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# *Anthopleura* and the phylogeny of Actinioidea (Cnidaria: Anthozoa: Actiniaria)

**M. Daly**[1] · **L. M. Crowley**[2] · **P. Larson**[1] · **E. Rodríguez**[2] · **E. Heestand Saucier**[1,3] · **D. G. Fautin**[4]

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**Abstract** Members of the sea anemone genus *Anthopleura* are familiar constituents of rocky intertidal communities. Despite its familiarity and the number of studies that use its members to understand ecological or biological phenomena, the diversity and phylogeny of this group are poorly understood. Many of the taxonomic and phylogenetic problems stem from problems with the documentation and interpretation of acrorhagi and verrucae, the two features that are used to recognize members of *Anthopleura*. These anatomical features have a broad distribution within the superfamily Actinioidea, and their occurrence and exclusivity are not clear. We use DNA sequences from the nucleus and mitochondrion and cladistic analysis of verrucae and acrorhagi to test the monophyly of *Anthopleura* and to evaluate the pattern of distribution of acrorhagi and verrucae. We find that *Anthopleura* is paraphyletic: although species of the genus cluster together, some groups also include members of genera like *Bunodosoma*, *Aulactinia*, *Oulactis*, and *Actinia*. This paraphyly is explained in part by the discovery that acrorhagi and verrucae are pleisiomorphic for the subset of Actinioidea studied.

**Keywords** *Anthopleura* · Actinioidea · Cnidaria · Verrucae · Acrorhagi · Pseudoacrorhagi · Atomized coding

*Anthopleura* Duchassaing de Fonbressin and Michelotti, 1860 (Cnidaria: Anthozoa: Actiniaria: Actiniidae) is one of the most familiar and well-known genera of sea anemones. Its members are found in both temperate and tropical rocky intertidal habitats and are abundant and species-rich when present (e.g., Stephenson 1935; Stephenson and Stephenson 1972; England 1992; Pearse and Francis 2000). Due to their diversity and abundance in predictable and accessible places, *Anthopleura* is the subject of many field studies of rocky intertidal ecology and physiology, including studies of thermal stress and nutrient transfer (e.g., Jennison 1978; Kruger and Griffiths 1998; Richier et al. 2008; Hiebert and Bingham 2012; Morar et al. 2011; Bingham et al. 2011; Quesada et al. 2014), the impact of pollution (e.g., Wicksten 1984), disease vectoring (e.g., Hopper et al. 2008), and the effect of local changes in habitat (e.g., Pineda and Escofet 1989; Haag and Dyson 2014). Specimens of species in this genus serve as model organisms for studying toxins and genomes (e.g., Hauck et al. 2007; Zhang et al. 2007; Xiang et al. 2008; Kohno et al. 2009; Peigneur et al. 2011, 2012; Alvarado et al. 2014; Macrander et al. 2015; Zhang and Zhu 2016, Ayala-Sumuano et al. 2017). Numerous studies have used species of *Anthopleura* to explore the symbiosis between anemones and microorganisms (e.g., Pearse 1974; Saunders and Muller-Parker 1997; Verde and McCloskey 2002; Weis et al. 2002; Bergschneider and Muller-Parker 2008; Letsch et al. 2009; McBride et al. 2009; Sanders and Palumbi 2011; Hiebert and Bingham 2012; Levine and Muller-Parker 2012;

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✉ M. Daly
daly.66@osu.edu

[1] Department of Evolution, Ecology, and Organismal Biology, Ohio State University, Columbus, OH 43212, USA

[2] Division of Invertebrate Zoology, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024, USA

[3] University of Louisiana at Lafayette, Lafayette, LA 70503, USA

[4] University of Kansas Natural History Museum, Lawrence, KS 66045, USA

Towanda and Thuesen 2012; Miura et al. 2014; Borbón et al. 2016; Dimond et al. 2017).

