

UNIVERSIDADE DE SÃO PAULO
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Taxonomy and Cladistic Analysis of Plakobranchidae
(Gastropoda: Sacoglossa)

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Original Version

Thesis presented to the Post-Graduate Program of the Museu de Zoologia da Universidade de São Paulo, to obtain the degree of Doctor of Sciences in Systematics, Animal Taxonomy, and Biodiversity

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ABSTRACT

The comprehension of the evolutionary history of the order Sacoglossa is fundamental to understand the adaptive radiation of Heterobranchia, the most morphologically heterogeneous group in Gastropoda. Sacoglossans form a well-supported monophyletic group, but the relationship of its internal clades is still confused. Plakobanchidae is the most diverse family and the main systematic problem of Sacoglossa since the lack of detailed descriptions provided many synonyms and invalid taxa from genus to species level. The family Plakobanchidae resulted in a monophyletic group only in few phylogenetic hypotheses of Sacoglossa. However, either molecular or morphological data were usually analyzed to reconstruct Sacoglossa phylogeny, while an integrative approach has never been applied for such purpose. A total 42 species, including 30 species of Plakobanchidae, 10 related species in Sacoglossa and other two heterobranchs, was analyzed to test the monophyly of Plakobanchidae and the relationship of its internal clades. A parsimonious phylogenetic analysis was performed in TNT considering 109 characters of external morphology, internal anatomy and ecology data. Molecular partial sequences of two mitochondrial (COI, 16S) and three nuclear (H3, 18S and 28S) genes were used to build a phylogenetic hypothesis under Bayesian Inference and Maximum Likelihood criteria. One final BI analysis was performed with a combined matrix with molecular, morphological and ecological data of all analyzed taxa. Plakobanchidae resulted as monophyletic only in morphological and combined data, but the monophyletic status of the genera *Plakobanchus* van Hasselt, *Thuridilla* Bergh and *Elysia* Risso, 1818 was fully supported by all analyses. Species within the multi-diverse *Elysia* form similar clades in all different analysis matching with prior synonymized names, meaning that old names might be resurrected for a better explanation of the natural history of this genus.

Keywords: Sea slugs; Heterobranchia; phylogeny; total evidence; morphology.

RESUMO

Compreender a evolução da ordem Sacoglossa é de fundamental importância para o entendimento da irradiação adaptativa de Heterobranchia, o grupo de Gastropoda mais morfológicamente diverso. Os sacoglossos formam um grupo monofilético bem suportado, mas as relações dos seus clados internos ainda é confusa. Plakobanchidae é a família mais diversa e com os principais problemas sistemáticos de Sacoglossa, uma vez que a ausência de descrições detalhadas contribuiu para muitas sinonimizagens e invalidações de táxons em níveis de gênero e de espécie. A família Plakobanchidae resultou em um grupo monofilético apenas em poucas hipóteses filogenéticas de Sacoglossa. Entretanto, apenas dados moleculares ou morfológicos foram analisados para reconstruir a filogenia de Sacoglossa, enquanto que uma abordagem integrativa nunca foi feita para esse propósito. Um total de 42 espécies, incluindo 30 espécies de Plakobanchida, 10 de espécies próximas em Sacoglossa e dois outros heterobrâncios, foram analisados para testar a monofilia de Plakobanchidae e as relações entre seus grupos internos. Uma análise filogenética de parcimônia foi realizada no TNT considerando 109 caracteres da morfologia externa, anatomia interna e dados ecológicos. Sequências moleculares de dois genes mitocondriais (COI, 16S) e três nucleares (H3, 18S e 28S) foram usadas para construir hipóteses filogenéticas sob os critérios de Inferência Bayesiana e Máxima Verossimilhança. Uma última análise bayesiana foi feita com os dados moleculares, morfológicos e ecológicos combinados em uma matriz. Plakobanchidae resultou como um grupo monofilético apenas nas análises com dados morfológicos e dados combinados, mas a monofilia dos gêneros *Plakobanchus*, *Thurudilla* e *Elysia* foram bem suportadas em todas as análises. Espécies do multi-diverso *Elysia* formam clados similares nas diferentes análises que coincidem com nomes sinonimizados do gênero, o que significa que nomes antigos do grupo podem ser reerguidos para explicar melhor a história evolutiva do grupo.

