai</i> .
Pectiniidae:	<i>Oxypora lacera</i> .
Poritidae:	<i>Goniopora stokesi</i> .
Trachyphyllidae:	<i>Trachyophyllia geoffroyi</i> .

Phylogenetic and molecular evolutionary analyses were conducted using MEGA6 (Tamura *et al.*, 2013) and Bayesian inference (BI) using MrBayes v. 3.12 (Huelsenbeck & Ronquist, 2001).

Bayesian phylogenetic analysis was conducted using the MrBayes v. 3.2.2 online version at CIPRES Portal v. 3.3 cluster at the San Diego Supercomputer Center (Miller, Pfeiffer & Schwartz, 2010). A general time-reversible model with a proportion of invariant sites and gamma-distributed rate heterogeneity (GTR + I + γ) was used to analyse the data set. Tree searching was performed running four linked chains initiated from random trees with a sequential heat of 0.05 (determined empirically) for 4 000 000 generations with trees sampled every 1000 generations. One quarter of the initially generated trees were discarded manually as it is not possible to automatically define the burn-in fraction. The search for trees was repeated four times and the majority rule consensus trees from each run were visually compared and then mixed to generate a majority rule consensus tree that was taken as the best representation of the posterior distributions of the tree topology and model parameters (Huelsenbeck & Ronquist, 2001).

Estimates of evolutionary divergence between sequences of *Galkinius* were conducted in MEGA6 (Tamura *et al.*, 2013). The analysis involved 119 nucleotide sequences of which 73 sequences were generated in the present study, and 46 were retrieved from GenBank.

RESULTS

HOST DIVERSITY

Table 1 presents the coral hosts on which specimens of the genus *Galkinius* were recorded by us. There are

20 genera and 36 species that host *Galkinius*. The host spectrum encompasses eight coral families: namely Merulinidae, Faviidae, Acroporidae, Fungiidae, Poritidae, Trachyphyllidae, Acroporidae, and Diploastreidae. Further information on records of coral species hosting *Galkinius* used in the present study is presented in Supporting Information Table S1.

OPERCULAR VALVE MORPHOLOGY

The morphology of the opercular valves is used as the main diagnostic feature of genera and species of pyrgomatids. We therefore compared the morphology of opercular valves of specimens of *Galkinius* of different sizes extracted from a range of host corals. We present scanning electron microscope (SEM) images of opercular valves from which we have more than one specimen from an individual coral colony. Our data include SEM images of valves from three colonies of *Hydnophora* (Fig. 1), three colonies of *Echinophyllia* (Fig. 2), three colonies from *Favia* (Fig. 3), two colonies of *Favites* (Fig. 4), and a single colony from each of the corals *Goniastrea* (Fig. 5), *Psammocora* (Fig. 6), *Platygyra* (Fig. 7), and *Acanthastrea* (Fig. 8). Valves from a single colony are grouped, each group is indicated by a letter, and the specimens by Roman numerals. It is clear that there are distinct differences in valve morphology amongst valves from different host species, different conspecific hosts and even within a single host colony. The most variable character is the shape of the spur and the groove that runs along it. In some specimens the spur is long and pointed, projecting beyond the basal margins of the tergal part of the fused valve. In other specimens the tergum is rhomboidal and the spur is broad and hardly projecting. The morphology of the spur is the main character used by Chan *et al.* (2013a, b) to separate the different species of *Galkinius*.

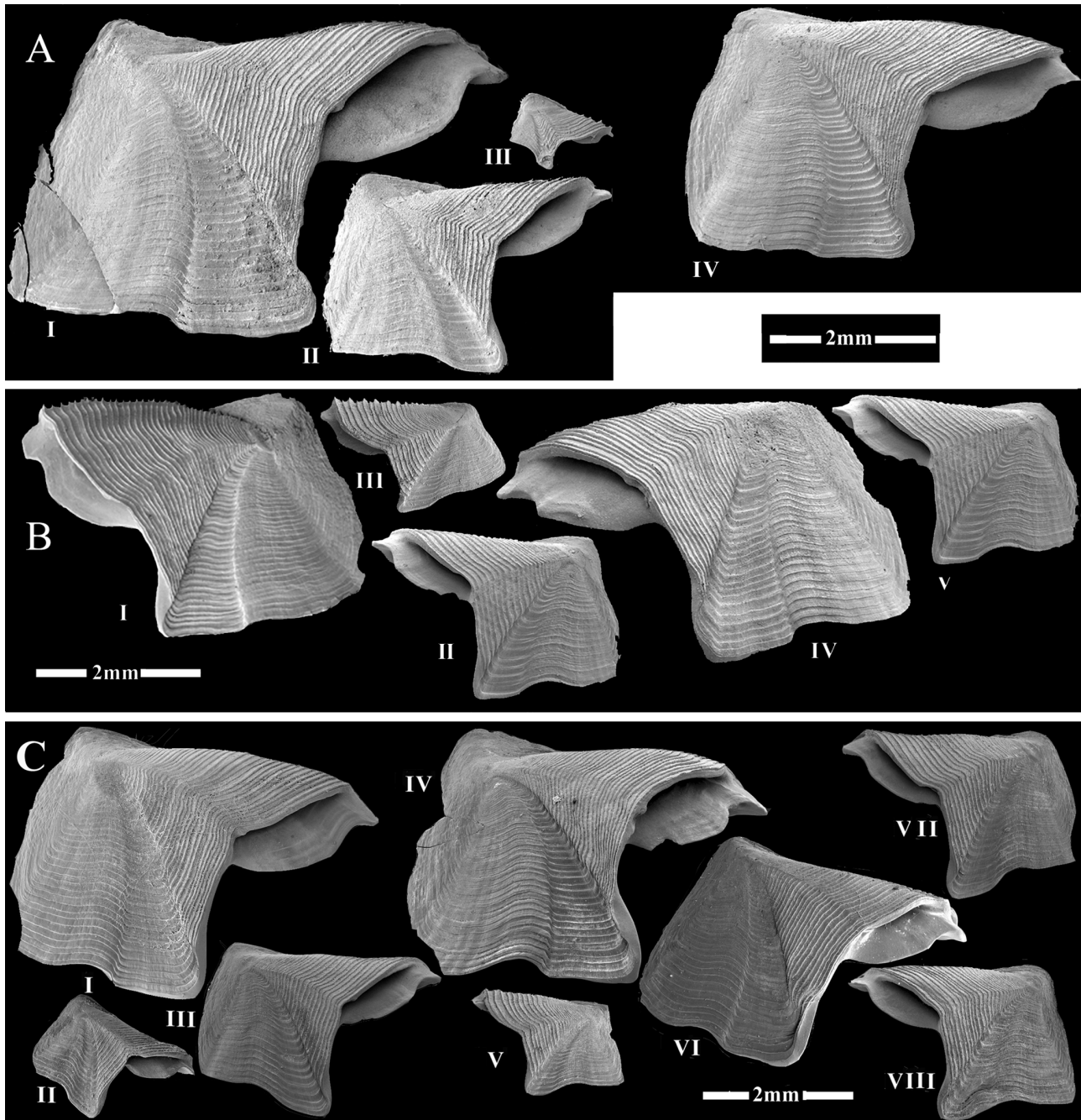


Figure 2. Opercular valves of *Galkinius* extracted from *Echinophyllia*: A, TAU-AR-29262; host: *Echinophyllia aspra*. B, TAU-AR29261; host: *Echinophyllia echinata*. C, TAU-AR (no data); host: *E. echinata*.