*Anthopleura* is of particular interest in part because its members have inducible structures called acrorhagi that are deployed as part of a complex intraspecific interaction (reviewed in Francis 1988). Acrorhagi are bulbous marginal structures densely packed with holotrichous nematocysts (Fig. 1d, g, h); they are inflated and applied to the column of a conspecific individual during aggressive interactions (reviewed in Daly 2003). Acrorhagi, which are characteristic of *Anthopleura* and several other genera within the family Actiniidae (Table 1), have been documented to play a role in intra- and interspecific interactions with other anemones (Francis 1973, 1976; Bigger 1988; Ayre and Grosberg 2005) and in allorecognition (Bigger 1980; Grosberg 1988). This last capacity is clearly tied to the initiation and precision of the intraspecific behaviors mediated by acrorhagi (Foster and Briffa 2014). Because acrorhagial interactions are critical to fitness (Rudin and Briffa 2011), it is plausible that these structures have played a role in their diversification. However, as acrorhagi-bearing anemones are most species rich in temperate

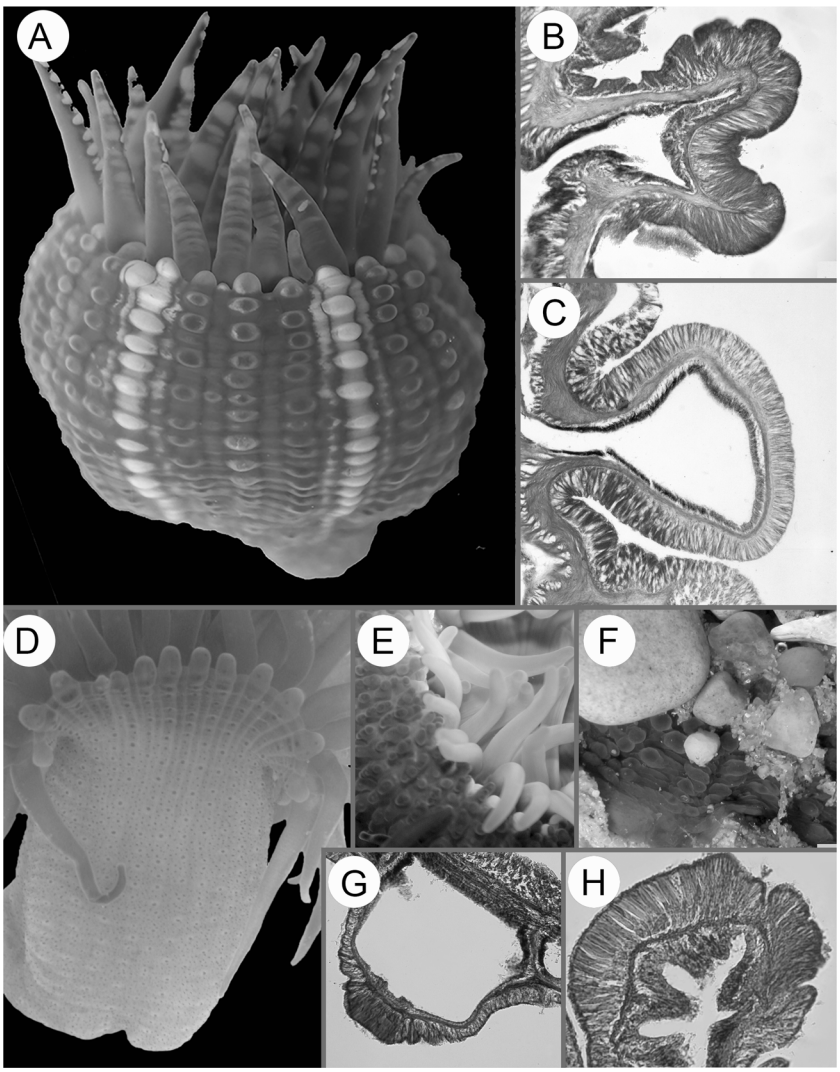



**Fig. 1** Anatomy and morphology of acrorhagi, verrucae, and vesicles. **a** External morphology of living, contracted specimen of *Bunodactis verrucosa*. Rows of verrucae on column extend to limbus and project over fosse. These marginal projections lack holotrichs and so are pseudoacrorhagi. **b** Longitudinal histological section through a verruca of *Anthopleura thallia*.**c** Longitudinal section through a vesicle of *Bunodosoma californicum*. Note that the vesicle lacks the dense glandular cells of the verruca in **b**. **d** External morphology of a living specimen of *Anthopleura ballii*. The marginal projections contain dense holotrichs on the surface facing the tentacles and fosse and so are acrorhagi. **e** External anatomy of vesicles on distal column of *Bunodosoma cavernatum*. Small white spherules visible between distal vesicles and tentacles are acrorhagi. **f** External morphology of the column of *Anthopleura biscayensis*, showing verrucae; some verrucae are holding gravel and are thus not visible. **g** Cross section through an acrorhagus of *Anthopleura pallida*. The holotrich-dense pad (H) in the center of the acrorhagus faces into the fosse in life. **h** Longitudinal section of an acrorhagus of *A. pallida*