Palavras-chave: Lesmas-do-mar; Heterobranchia; filogenia; evidência total; morfologia.

1. INTRODUCTION

1.1. GENERAL CLASSIFICATION

Phylum Mollusca is a highly diverse animal group with about 200.000 extant and 30.000 extinct species (PONDER & LINDBERG, 2008). Being the second most species animal group, Mollusca comprises 8 living classes with distinct body organizations, comprising interstitial worm-like specimens, chitons, bivalves, octopuses, snails, limpets and terrestrial slugs. The diversity of body plans allowed the mollusks to explore different habitats in marine, aquatic and terrestrial environments (PONDER & LINDBERG, 2008).

The class Gastropoda is the one with more successful adaption in distinct environments and represents the richest class in Mollusca (BIELER, 1992). Gastropods are usually the most common mollusks in all marine ecosystems. In addition, they are the only group to have invaded the land and freshwater habitats on all continents (AKTIPIIS *et al.*, 2008).

The traditional division of Gastropoda in Prosobranchia, Opisthobranchia and Pulmonata has changed drastically since phylogenetic analyses indicated the paraphyly of these groups (PONDER & LINDBERG, 1997, ZAPATA *et al.*, 2014). After the first work to use cladistic methods, the groups Opisthobranchia, Pulmonata and some prosobranchs were included in the clade Heterobranchia, and the remaining prosobranchs were grouped in the clades Patellogastropoda, Cocculiniformia, Neritimorpha, Vetigastropoda and Caenogastropoda (PONDER & LINDBERG, 1997; BOUCHET & ROCCOI, 2005; SIMONE, 2011).

Within the gastropods, Heterobranchia is the most morphologically diverse clade, represented by up to 40.000 species inhabiting pelagic, benthic, terrestrial and freshwater habitats (WÄGELE *et al.*, 2008; MORDAN & WADE, 2008). The synapomorphies of Heterobranchia includes the sinistral larval shell, absence of ctenidium, simple esophagus and lack of odontophoral cartilages (PONDER & LINDBERG, 1997). Both morphological (PONDER & LINDBERG, 1997; DAYRAT & TILLIER, 2002; SIMONE, 2011) and molecular phylogenies (GRANDE *et al.*, 2004; DINAPOLI &

KLUSSMANN-KOLB, 2010) have recovered Heterobranchia as monophyletic as proposed by Hazsprunar (1985).

Heterobranchia presents the internal clade Euthyneura, which is composed mostly by previous opisthobranch and pulmonate groups and is characterized by different degrees of detorsion on nervous system and nerve concentration (HASPRUNAR, 1985). The monophyly of Euthyneura was largely corroborated by morphological based phylogenies (PONDER & LIINDBERG, 1997; DAYRAT & TILLIER, 2002; WÄGELE & KLUSSMANN-KOLB, 2005), but was changed after the inclusion of Glacidorboidea and Pyramidelloidea in molecular based analyses (GRANDE *et al.*, 2008; DINAPOLI & KLUSSMANN-KOLB, 2009; JÖRGER *et al.*, 2010).

Relationships among opisthobranch and pulmonate groups has changed after the inclusion of more “lower heterobranchs” groups in phylogenetic analysis of Heterobranchia. A new classification of Euthyneura was proposed by Jörger *et al.* (2010) as following: Nudipleura (Nudibranchia and Pleurobranchomorpha), Euopisthobranchia (Umbraculida, Cephalaspidea *s.s.*, Aplysiomorpha and Pteropoda), and Panpulmonata. The latter is composed by the former opisthobranch clades Sacoglossa and Acochliidae, lower heterobranch groups (Pyramidelloidea e Glacidorboidea) and all former pulmonated groups. A monophyletic group was named as Eupulmonata including the pulmonate clades Stylommatophora, Systellomatophora, Ellobioidea, Otiinoidea and Trimusculoidea. Other authors found similar relationship among opisthobranch clades, but not for pulmonates (GOBBELER & KLUSSMANN-KOLB, 2010; SCHRÖDL *et al.*, 2011; DAYRAT *et al.*, 2011). Medina *et al.*, (2011) was the only analysis that recovered Opisthobranchia as monophyletic.