Small specimens extracted from *Hydnophora* (Fig. 1A_I; III) have a narrow spur extending beyond the basal margin of the tergum, with the scutal margins of the spur being about twice as long as the carinal margins. In medium-sized valves extracted from another colony (Fig. 1B_{II}; III) the spur is pointed and triangular; in larger valves from the same colony (Fig. 1B_{IV}) and from a different one (C_{II}) the spur is shorter and in some cases

nearly indistinct (Fig. 1A_{II}; IV). In larger specimens from *Hydnophora* (Fig. 1A_{II}; IV; V; B_{IV}; C_I) the spur is shorter and the width of the tergal furrow increases from the apex to the spur base. In the largest valve extracted from *Hydnophora* (Fig. 1B_I) the tergum occupies the greater part of the valve and the spur is short and hardly projects beyond the basal margin of the tergum.

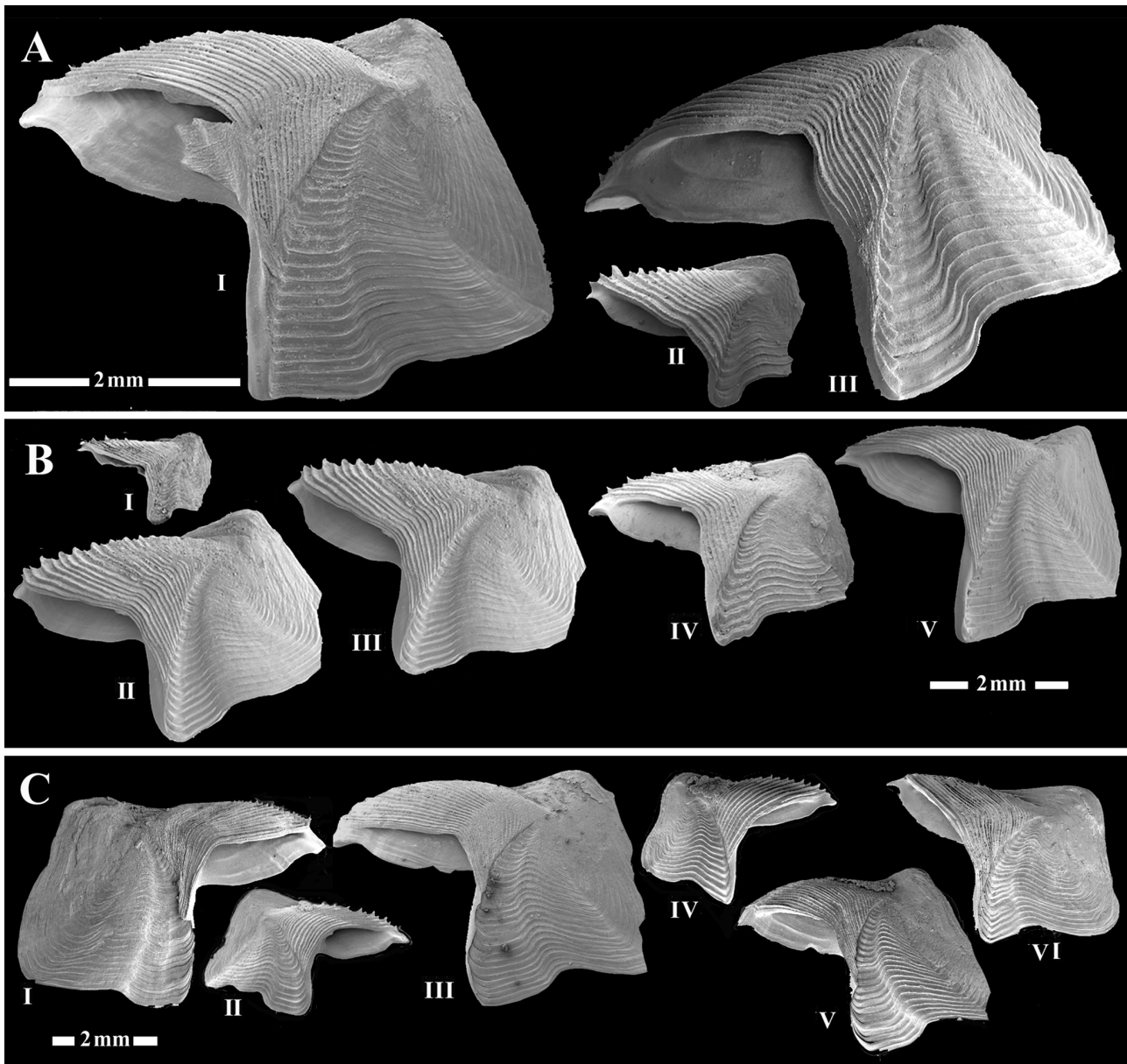


Figure 3. Opercular valves of *Galkinius* extracted from *Favia*: A, TAU-AR29262 host: *Favia pallida*. B, TAU-AR29264 host: *Favia* sp. C, TAU-AR29263; host: *Favia rosaria*.

In valves of *Galkinius* retrieved from *Echinophyllia*, the tergal apex is generally higher than that of the scutum. The spur of small specimens (Fig. 2A_{III}; B_{III}; C_{II}; v) is a pointed triangle; in the large specimens (Fig. 2A_I; IV; B_I) the spur is broad and hardly projects beyond the basal margins of the tergal part of the fused valve. In specimens of intermediate sizes (Fig. 2A_{II}; B_{II}; v; C_{III}; VII; VIII) the spur is distinct but relatively shorter than in the small ones, thus showing intermediate morphology.

In valves extracted from different species of *Favia* the tergal apex is relatively high and there is vari-

ability in the shape of the spur. In valves from small specimens of *Favia pallida* (Dana) (Fig. 3A_{II}), the spur is narrow and projects from the basal margins. In the large valves from *F. pallida* (Fig. 3A_I) the broad spur hardly projects beyond the basal margin. The valves from two small specimens extracted from *Favia rosaria* are different; in one (Fig. 3C_{II}) the spur is broad and in the other (Fig. 3C_{IV}) the spur is pointed. In larger specimens (Fig. 3A_I; C_I) the spur is broad and nearly indistinct.

In *Favites halicora* (Ehrenberg) the spur of valves retrieved from small barnacles (Fig. 4B_{II}) is relatively