**Table 1** Actinioidean genera characterized as having acrorhagi, pseudoacrorhagi, verrucae, or vesicles in focal genera, with source citations

| Genus | Marginal structure | Column structure | Sources |
|---|---|---|---|
| ***Actinia*** | Acrorhagi | Smooth | Stephenson (1935) |
| ***Anemonia*** | Acrorhagi and pseudoacrorhagi | Smooth | Stephenson (1935); Häussermann and Försterra (2001) |
| ***Anthopleura*** | Acrorhagi | Verrucae | Daly (2004b) |
| ***Anthostella*** | Acrorhagi and pseudoacrorhagi | Solid spots | Carlgren (1938, 1949) |
| ***Aulactinia*** | Pseudoacrorhagi | Verrucae | Fautin and Chia (1986) |
| ***Bunodactis*** | Pseudoacrorhagi | Verrucae | Stephenson (1935) |
| ***Bunodosoma*** | Acrorhagi | Non-adhesive vesicles | Daly (2004a) |
| *Condylactis* | None | Smooth or with verrucae | Cairns et al. (1986) |
| ***Dactylanthus*** | None | Vesicles | Dunn (1983); Rodríguez and López-González (2013) |
| ***Epiactis*** | None | Smooth or with verrucae | Fautin and Chia (1986) |
| ***Gyractis*** | Pseudoacrorhagi | Verrucae | Fautin (2005) |
| ***Heteractis*** | None | Smooth or with verrucae | Dunn (1981) |
| *Isactinia* | Pseudoacrorhagi | Smooth | Carlgren (1949) |
| ***Isoaulactinia*** | Pseudoacrorhagi | Vesicles | Belém et al. (1996); Daly (2004a) |
| ***Isotealia*** | Pseudoacrorhagi | Smooth | Carlgren (1949); Riemann-Zürneck (1980) |
| ***Macrodactyla*** | None | Verrucae | Dunn (1981) |
| *Oulactis* | Acrorhagi | Verrucae | Häussermann (2003) |
| *Paracondylactis* | Pseudoacrorhagi | Smooth or rarely with verrucae or vesicles | Carlgren (1949) |
| *Parantheopsis* | None | Verrucae | Carlgren (1949) |
| ***Phlyctenactis*** | None | Vesicles | Parry (1951) |
| ***Phymactis*** | Acrorhagi | Vesicles | Häussermann (2004) |
| ***Phymanthus*** | Pseudoacrorhagi | Verrucae | González-Muñoz et al. (2015) |
| ***Preactis*** | None | Vesicles | England and Robson (1984); Cappola and Fautin (2000) |
| ***Pseudactinia*** | Acrorhagi | Vesicles | Carlgren (1949) |
| *Telianthus* | Pseudoacrorhagi | Smooth | Carlgren (1927) |
| ***Stichodactyla*** | None | Verrucae | Dunn (1981) |
| ***Urticina*** | None | Smooth or with verrucae | Stephenson (1935); Hand (1955) |

Refer to text for definitions of acrorhagi, pseudoacrorhagi, verrucae, and vesicles and to Table 3 for coded matrix of characters for species included in analyses. Genera included in the present study are in bold text

regions with hard substrate habitats (Fautin 2013), historical and ecological contingency cannot easily be disentangled as explanations: this habitat might favor the retention or re-evolution of acrorhagi, or this group may have undergone a radiation in this habitat because they have these structures (or both).

Beyond their critical role in the ecology and biology of the anemones that bear them, acrorhagi are key diagnostic and taxonomic features (Stephenson 1935; Carlgren 1949). Nonetheless, practical and conceptual problems plague their application as a taxonomic feature: most critically, in at least some groups, acrorhagi do not manifest under all ecological circumstances or in all individuals, being induced through contact with conspecifics (reviewed by Daly 2003). Furthermore, because identification of an acrorhagus relies on relatively high-powered microscopy to differentiate between the types of nematocysts contained within the tissue, acrorhagi may not be correctly documented in species described before the middle of the twentieth century. Acrorhagi are sometimes confused with "pseudoacrorhagi," bulbous structures that protrude from the distal column margin but that lack the nematocysts and behaviors associated with acrorhagi (Fig. 1a; see Daly 2003). Confusion happens because acrorhagi and pseudoacrorhagi are similar in external appearance and because the two structures may co-occur, mistakenly leading to the inference that only one is present.