One of the most intriguing findings with new molecular framework in Heterobranchia was the relationship between the opisthobranch group Sacoglossa and Siphonarioidea. Both groups were recovered as a clade (JÖRGER *et al.*, 2010) or separated (GOBBELLER & KLUSSMANN-KOLB, 2011) at the base of Panpulmonata, or forming a clade related to other opisthobranch groups (MEDINA *et al.*, 2011). Sacoglossans have small size, generally ranging from 5 to 40 mm, and body characteristics resembling other marine (e. g., Cephalaspidea and Nudibranchia) and terrestrial groups (Pulmonata) (JENSEN, 1996, 1997). Thus, the order occupies a strategic position for understanding the evolutionary history of Heterobranchia.

1.2. THE ORDER SACOGLOSSA: GENERAL FEATURES

There are up to 300 described species of Sacoglossa, but many undescribed ones have been reported in sea slugs field guides and website forums from different parts of the world (COLEMAN, 2001; YONOW, 2008; VALDÉS, HAMANN & BEHRENS, 2006; GOSLINER, BEHRENS & VALDÉS, 2008; GOSLINER, VALDÉS & BEHRENS, 2015). Some groups present a small and fragile shell, varying in shape from slightly elongated to bulloid and bivalve forms, but in the more diverse groups the shell is completely lacking in adults (JENSEN, 1996).

Sacoglossans are highly specific herbivores with body size reaching up to 4 cm, living cryptically associated to marine plants (JENSEN, 1996). Many species feed on siphonalean green algae (Caulerpales, Codiales and Dasycladales), few specialized on brown algae, diatoms and sea grasses (JENSEN, 1997), and three non-herbivorous species feed on egg masses of other opisthobranchs (JENSEN, 1986, 1997; COELHO *et al.*, 2006).

Most genera of Sacoglossa are restricted to feed on one green algal group, and a wide variety of diets among different species is apparently an evidence that the speciation process in sacoglossans has been strongly driven by diet changes (JENSEN, 1997). This high specific diet has also limited the species distributions to the oceanic photic zone (down to 100m depth), with the highest number of sacoglossans occurring on tropical areas of the globe, such as Central Pacific and Caribbean Sea (JENSEN, 2007).

As one of few specialized herbivores in marine environment, the sacoglossans have developed a digestive system morphologically and physiologically adapted to feed on this group of algae (MARÍN & ROS, 2004). The buccal apparatus is equipped with an uniseriate radula that pierce the marine plants' cells, while a muscular and modified buccal mass suck the algal cytoplasm, that is later absorbed by the large internal surface of the digestive tract (JENSEN, 1997).

In general, the algae that sacoglossans feed on are rich of secondary metabolites capable of keep generalist herbivores away (MARÍN & ROS, 2004). However, sacoglossans can sequester the algal secondary metabolites and use them for their own defense, either by bioaccumulation or even modifying them in new compounds

(biotransformation) (GAVAGNIN *et al.*, 2000). Those chemical defense strategies are commonly observed in shelled sacoglossans, while shell-less sacoglossans produce their own deterrent chemicals (usually polypropionates) by *de novo* bio-synthesis (MARÍN & ROS, 2004). This can be an evidence of a parallel evolutionary pathway between morphological change from a shell to shell-less body types, and a switch on chemical defense strategy from bioaccumulation and biotransformation to bio-synthesis of new compounds (CIMINO, FONTANA & GHISELIN, 1999).

The evolution of Sacoglossa is also related to the ability of members of the clade to keep functional plastids from the algae they consume within their digestive gland cells, a biological phenomenon known as kleptoplasty (RUMPHO *et al.*, 2007). This physiological adaptation is common in ciliate and foraminifera protists. The order Sacoglossa, however, is the only metazoan group known to retain chloroplasts intracellularly for different amount of time (RUMPHO, SUMMER & MANHART, 2010).

The species of shelled sacoglossans in the genera *Volvatella* Pease, 1860 and *Ascobulla* Ev. Marcus, 1972 are incapable to keep chloroplasts alive in their digestive tract. However, other shelled genera such as *Oxynoe* Rafinesque, 1814 and *Lobiger* Krohn, 1847, retain functional chloroplasts intact for up to four days, although with no carbon fixation (see RUMPHO *et al.*, 2007). On the other hand, functional kleptoplasty has evolved in two lineages of shell-less sacoglossans, the cerata-bearing genus *Costasiella* Pruvot-Fol, 1951 and the highly diverse clade Plakobranchoidea, which comprises all parapodia-bearing species (CHRISTA *et al.*, 2014).