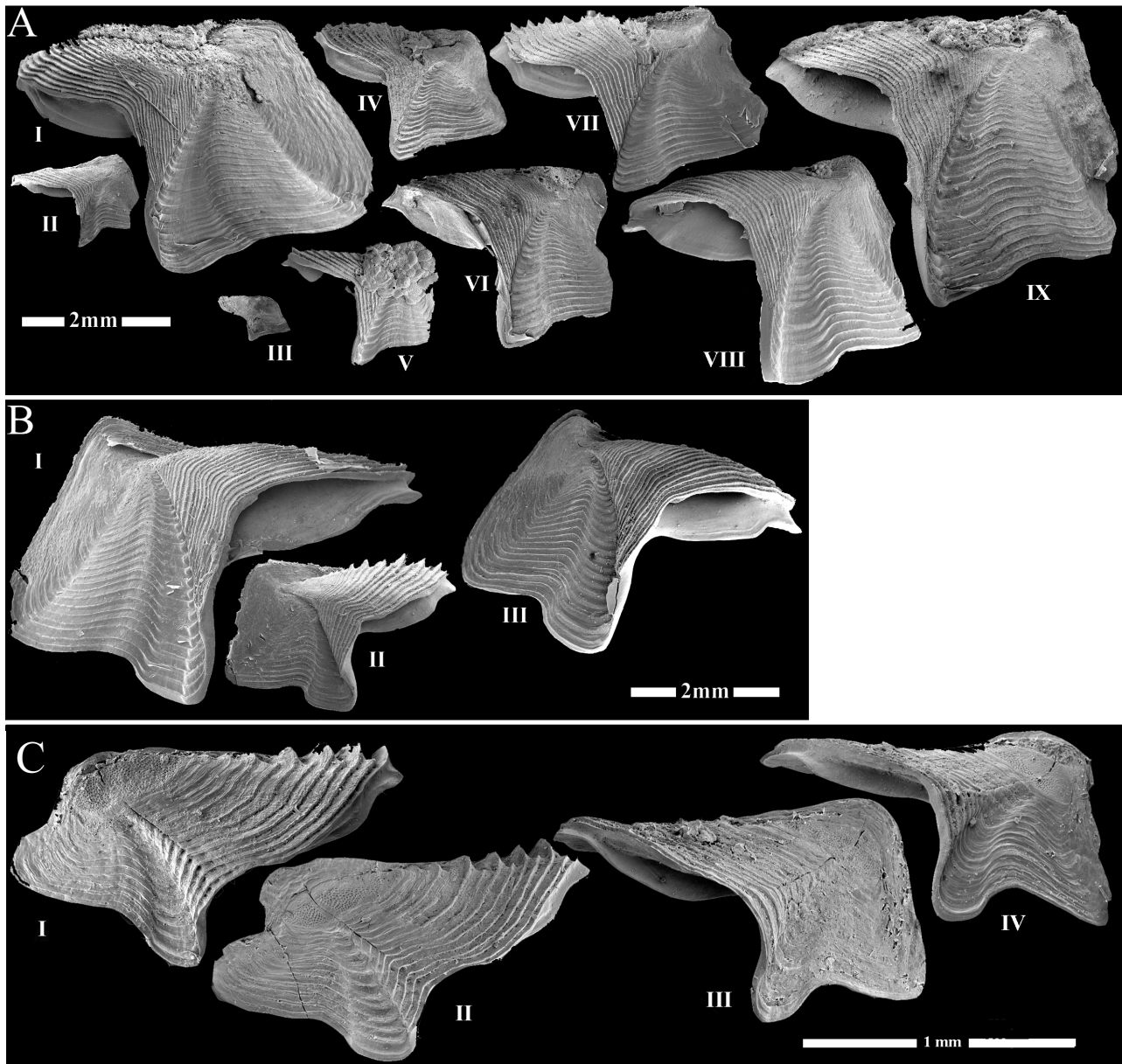


Figure 4. Opercular valves of *Galkinius* extracted from *Favites*: A, TAU AR29315; host: *Favites* sp. 5. B, NHM 2013 763–767; host: *Favites halicora*. C, NHM 2013. 769–773; host: *Favites pentagona*.

slender and longer than those found in larger specimens (Fig. 4B_{I, III}).

The valves extracted from *Goniastrea* (Fig. 5) are distinct by the exceptionally high tergal apex, which is higher than the scutum. The spur is narrow in small specimens (Fig. 5_{I, V}) and is relatively slender compared with those of larger specimens. Generally, the spurs of valves of *Galkinius* from this host are longer and more slender than those from the other hosts.

In specimens extracted from *Psammocora* (Fig. 6), the scutal part is distinct because of the wide adductor plate that extends below the basal margins, and is most

conspicuous in the large specimen (Fig. 6_{III}). The tergal part is rhomboidal and a tergal furrow runs in the middle of the tergum. This agrees with Darwin's (1854) description of *Creusia spinulosa* var. 10 (Fig. 6B) and was assigned by us to *G. decima*.

The tergum of the unidentified *Galkinius* extracted from *Platygyra pini* Chevalier (Fig. 7) is rhomboidal with the basal part of it being triangular; the tergal furrow is wide and can occupy most of the tergal part of the fused valve, resulting in an indistinct spur and only slightly projecting from the basal margins. In small specimens (Fig. 7_{II, III, V}) the rhomboid shape of the tergal

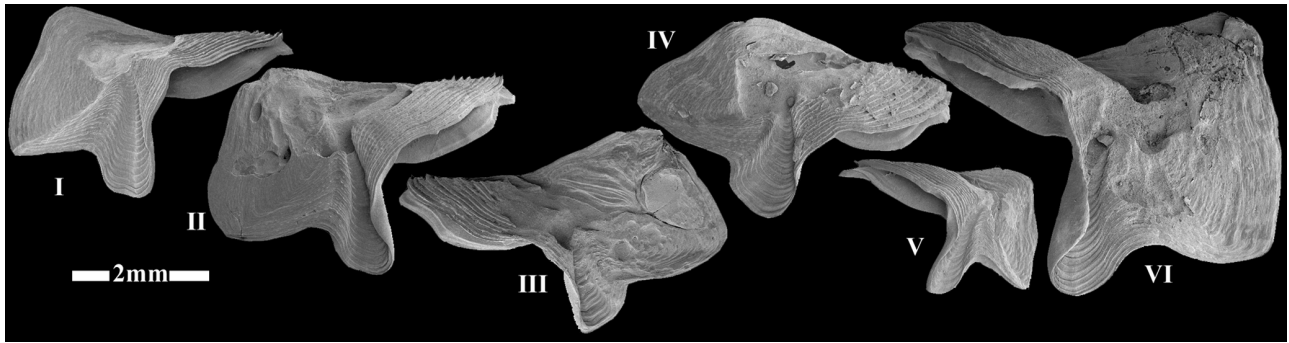


Figure 5. Opercular valves of *Galkinius* extracted from *Goniastrea*: NHM 2013 784–788; host: *Goniastrea* sp.

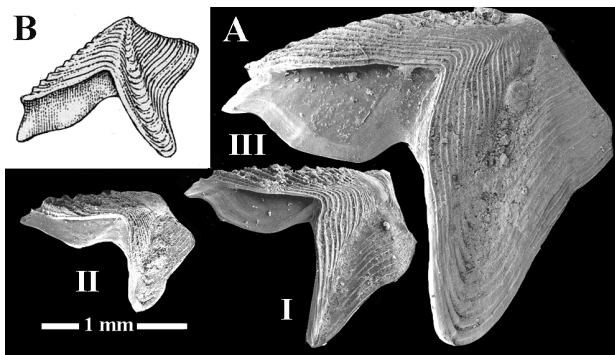


Figure 6. A, opercular valves of *Galkinius* extracted from *Psammocora*: TAU AR29344; host: *Psammocora* sp. B, *Galkinius decima*, after Darwin (1854: plate XIV, 6t; *Creusia spinulosa* var. 10).

part is distinct, whereas in larger specimens (Fig. 7, IV, VI) the carinal axis is more elongated and the tergal part is more rectangular.

There are also high levels of morphological variability in valves extracted from *Acanthastrea* (Fig. 8). Differences were found in valves from *Acanthastrea hillae* Wells. In the small specimens from this coral colony the spur is narrow but in the large specimen the spur is relatively short, the tergal furrow is wide, and the apex of the tergal part is high. The valves from *Acanthastrea lordhowensis* Veron & Pichon are similar in size but show slight differences in morphology, mainly in the relative length of the spur.

ONTOGENY OF THE OPERCULAR VALVES

Examination of the opercular valves from small specimens of *Galkinius* (Fig. 9) revealed the presence of primordial separate scutum and tergum. These primordial valves are characterized by a series of pits. The primordial scutum is elongated and the tergum is triangular. A suture between the tergum and scutum runs from the apex of the composite operculum to the basal margin. This suture marks the articulation of the

tergum and scutum. With time and age, abrasion obliterates the primordial valves. After erosion of the primordial valves, in the small specimens the suture that divides the scutal and tergal part of the fused valve can still be located. Towards the basal margins the suture is indistinct but its position can be traced by a change in direction of the growth lines that form a ridge on the outer face of the valve. This ridge line indicates the boundary between the scutum and tergum.

MOLECULAR ANALYSIS

The final aligned and trimmed database contains 61 newly generated sequences of *COI* of *Galkinius* and of *Creusia spinulosa* and 12 randomly selected sequences of the nine species of *Galkinius* described by Chan *et al.* (2013a, b). We included in our analyses a sequence of *Cantellius pallidus* (Broch) as an outgroup. There was a total of 637 positions in the aligned data set. The evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013). A GTR + I + γ model was selected that had an Akaike information criterion value of 8847.724 (Akaike, 1974).