The problems associated with acrorhagi as a diagnostic feature weigh heavily on systematic studies of *Anthopleura* because this feature is one of two that define

the genus (Stephenson 1935; Carlgren 1949; Daly and den Hartog 2004). *Anthopleura* is differentiated from other members of Actiniidae in having both acrorhagi and protrusive, adhesive column structures called verrucae (Fig. 1b, f; see also Daly 2004a; Daly and den Hartog 2004). As for acrorhagi, some functions have been identified for verrucae, including reducing water loss at low tide (Hart and Crowe 1977) and retarding UV exposure (Dykens and Shick 1984). Verrucae are poorly defined in terms of anatomy and can be difficult to identify in preserved animals (reviewed in Häussermann 2004). The distinction between verrucae and so-called vesicles—non-adhesive, hollow outgrowths of the column (Fig. 1c, e)—is unclear (Stephenson 1928; Häussermann 2004), and the term vesicle may be overly general, referring to multiple structures. These inconsistencies make vesicles a problematic taxonomic feature: for example, both *Phymactis* and *Bunodosoma* are defined as having acrorhagi and non-adhesive vesicles (Table 1; see Gomes et al. 2012); as currently defined, these genera cannot be differentiated.

The taxonomic problems caused by the imprecise way in which acrorhagi and verrucae are defined extend beyond *Anthopleura* because acrorhagi and verrucae are also characteristic of groups other than *Anthopleura* (Table 1). The other genera are characterized either by the occurrence of only one of these features (e.g., verrucae but no acrorhagi in *Aulactinia*; acrorhagi but no verrucae in *Actinia*) or by the additional presence of additional anatomical structures (e.g., verrucae, acrorhagi, and marginal frills in *Oulactis*). In addition to taxonomic confusion, the evolutionary relationship between acrorhagi and pseudoacrorhagi (or between verrucae and vesicles) remains unknown: it is possible that acrorhagi and pseudoacrorhagi (or verrucae and vesicles) represent alternate manifestations of a homologous structure or that they are distinct and have no direct historical relationship (Daly 2003; Daly and den Hartog 2004).

We evaluate phylogenetic relationships among actinioidean sea anemones, with the goal of interpreting the phylogenetic, evolutionary, and taxonomic value of acrorhagi and verrucae. We include representatives of all genera characterized as having one or both of these features (Table 1), with especially dense sampling of *Anthopleura* (Table 2). Previous molecular phylogenies (Geller and Walton 2001; Daly et al. 2008; Rodríguez et al. 2014) and a morphological phylogeny (Daly 2004a) have demonstrated that *Anthopleura* is polyphyletic with respect to allied genera like *Bunodosoma*, *Aulactinia*, and *Gyractis*. Our more densely sampled analysis concurs in finding a non-monophyletic *Anthopleura*, although our trees indicate that the genus is paraphyletic with respect to other genera (and groups of genera) within Actiniidae rather than polyphyletic. Furthermore, we find that Actiniidae is paraphyletic with respect to other actinioidean lineages. Regardless of how we code or optimize acrorhagi or verrucae, we find both to be primitively present in our sample of taxa. Verrucae and vesicles are more labile than acrorhagi, evolving multiple times. Although our analyses support the interpretation of acrorhagi as homologous in the animals that bear them, they are inappropriate for recognizing subgroups within Actiniidae because they appear to be a shared primitive feature, rather than uniquely derived in any group. Our trees include the broadest taxonomic sample of the superfamily Actinioidea to date and so provide perspective on broader patterns of evolution in this major lineage of Actiniaria.

## Material and methods

### Taxonomic sampling and data collection

We include 27 representatives of *Anthopleura*: 23 nominal species, with two representatives of the widely distributed *Anthopleura kurogane* and *Anthopleura nigrescens* and two undescribed species (*Anthopleura* sp. "Green" and *Anthopleura* sp. South Africa, from Oman and South Africa, respectively). We broadly sample allied groups within Actinioidea, including representatives of 32 genera spanning eight families (Table 2) and the majority of species in Actinioidea that have verrucae or acrorhagi (Table 1). Guided by Rodríguez et al. (2014), we use the metridioideans *Metridium senile* and *Diadumene leucolena* as our chosen outgroups. Sequences from GenBank were included as appropriate (Table 2). We have generally only included those taxa from which we were able to amplify at least three of the five markers from a single specimen, and thus have analyzed a total of 328 sequences for 78 terminal taxa.