Functional kleptoplasty is considered like short-term in the great majority of species, in which the chloroplast is functional up for 14 days (HÄNDELER *et al.*, 2009). When kleptoplasty is considered as long-term, intact chloroplasts might last few weeks to months without being digested by the sea slug. Only few species of different independent lineages are capable of such rates of intracellular plastid survival, which is an evidence that long-term retention might have evolved independently at least five times in Sacoglossa (CHRISTA *et al.*, 2013; 2014).

1.3. THE ORDER SACOGLOSSA: THE EARLY CLASSIFICATION AND PHYLOGENETIC FRAMEWORK

The taxon Sacoglossa was erected by von Ihering (1876) to group shelled and non-shelled sea slugs by sharing a modified gill, a central nervous system with seven closely spaced ganglia and an uniseriate radula, which part of it is stored at a ventral pouch on buccal mass. After that, the status of natural group of Sacoglossa was rarely debated, but the hypotheses of relationship among internal groups were quite different in the first classifications (see JENSEN, 1996).

Major taxonomic changes in Sacoglossa include the insertion of other groups of gastropods after further morphological investigations. The former cephalaspidean group Volvatellidae, the bivalve gastropods group Juliidae and the worm-like group Platyhedylidae were transferred to Sacoglossa based on radula and buccal mass morphology (EVANS, 1950; BABA, 1961; WAWRA, 1979; MARCUS, 1982).

Sacoglossa is subdivided in two major morphological groups: the shelled suborder Oxynoacea and the shell-less suborder Plakobranchea (JENSEN, 1996). Both groups have the monophyly well-supported, although the relation of the genus *Cylindrobulla* P. Fischer, 1857 with the other sacoglossans is still widely debated (MIKKELSEN, 1998; LAETZ *et al.*, 2014). This former cephalaspidean genus was considered as sister to all Sacoglossa in the first phylogenetic analysis of the order (JENSEN, 1996) and some molecular based phylogenies (HÄNDELER & WÄGELE, 2007; HÄNDELER *et al.*, 2009), but also placed in a more derived position within Sacoglossa as sister to all shelled groups by both morphological and molecular phylogenetic hypothesis (MIKKELSEN, 1998; LAETZ *et al.*, 2014).

The suborder Oxynoacea is represented by headshielded species (Cylindrobullidae and Volvatellidae), bivalve shelled species (Juliidae) and species with bulloid shell and autotomic body parts (Oxynoidea). Plakobranchea is subdivided in two shell-less groups, the cerata-bearing superfamily Limapontioidea and the parapodia-bearing superfamily Plakobranchoidea (JENSEN, 1996).

The superfamily Limapontioidea is a cerata-bearing group in Sacoglossa with a large variety of morphological traits and food habits. The rhinophore can be simple, rolled, grooved or absent in different genera, while the radular shape, anus position and food preference can be variable in the same genus (JENSEN, 1997b). The morph diversity made the Limapontioidea unsatisfactory classified for many authors in the past (PRUVOT-FOL, 1954, BABA & HAMATANI, 1970, GASCOIGNE, 1976). Furthermore,

phylogenetic reconstructions based on morphological (Jensen, 1996) and molecular evidences (HÄNDELER & WÄGELE, 2007, CHRISTA *et al.*, 2014) have not recovered Limapontioidea as monophyletic.

The family Platyhedylidae, later synonymized with Gascoignellidae by Jensen (1996), has only two genera, both with characters that resemble less derived Sacoglossa. *Platyhedyle* Salvini-Plawén, 1973 and *Gascoignella* Jensen, 1985 are interstitial worm-like animals with a highly flattened body and no parapodia or rhinophores. The group were placed in Limapontioidea in early classifications and transferred later to Plakobranchoidea by morphological phylogenetic analysis (JENSEN, 1996; MIKKELSEN, 1998). However, molecular based analysis placed Platyhedylidae back to Limapontioidea (KRUG *et al.*, 2015) or sister to all other shell-less Sacoglossa (CHRISTA *et al.*, 2014).