Figure 10 presents a maximum likelihood (ML) tree; its highest log-likelihood score is 4293.10. In this condensed tree the branches with significance values lower than 80% were cut off. Bootstrap values and Bayesian support values higher than this are indicated. Branches of low statistical significances were eliminated to form a condensed tree that emphasizes the reliable portions of branching patterns.

Clustering of the sequences was identical in the three methods of analysis, i.e. MP, NJ, and ML. However, the lengths of the branches and inner arrangements of sequences within clades are slightly different in the cladograms generated by the different approaches.

The sequences from specimens extracted from *Montipora* and *Acropora*, morphologically identified as *Creusia spinulosa*, cluster in a distinct clade. This clade is a sister group to all other *Galkinius* clades. Within the *Galkinius* clade there are several nodes with different levels of bootstrap support, some reaching up

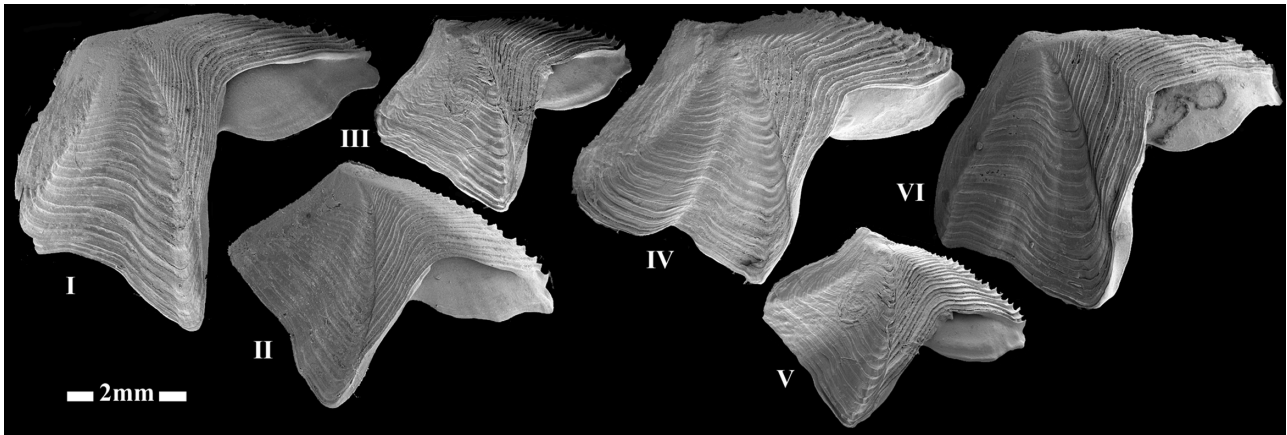


Figure 7. Opercular valves of *Galkinius* extracted from *Platygyra* NHM 2013 803–808; host: *Platygyra pini*.

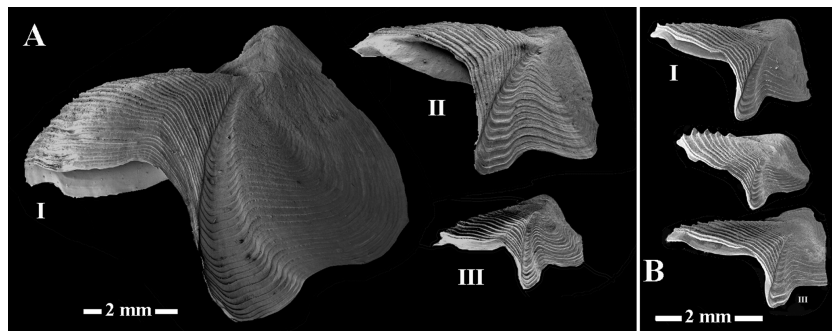


Figure 8. Opercular valves of *Galkinius* extracted from *Acantastrea*: A, WAM C55434; host: *Acantastrea hillae*. B, TAU AR36552; host: *Acantastrea rutondoflora*.

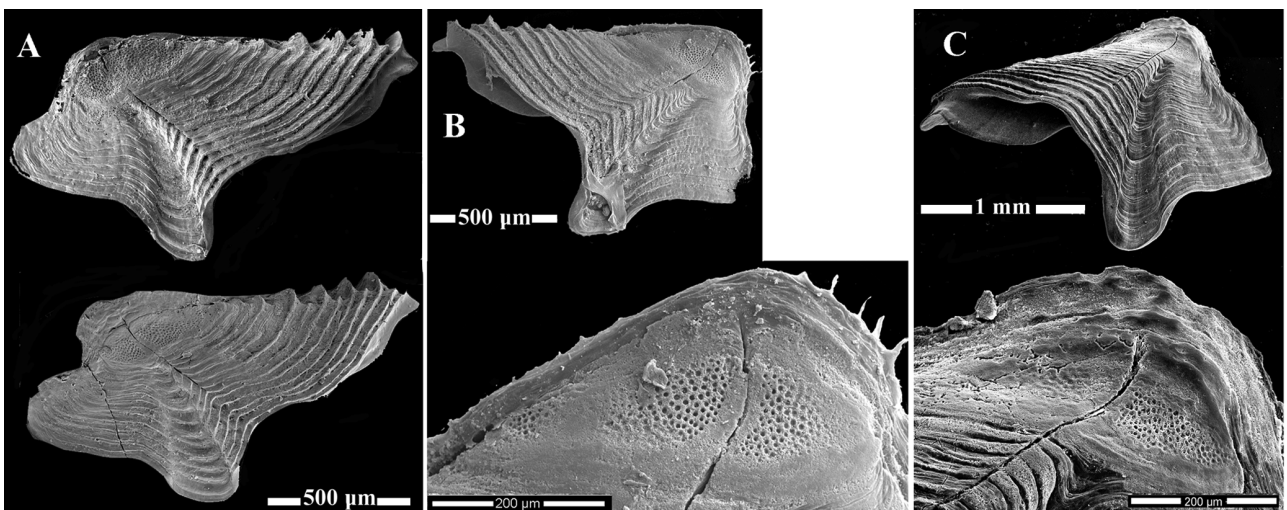


Figure 9. Fused opercular valves of *Galkinius* showing primordial scuta and terga and the suture in the fused valve marking the margins of the two parts of the valve. A, host: *Favites* sp. B, host: *Acantastrea*. C, host not known.