Specimens were collected by hand intertidally or during scuba dives. All specimens were identified using polyp anatomy and the distribution and size of cnidae in various regions of the polyp. Voucher specimens in formalin have been deposited at the American Museum of Natural History (AMNH), the Bavarian State Collection of Zoology (ZSM), the collection of Biodiversidad y Ecología de Invertebrados Marinos (BEIM) at the University of Seville, the California Academy of Sciences (CAS), the University of Kansas Natural History Museum (KUNHM), and the US National Museum of Natural History (USNM).

Genomic DNA was isolated from tentacle or column tissue using the Qiagen DNAeasy® Kit or by standard CTAB extraction. Template DNA was amplified from genomic samples using published primers and standard techniques. We sequenced three mitochondrial markers (partial 12S ribosomal RNA (rRNA) (12S), 16S rRNA (16S),

**Table 2** Species included in these analyses

| Family | Species | CO3 | 12 | 16 | 28 | ITS |
|---|---|---|---|---|---|---|
| Actiniidae | *Actinia fragacea* | GU473334.1 | EU190714 | EU190756 | KJ483085 | KT852191 |
| Actiniidae | *Actinia tenebrosa* | KT852330 | KT852045 | KT852111 | – | KT852239 |
| Actiniidae | *Anemonia erythraea* | **KY789271** | **KY789302** | **KY789335** | – | **KY789335** |
| Actiniidae | *Anemonia natalensis* | KJ482987 | KJ482920 | KJ482958 | KJ483117 | – |
| Actiniidae | *Anemonia viridis* | GU473335 | EU190718 | EU190760 | KJ483095 | **KY789405** |
| Actiniidae | *Anthopleura annae* | **KY789293** | **KY789327** | **KY789360** | **KY789392** | **KY789426** |
| Actiniidae | *Anthopleura artemisia* | KT852300 | KT852015 | KT852081 | – | KT852210 |
| Actiniidae | *Anthopleura atodai* | KT852275 | KT851993 | KT852055 | KT852247 | KT852185 |
| Actiniidae | *Anthopleura ballii* | **KY789281** | **KY789311** | **KY789346** | **KY789376** | **KY789409** |
| Actiniidae | *Anthopleura biscayensis* | **KY789284** | **KY789315** | **KY789350** | **KY789380** | **KY789412** |
| Actiniidae | *Anthopleura buddemeieri* | – | **KY789316** | **KY789351** | **KY789381** | **KY789414** |
| Actiniidae | *Anthopleura dixoniana* | **KY789276** | **KY789307** | **KY789341** | – | **KY789406** |
| Actiniidae | *Anthopleura dowii* | **KY789286** | **KY789318** | **KY789353** | **KY789383** | **KY789416** |
| Actiniidae | *Anthopleura elegantissima* | GU473333 | EU190713 | EU190755 | KJ483104 | **KY789400** |
| Actiniidae | *Anthopleura fuscoviridis* | **KY789272** | **KY789303** | **KY789336** | **KY789369** | **KY789402** |
| Actiniidae | *Anthopleura handi* | KT852298 | KT852013 | KT852079 | **KY789387** | KT852208 |
| Actiniidae | *Anthopleura* sp. "*inornata*" | **KY789274** | **KY789304** | **KY789338** | **KY789371** | **KY789403** |
| Actiniidae | *Anthopleura insignis* | **KY789297** | **KY789331** | **KY789364** | **KY789395** | **KY789429** |
| Actiniidae | *Anthopleura japonica* | **KY789280** | **KY789310** | **KY789345** | – | – |
| Actiniidae | *Anthopleura krebsi* | **KY789275** | **KY789305** | **KY789339** | **KY789372** | **KY789404** |
| Actiniidae | *Anthopleura kurogane* Japan | – | **KY789323** | **KY789356** | – | – |
| Actiniidae | *Anthopleura kurogane* Korea | **KY789288** | **KY789321** | **KY789355** | **KY789385** | **KY789418** |
| Actiniidae | *Anthopleura midori* | **KY789289** | **KY789324** | – | **KY789388** | **KY789424** |
| Actiniidae | *Anthopleura nigrescens* Galapagos | **KY789278** | – | **KY789343** | **KY789373** | **KY789408** |
| Actiniidae | *Anthopleura nigrescens* Hawaii | – | **KY789306** | **KY789340** | **KY789374** | – |
| Actiniidae | *Anthopleura pacifica* | **KY789279** | **KY789309** | **KY789344** | **KY789375** | – |
| Actiniidae | *Anthopleura pallida* | **KY789277** | **KY789308** | **KY789342** | – | **KY789407** |
| Actiniidae | *Anthopleura* sp. Green | **KY789273** | – | **KY789337** | **KY789370** | – |
| Actiniidae | *Anthopleura sola* | – | – | **KY789365** | – | – |
| Actiniidae | *Anthopleura* sp. South Africa | **KY789295** | **KY789329** | **KY789362** | **KY789393** | – |
| Actiniidae | *Anthopleura thallia* | **KY789300** | **KY789333** | **KY789366** | **KY789397** | **KY789432** |
| Actiniidae | *Anthopleura waridi* | **KY789270** | **KY789301** | **KY789334** | **KY789368** | **KY789399** |
| Actiniidae | *Anthopleura xanthogrammica* | – | – | **KY789367** | **KY789398** | – |
| Actiniidae | *Anthostella stephensoni* | JQ810726 | JQ810719 | JQ810721 | KJ483132 | **KY789427** |
| Actiniidae | *Aulactinia incubans* | KT852299 | KT852014 | KT852080 | KT852256 | KT852209 |
| Actiniidae | *Aulactinia reynaudi* | – | KT852041 | KT852106 | KT852260 | KT852234 |
| Actiniidae | *Aulactinia stella* | KT852329 | KT852044 | KT852110 | KT852263 | KT852238 |
| Actiniidae | *Aulactinia veratra* | KT852283 | KT852001 | KT852063 | – | KT852194 |
| Actiniidae | *Bolocera kerguelensis* | KJ482985 | KJ482925 | KJ482965 | – | – |
| Actiniidae | *Bunodactis verrucosa* | FJ489484.1 | EU190723.1 | EU190766.1 | KT852250 | – |
| Actiniidae | *Bunodosoma californicum* | – | **KY789312** | **KY789347** | **KY789377** | – |
| Actiniidae | *Bunodosoma capense* | **KY789298** | **KY789332** | – | **KY789396** | **KY789430** |
| Actiniidae | *Bunodosoma cavernatum* | **KY789282** | **KY789313** | **KY789348** | **KY789378** | **KY789410** |
| Actiniidae | *Bunodosoma grande* | GU473336 | EU190722 | EU190765 | KJ483083 | **KY789413** |
| Actiniidae | *Bunodosoma granuliferum* | **KY789283** | **KY789314** | **KY789349** | **KY789379** | **KY789411** |
| Actiniidae | *Bunodosoma* sp. South Africa | **KY789296** | **KY789330** | **KY789363** | **KY789394** | **KY789428** |
| Actiniidae | *Epiactis japonica* 1 | **KY789285** | **KY789317** | **KY789352** | **KY789382** | **KY789415** |
| Actiniidae | *Epiactis japonica 2* | KT852311 | KT852026 | KT852091 | – | – |
| Actiniidae | *Epiactis lisbethae* | KT852289 | KT852006 | KT852069 | KT852253 | KT852202 |