The superfamily Plakobranchoidea is represented by the monogeneric family Bosellidae and the hyper diverse family Plakobranchoidea. The genus *Bosellia* Trinchese, 1891 was previously classified as Polybranchidae (a cerata-bearing group) and Plakobranchoidea, but later Marcus (1982) erected the family Bosellidae due to absence of parapodia and lower number of chromosome (n=7) comparing to other sacoglossans (JENSEN, 1997a). The family was later considered paraphyletic by a molecular phylogenetic hypothesis, because *Bosellia marcusii* Marcus, 1972 was recovered in Plakobranchoidea (BASS & KARL, 2006), and later considered as a derived *Elysia* Risso, 1818 with reduced parapodia (see CARMONA *et al.*, 2011).

Plakobranchoidea is the most diverse family by far, covering almost half of the Sacoglossa richness (JENSEN, 2007). This group encompasses the genus *Elysia* Risso, 1818, which includes 90 valid species and a list of synonymies with nearly 20 names (KRUG *et al.*, 2016). This vast synonymic list is mainly due to the large number of taxa created based on one specific morphological character of one single species, such as in the genera *Tridachia* Deshayes, 1857 and *Tridachiella* MacFarland, 1924 (KRUG *et al.*, *op. cit.*), which were created to place species with highly undulated parapodial margins.

The members of the Plakobranchoidea family are especially notable by the presence of many lineages that display short and long-term kleptoplasty, as well as a wide variety of diets (RUMPHO *et al.*, 2000; EVERTSEN *et al.*, 2007; JENSEN, 1996, 1997). Moreover, species within Plakobranchoidea have been subjects of pharmaceutical

studies (CIMINO & GAVAGNIN, 2007) and proposed as potential bioinvasion control models (THIBAUT *et al.*, 2001).

In one of the main systematic considerations of Plakobrachidae Jensen (1992) proposed the validity of the genera *Plakobranchus* van Hasselt, 1824, *Pattyclaya* Ev. Marcus, 1982, *Elysiella* Bergh, 1871, *Thuridilla* Bergh, 1872 and *Elysia*, inserting *Tridachia* and *Tridachiella* in *Elysia*. However, the first cladistic analyses of the group proposed the monophyly of *Elysia* after insertion of *Pattyclaya* and *Elysiella* or subdivision of the genus into smaller groups (GOSLINER, 1995; JENSEN, 1996).

Molecular-based analyses suggest that *Elysia* could be monophyletic after the inclusion of *Pattyclaya*, *Elysiella*, *Tridachia* and *Tridachiella* (BASS & KARL, 2006; HÄNDELER & WÄGELE, 2007; CHRISTA *et al.*, 2014, 2015; KRUG *et al.*, 2016). Furthermore, the paraphyletic status of the family Plakobrachidae has also been proposed (MAEDA *et al.*, 2010; CHRISTA *et al.*, 2014, 2015).

Species description of Plakobrachidae are almost exclusively restricted to external morphology and few details of reproductive system. Hence, diagnoses of the genera, even when based on phylogenetic systematics, are incomplete. Despite the efforts of the aforementioned authors, there is a large gap in the knowledge of morphological diversity in Plakobrachidae. Also, patterns of evolution and diversification in different lineages. Therefore, clarifying some of these gaps can help elucidate more of the poorly known internal relationship of Plakobrachidae.

2. CONCLUSIONS

1. The family Plakobanchidae resulted as a well-supported monophyletic group in all morphological analysis;
2. Plakobanchidae has not resulted as a natural group in molecular phylogenies, but its monophyletic status was recovered in total evidence analysis;
3. All the three valid genera within Plakobanchidae are monophyletic in analyses with distinct criteria and data set;
4. *Thuridilla* and *Plakobanchus* are well-supported in all scenarios and, except for the consensus topology with prior weights, resulted as sister groups;
5. The genus *Elysia* are low supported in all analysis, and its internal clades are grouped slightly different among analyses;
6. Morpho groups in *Elysia* has been recovered as clades in most analyses, which clearly reflects distinct evolutionary pathways, and meaning that a future subdivision of the genus is necessary for a better understanding of their natural history;
7. Despite similar external morphology, plakobanchids present a large variation on their internal morphology in all systems studied herein;

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