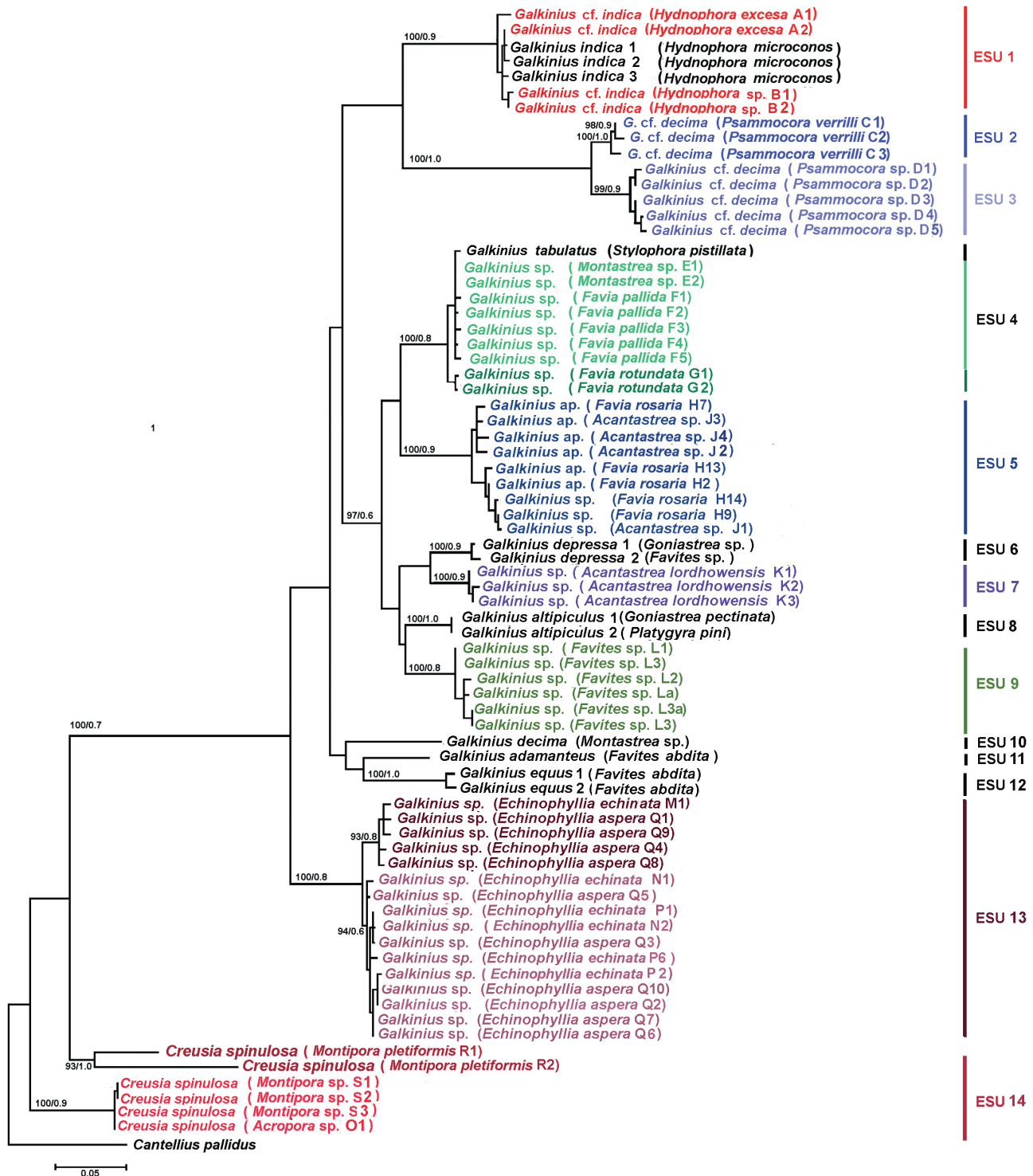


Figure 10. Maximum likelihood phylogenetic tree of *Galkinius* and *Creusia* based on cytochrome *c* oxidase subunit I. The outgroup is *Cantellius pallidus*. The analysis involved 74 nucleotide sequences, of which 13 sequences of *Galkinius* are from species described by Chan *et al.* (2013a) and 60 are new sequences. Host names are in parentheses. Barnacles extracted from a single coral colony are indicated by the same capital letter. Bootstrap support of nodes with more than 90% associated with sequences that clustered together is shown next to the branches with their Bayesian support. The tree is drawn to scale, with branch lengths presenting the number of substitutions per site. Abbreviation: ESU, evolutionarily significant unit.

to 99–100%. These nodes are regarded as distinct evolutionarily significant units (ESUs) and therefore are referred to as the *Galkinius* complex.

The evolutionary divergence between sequences of *Galkinius* was estimated using MEGA6 (Tamura *et al.*, 2013). The resulting detailed data matrix is presented in Supporting Information Table S3. The mean values of divergence for sequences extracted from the same colony are presented in Table 2. The numbers of base substitutions per site between sequences are shown below the diagonal. SE estimates are shown above the diagonal. Analyses were conducted using the maximum composite likelihood model (Tamura *et al.*, 2004). The rate variation amongst sites was modelled with a gamma distribution (shape parameter = 1). The analysis involved 119 nucleotide sequences, of which 73 were generated in the present study and 46 were retrieved from GenBank. Overall, six identified species were represented. All positions containing gaps and missing data were eliminated. There was a total of 510 positions in the final data set.

The within averages divergence of sequences extracted from the same colony and of those morphologically identified as belonging to the same species (Chan *et al.*, 2013a, b) ranged between 0.012 and 0.002 (Table 2B). The average amongst-species divergence ranged between 0.046 and 0.173 (Table 2A).

DISCUSSION

HOST DIVERSITY

Barnacles of the genus *Galkinius* are one of the most widely distributed coral barnacles and occupy a large spectrum of host corals. In the table compiled by Ogawa & Matsuzaki (1992) *Galkinius indica* was recorded from 27 coral species; our examination of four coral collections (Supporting Information Table S1) revealed another 34 species of host corals for *Galkinius*. This finding means that *Galkinius*, together with *Cantellius*, are the least host-specific genera within the Pyrgomatidae. One of the shared features of these two genera is a shell made of separate compartments. This generalized character, found in balnomorphans, is regarded as a plesiomorphic trait in the Pyrgomatidae, whereas fused shell plates is the advanced trait and found only in the Pyrgomatidae. This high diversity of hosts correlates with the statement of Newman & Jumars (1976) that structurally generalized genera exploit a wider variety of hosts than specialized ones. It should be noted that host specificity is a common feature within the Pyrgomatidae, at the genus and the species levels. For example, *Pyrgoma* is restricted to *Turbinaria*, *Hiroa* and *Cionophorus* to *Astreopora*, and the tribe Hoekiniini to *Hydnophora*.

Galkinius decima was not listed in the table of Ogawa & Matsuzaki (1992), although later Ogawa, Matsuzaki

& Kawasaki (1998) reported its presence on *Cyphastrea microphthalmia* (Lamarck) and Ogawa (2000) on *Favia matthai* (Vaughan). In fact, it seems that *G. decima* is a rare species and that apart from the above reports it has only been recorded by Darwin (1854), as *Creusia spinulosa* var. 10. In a recent survey (unpublished data), we found this species on three other host genera, *Psammocora*, *Montastrea*, and *Acropora*.

OPERCULAR VALVE MORPHOLOGY

Separation of the different species, varieties, forms, and phases of *Galkinius* is based on the morphological variability of the hard parts, mainly the opercular valves. Darwin (1854) noted two separate varieties of *Creusia* var. 10 and 11, which were elevated by Ross & Newman (1973) to specific rank and recognized as *G. decima* and *G. indica*, respectively. Based on the shape of the spur, Broch (1931) and Ogawa (2000) recognized, respectively, the species *G. angustiradiata* and *G. supraspinulosa*. Chan *et al.* (2013a, b) described the morphology of the opercular valves, cirri, and trophi and distinguished nine species of *Galkinius* from Taiwan. Opercular valve morphology was the major character for separation of species. Figure 11 is modified from Chan *et al.* (2013b) and shows the opercular valves of these nine species.

It is apparent that valves of *Galkinius* exhibit morphological variation amongst different host colonies within a genus, host colonies of the same species, and within an individual host colony. The differences are noticeable mainly in the shape of the tergal spur and the furrow that runs from the apex of the coalesced valve to the extremity of the spur. Using the key of Chan *et al.* (2013a, b), we found that specimens from the same host colony or the same host species can be assigned to different *Galkinius* species.

Chan *et al.* (2013a, b) referred to *Galkinius* from *Hydnophora* as *G. indica* (Fig. 11J, K), in which the tergum is characterized by a spur 'narrow and short reaching 1/2 width of basal margins of tergum'. However, this description does not apply to many of the valves extracted by us from *Hydnophora*. In large specimens the spur is short and hardly projects beyond the basal margins of the tergum (Fig. 1A_{II}, IV, V; B_I, IV; C_{IV}, II D_I, II). Some of the medium- and small-sized valves (Fig. 1A_I, III; B_{III}; C_I, III, V) agree with the description of the valves of *G. indica* (Fig. 11J, K; Chan *et al.*, 2013a, b). Other small valves with narrow and relatively long spurs (Fig. 1 A_I; D_{II}) agree with the description of *Galkinius trimegadonta* (Fig. 11G).

Using the key of Chan *et al.* (2013a, b) we found that the larger valves of *Galkinius* from *Echinophyllia echinata* (Saville-Kent) (Fig. 2A_I, II, IV) were assigned to *G. altiapiculus* (Fig. 11A, B), but those from small

Table 2. Mean estimates of evolutionary divergence between sequences of *Galkinius*. A, the mean number of base substitutions per site between sequences of different evolutionarily significant units (ESUs). SE estimates are shown above the diagonal; B, average divergence within each ESU; d, average overall sequence distance

	ESU1	ESU2	ESU3	ESU4	ESU5	ESU6	ESU7	ESU8	ESU9	ESU10	ESU11	ESU12	ESU13	ESU14
A														
ESU1		0.036	0.039	0.030	0.028	0.030	0.025	0.029	0.030	0.030	0.031	0.030	0.034	0.046
ESU2	0.157		0.013	0.038	0.032	0.033	0.036	0.040	0.039	0.029	0.035	0.031	0.035	0.039
ESU3	0.169	0.045		0.041	0.037	0.037	0.038	0.040	0.040	0.035	0.035	0.039	0.043	0.043
ESU4	0.132	0.159	0.174		0.026	0.020	0.018	0.023	0.020	0.024	0.030	0.023	0.030	0.042
ESU5	0.122	0.142	0.159	0.106		0.028	0.020	0.027	0.025	0.024	0.032	0.030	0.033	0.045
ESU6	0.128	0.141	0.156	0.088	0.115		0.013	0.020	0.018	0.018	0.027	0.027	0.029	0.046
ESU7	0.100	0.157	0.161	0.072	0.077	0.047		0.018	0.015	0.016	0.023	0.023	0.026	0.047
ESU8	0.134	0.171	0.172	0.100	0.118	0.083	0.067		0.017	0.024	0.028	0.030	0.034	0.048
ESU9	0.134	0.169	0.174	0.085	0.105	0.072	0.057	0.068		0.021	0.028	0.027	0.030	0.044
ESU10	0.123	0.116	0.147	0.100	0.099	0.067	0.056	0.104	0.092		0.019	0.019	0.020	0.043
ESU11	0.137	0.156	0.151	0.128	0.135	0.112	0.091	0.123	0.121	0.075		0.018	0.030	0.047
ESU12	0.134	0.135	0.170	0.098	0.132	0.117	0.099	0.136	0.122	0.079	0.072		0.029	0.044
ESU13	0.149	0.153	0.190	0.132	0.148	0.130	0.118	0.155	0.138	0.088	0.136	0.132		0.045
ESU14	0.194	0.165	0.190	0.188	0.196	0.196	0.201	0.210	0.187	0.179	0.195	0.183	0.185	
B														
d	0.008	0.005	0.009	0.007	0.010	0.001	0.002	0.004	0.012	n/c	n/c	0.014	0.014	0.000
SE	0.003	0.003	0.004	0.003	0.004	0.001	0.002	0.001	0.004	n/c	n/c	0.005	0.004	0.000

Analyses were conducted using the maximum composite likelihood model (Tamura *et al.*, 2004). The analysis involved 117 nucleotide sequences. There was a total of 288 positions in the final data set. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

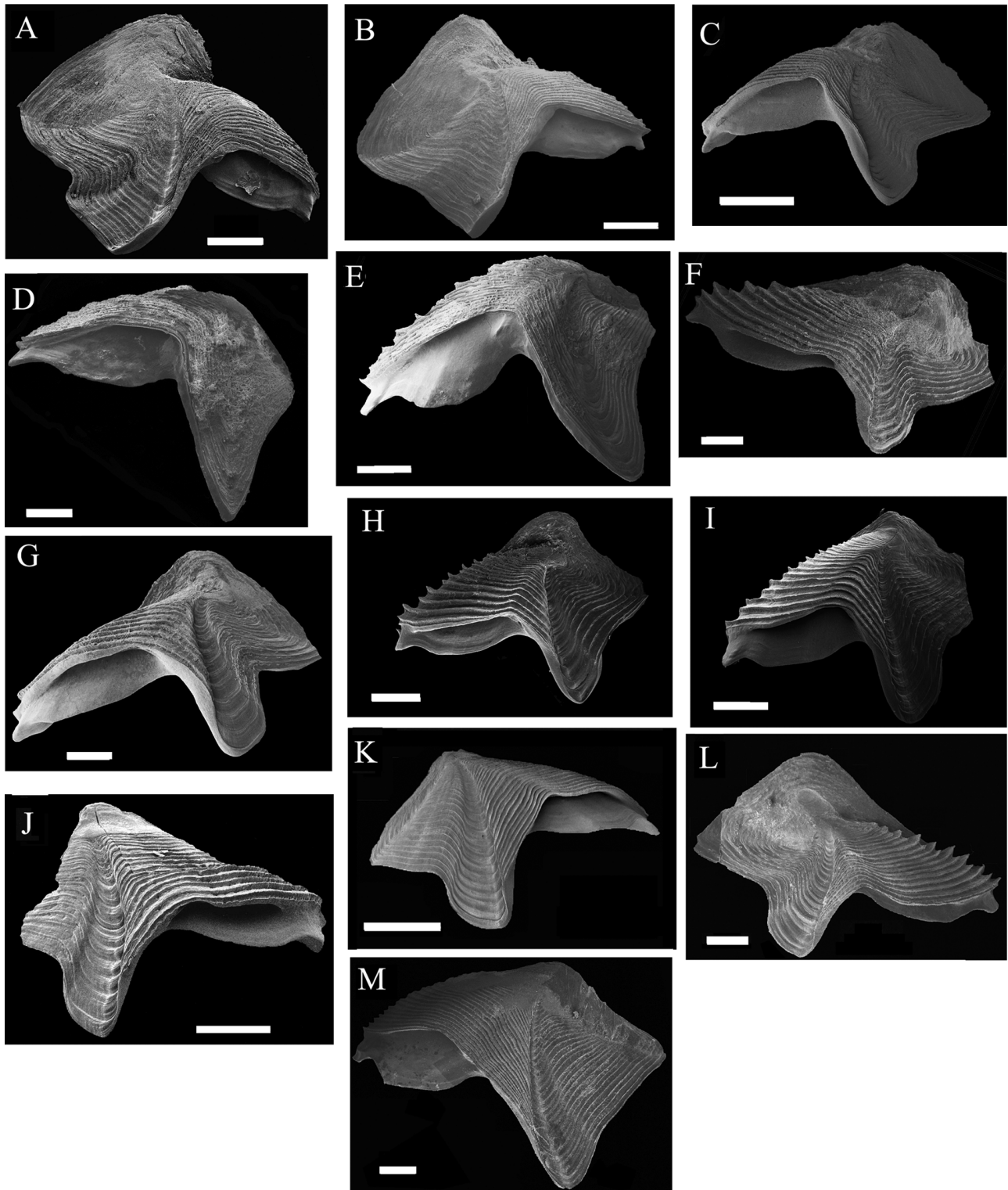


Figure 11. External view of fused scuta and terga of the different species of *Galkinius* adapted from Chan *et al.* (2013a, b). A, *Galkinia altiapiculus* from *Platygyra pini*; B, *G. altiapiculus* from *Goniastrea pectinata*; C, *Galkinia tabulatus* from *Stylophora pistillata*; D, *Galkinia decima* from *Montastrea* sp.; E, *G. decima* from *Montastrea* sp.; F, *Galkinia trimegadonta* from *Platygyra* sp.; G, *G. trimegadonta* from *Platygyra* sp.; H, *Galkinia equus* from *Favites abdita*; I, *G. equus* from *Favites abdita*; J, *Galkinia indica* from *Hydnophora microconos*; K, *G. indica* from *Hydnophora microconos*; L, *Galkinia depressa* from *Favites abdita*; M, *Galkinia adamanteus* from *Favites abdita*. Scale bar for all images = 1 mm.

specimens (Fig. 2A_{III}) were allocated to *G. equus* (Fig. 11H, I). In *Echinophyllia aspra* (Ellis & Solander), the tergal apex is higher than the apex of scutum (Fig. 2B_{I, II, V}) as in *G. altiapiculus*, but the spur is narrower, whereas in specimen B_{IV} the tergal apex matches the description of *G. adamanteus* (Fig. 11M) but the spur is different from that depicted in Figure 11M. The small specimen from *E. aspra* (Fig. 2B_{III}) is most similar to *G. adamanteus* but the adductor plate is narrow and does not extend below the basal margin of the scutum. Specimens extracted from another colony of *Echinophyllia* (Fig. 2C) demonstrate the same pattern as in *E. aspra*; the larger specimens (C_{I, III, V}) resemble *G. altiapiculus* whereas the smaller (C_{II, IV, VI, VII}) do not match any described species of *Galkinius*.

The three opercular valves extracted from the colony of *Favia pallida* (Fig. 3C) show variation within the colony. Specimen A_I, with a wide tergal spur, is comparable to *G. altiapiculus* (Fig. 3A), whereas specimen A_{III} resembles *G. indica* (Fig. 11J, K). It is hard to associate the opercular valves extracted from *Favia* sp. (Fig. 3B) and from *F. rosaria* to any of the species described by Chan *et al.* (2013a, b) because their spur is short and looks like that of *G. altiapiculus* but it is missing the characteristic high apex of *G. altiapiculus*.

The valves of *Galkinius* extracted from *Favites* (Fig. 4A) show wide variations. Small specimens (Fig. 4A_{I, II, IV, VII, V}) are similar to those identified as *G. equus* (Fig. 11G, H), bigger specimens from the same colony (Fig. 4A_{III, VIII, IX}) can be assigned to *G. altiapiculus* or are intermediate between these two species (Fig. 4A_{VI}). The valves of *Galkinius* from *Favites halicora* (Fig. 4B) exhibit intermediate morphology between that of *G. indica* (Fig. 11I, J) and that of *G. altiapiculus* (Fig. 11A). Opercular valves from *Favites pentagona* (Esper) (Fig. 4C_{I, III, IV}) have a narrow adductor plate, similar to that in *G. depressa*.

The opercular valves extracted from *Goniastrea* (Fig. 5) are conspicuous by the very high tergal apex and a spur with a deep furrow. The scutal part of the valve is low. The relative length and width of the spur differs amongst specimens. There is no analogous species in the previously described species of *Galkinius*.

Valves of specimens extracted from *Psammocora* (Fig. 6) were assigned by us to *G. decima*. Chan *et al.* (2013a) allocated specimens extracted from *Montastrea* to the species *G. decima*.

Opercular valves extracted from specimens found on *Platygyra pini* (Fig. 7) are characterized by a rhomboidal tergum with a triangular basal part. Such valves were also described for *G. adamanteus* (Fig. 11L) extracted from *Favites abdita* (Chan *et al.*, 2013a).

Whereas small specimens of *Galkinius* from *Acanthastrea* (Fig. 8) can be assigned to *G. indica* (Fig. 8A_{III}; B_{I, III}) or *Galkinius tabulatus* (Fig. 8A_{II}; B_{II}) as they have a pointed spur, larger valves extracted

from the same host (Fig. 8A_I) resemble those of *G. altiapiculus*, with a short, wide spur.

The variation and host selection of specimens that morphologically can be assigned to different species but extracted from the same coral colony can be explained by two alternative propositions. Based on valve morphology, host specificity in *Galkinius* species may be low and a single colony can be inhabited by multiple species. Alternatively, it may be that there is very high morphological diversity within genetically identical barnacles inhabiting a single host colony. It should be possible to resolve this question using molecular analyses of morphologically different specimens extracted from a single colony or from the same coral species.

ONTOGENY OF THE OPERCULAR VALVES

Fused opercular valves are rare in the Sessilia. In free-living sessilians they are known only in *Nesochthamalus intertextus* (Darwin) (Darwin, 1854). However, in the family Pyrgomatidae there are several genera with fused opercular valves. Previous phylogenetic analyses (Simon-Blecher, Huchon & Achituv, 2007; Malay & Michonneau, 2014; Tsang *et al.*, 2014) have indicated that separate opercular valves is a plesiomorphic character within the Pyrgomatidae. The ontogeny of the valves supports this hypothesis. The present observations show that the primordial valves are separate and that in an early stage of development there is a suture between the two fused parts of the valve.

Our observations agree with those of Utinomi (1943) describing the ontogeny of *Galkinius*, formerly known as *Creusia spinulosa* f. *angustiradiata*. He showed that in small spats (0.41 mm rostro-carinal diameter) the scutum and tergum are still separate with the scutum transversely elongated and the tergum triangular. However, at the size of 1.7 mm the opercular valves on each side are fused and the line of the junction is discernible. He did not mention the presence of primordial valves or their imprint in small specimens. Indeed, using the optical means available in 1943, it was probably not possible to distinguish the primordial valves and the suture between the scutal and tergal parts of the fused valves. The use of SEM (Fig. 9A–C) enables the distinction of these structures. The presence of primordial valves, or their imprints, in coral barnacles was noticed by Achituv, Tsang & Chan (2009) and by Chan *et al.* (2013b) in other coral-inhabiting barnacles: *Cantellius sextus* (Hiro), *Cantellius arcuatus* (Hiro), *Cantellius septimus* (Hiro), and *Cantellius hoegi* Achituv, Tsang & Chan. Darwin (1851: 22; 1854: 129) described chitinous primordial valves in lepadomorphs. When calcification commences, the shell is deposited under and around the primordial valves. Therefore, it seems that the honeycomb structure is the 'imprint' of primordial valves in calcareous matter, and in mature

specimens these structures are lost because of erosion. Similar structures were described by Newman (1987) in the early stages of *Chionelasmus darwini* (Pilsbry) development.

In the morphological description of the fused valve, it is accepted that the boundary between the scutal and the tergal parts is found along the outer margins of the spur furrow. However, the position of the suture shows that the ridge line that replaces it denotes the boundary between the scutum and tergum. The part between the furrow and this ridge is part of the tergum.

Based on examination of opercular valves of different sizes it appears that the growth of the valves is allometric. Proportionally, the transverse axis increases more than the basal-occludent axis. The tergal part of the fused plate and its furrow are widening. The spur hardly increases in length and becomes wider and relatively shorter, and is barely distinct. The relative size of the scutum in *Galkinius* is reduced during growth. Owing to the allometric growth, the relative length of the spur of specimens of different sizes, even when collected from the same coral colony, is variable. Generally, in small individuals the spur is narrow and more pointed than in large individuals. We therefore suggest that in many cases these morphological differences represent differences in the ontogeny of the valves rather than systematic differences.

MOLECULAR ANALYSIS AND VALVE MORPHOLOGY

According to the constructed genealogical relationship based on cytochrome c oxidase subunit I, *Galkinius* is a monophyletic group. The morphological different taxon *Creusia spinulosa* is paraphyletic with respect to *Galkinius*.

Species are defined using a variety of different operational techniques, but, in most cases morphological parameters are used in the description and definition of species. Nixon and Wheeler (1990) defined phylogenetic species as the smallest aggregation of sexual populations or asexual lineages diagnosable by a unique combination of character states. To reveal these phylogenetic units, cladistic methods and interpretations are used. This approach also exposes the evolutionary history of taxa and processes of speciation. Another term with which to define phylogenetic units is the ESU. This was coined by Ryder (1986) to accommodate a group of organisms that has been isolated from other conspecific groups for sufficient time to have undergone meaningful genetic divergence from those other groups. Moritz (1994) suggested that ESUs could be recognized by using mitochondrial DNA. This tool can be used in the absence of appropriate morphological characters. The ESU approach has been used mainly in bacteria in which 'species' cannot be recognized morphologically but as a group of strains that is genetically well separated from its

phylogenetic neighbours. In bacteria a pragmatic approach was taken to define a species by a polyphasic approach, in which a DNA reassociation value of about 70% plays a dominant role. Stackebrandt & Goebel (1994), in the absence of phenotypic coherency amongst strains, delineated species at the level of either 60 or 80% DNA-DNA similarity.

This approach was also applied to eukaryotes in a range of phyla. In crustaceans, Knowlton (2000) affirmed that genetic analyses of species boundaries may reveal or confirm the existence of cryptic species, some of which are distinguished by genetic differences given their morphological similarity. Cracraft (1989) argued that the degree of genetic differentiation can be used to determine whether allopatric taxa merit recognition at the species level. Thorpe (1983) suggested that genetic divergence provides a reliable criterion that can be adopted to make taxonomic decisions for allopatric taxa. Herbert *et al.* (2003) stated that sequence divergences of COI can be used to resolve species boundaries.

The pairwise divergence values between specimens of our material and those of the species erected by Chan *et al.* (2013a, b) are presented in Supporting Information Table S3; the average values are presented in Table 2. These values enabled us to set boundaries between ESUs. The within-group pairwise divergence in the six morphologically identified species of *Galkinius* does not exceed 0.025 (ESU1). The lowest between-group pairwise divergence is 0.075 between ESU10 and ESU11 (Supporting Information Table S3). We therefore propose that sequences with a divergence level lower than the midpoint of these values (0.040) should be assigned to the same clade within an independent ESU. Based on these parameters, we identified 14 ESUs, one comprises *Creusia spinulosa*, whereas the others are grouped within the *Galkinius* genus, of which seven were identified by Chan *et al.* (2013a, b) as distinct species.

Within the *Galkinius* group, in most cases, specimens extracted from a single host are clustered in distinct clades. The clades presented by Chan *et al.* (2013a, b) are also supported by our analyses. Those extracted by us from *Hydnophora* cluster with those extracted from the same genus by Chan *et al.* (2013a, b) and identified as *G. indica*. The average divergence between sequences from *Hydnophora* range between 0.000 and 0.025, with a mean value of 0.008 (Table 2); hence, they are included in a single ESU. However, as shown above, morphologically not all valves from *Hydnophora* conform to the morphological description of *G. indica* by Chan *et al.* (2013b). Our results and those of Chan *et al.* (2013b) suggest host specificity of *G. indica* over a wide geographical distribution covering the West Pacific and the Indian Ocean. Similarly, using the divergence criteria of 0.040, each clade in our analyses represents

an independent ESU (Supporting Information Table S3; Table 2). However, using morphological characters of opercular valves that affiliated to a single ESU it seems that they should be assigned to different species.

There are several cases in the present study in which specimens assigned to a single morphological species are clustered in separate clades and affiliated to different ESUs. For example, the genealogical analyses placed specimens identified by us as *G. decima* from *Psammocora* (ESU2 and ESU3) and the single sequence from *Montastrea* (ESU10), identified as *G. decima* by Chan *et al.* (2013a, b), in different clades. The divergence range between these clades is 0.116 and 0.147, indicating that these are two different ESUs.

By contrast, the divergence between the single specimen identified as *G. tabulatus*, from *Stylophora pistillata* (Esper) from Taiwan (Chan *et al.*, 2013a), and those obtained from *Favia pallida*, *Montastrea* sp., and from *Favia rotundata* (Veron & Pichon) ranges between 0.007 and 0.021, which, according to our delimiting criteria, means that these species belong to the same ESU. However, the opercular valves from *G. tabulatus* do not conform morphologically to those obtained from the other three hosts. Chan *et al.* (2013a, b) found that *G. altiapiculus* from Taiwan occupies three different hosts *Platygyra pini*, *Goniastrea pectinata* (Ehrenberg), and *Merulina ampliata* (Ellis & Solander). These hosts reside in two coral families, Merulinidae and Faviidae.

Our results cast shadow on the validity of the use of opercular valve morphology as a suitable character for separating species of *Galkinius*. There are distinct differences in valve morphology between valves of a single ESU, from different host species, and different conspecific hosts. The morphological differences may result from high plasticity or allometric growth of the opercular valves and the changes in the morphology during the life time of *Galkinius* species.

HOST SPECIFICITY IN *GALKINIUS*

Host specificity seems to be a common feature within the pyrgomatids. Mokady *et al.* (1999) suggested, based on mt-12s ribosomal RNA sequences, that barnacles previously assigned to a single species, *Trevathana dentata* (Darwin), group into different sibling species, each corresponding to a different host coral species. This was later supported by Malay (2007) who suggested that speciation in *Trevathana* species occurred via host switching. Brickner *et al.* (2010), using both molecular markers and morphology, described five species of *Trevathana* from the Red Sea, each from a different host coral. At the genus level it is known that species of pyrgomatids are restricted to a single host e.g. *Pyrgoma* to *Turbinaria*, the monospecific genus *Hiroa* Ross & Newman (Achituv & Newman 2002) and *Cionophrous* Ross & Newman to *Asteropora*, *Pyrgopsella annandalei* (Gruvel) to

Symphyllia (Achituv & Simon-Blecher, 2006), and the tribe Hoekiniini to *Hydnophora*. Others, like *Cantellius*, *Savignium*, *Darwiniella*, and *Nobia*, occupy a range of hosts. It is of interest to examine if this is a rule in the pyrgomatids.

In *Galkinius* there is a varied range of host specificity. *Galkinius altiapiculus* was obtained from three host corals. *Galkinius tabulatus*, in addition to its type host *Stylophora pistillata*, is associated with *Galkinius* found on three other host corals. Barnacles from *F. rosaria* Veron are clustered with those from *Acantastrea* sp. in another distinct ESU. By contrast, out of the 14 ESUs identified by us, ten are related to a monospecific host. It should be kept in mind that this is likely to result from sampling error because one of the main drawbacks to studying coral-inhabiting barnacles is the limited availability of material suitable for molecular study. Most of the information regarding coral barnacles originates from dry corals in museum collections. In addition, in many cases the data on barnacles obtained from museum collections of Cirripedia do not include enough details on the host coral. We thus stress the importance of preserving both barnacles and their hosts in a manner suitable for molecular identification, as well as all related information.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Records of corals species hosting *Galkinia* used in the present study. Origin of samples and deposition of samples is indicated. Abbreviations of collections: ASIZCR, Biodiversity Research Museum of Academia Sinica, Taipei, Taiwan; CEL, Coastal Ecology Lab, Academia Sinica, Taiwan; MNHN, Museum Nationale d'Histoire Naturelle, Paris; NHM, Natural History Museum, London; NMNS, National Museum of Natural Science, Taichung, Taiwan; RMNH, Naturalis Biodiversity Center of the Rijksmuseum van Natuurlijke Historie, Leiden, the Netherlands; TAU, Tel Aviv University Zoological Museum, Steinhardt National Collections of Natural History; WAM, Western Australian Museum crustacean collection and coral collection (some of the barnacles from the crustacean collection of WAM do not have the host coral in which case only the crustacean collection number, indicated by C-, is provided).

Table S2. A, cytochrome *c* oxidase subunit I (*COI*) sequences of *Galkinius* used for construction of the maximum likelihood tree. The data include sequences generated in the present study (GenBank accession numbers KP702762–KP702820) and randomly selected sequences of species of *Galkinius* described by Chan *et al.* (2013b). Barnacles extracted from a single coral colony are indicated by the same capital letter, numerals indicate a single barnacle.

Table S3. Estimates of evolutionary divergence between sequences of *Galkinius*. The number of base substitutions per site from between sequences is shown. Standard error estimates are shown above the diagonal. The analysis involved 118 sequences of *COI*, 73 sequences were generated in the present study, 46 were retrieved from the GenBank presenting 6 identified species.