**Table 2** (continued)

| Family | Species | CO3 | 12 | 16 | 28 | ITS |
|---|---|---|---|---|---|---|
| Actiniidae | *Epiactis prolifera* | **KY789287** | **KY789320** | **KY789354** | **KY789384** | **KY789417** |
| Actiniidae | *Epiactis thompsoni* | KT852294 | KT852011 | KT852074 | – | KT852206 |
| Actiniidae | *Glyphoperidium bursa* | KJ482982.1 | KJ482923.1 | KT852076 | KJ483136 | – |
| Actiniidae | *Gyractis sesere* | KT852297 | KT852012 | KT852078 | **KY789386** | **KY789419** |
| Actiniidae | *Gyractis* sp. Oman | **KY789290** | **KY789325** | **KY789357** | **KY789390** | – |
| Actiniidae | *Isosicyonis alba* | KJ482981 | – | KJ482959 | KJ483134 | – |
| Actiniidae | *Isosicyonis striata* | FJ489493 | EU190736 | EU190781 | – | **KY789422** |
| Actiniidae | *Isotealia antarctica* | JQ810727 | JQ810720 | JQ810722 | – | – |
| Actiniidae | *Korsaranthus natalensis* | KJ482987 | KJ482920 | KJ482958 | KJ483117 | – |
| Actiniidae | *Macrodactyla doreensis* | GU473342 | EU190739 | EU190785 | KJ483049 | – |
| Actiniidae | *Oulactis muscosa* | KT852317 | KT852033 | KT852097 | **KY789391** | KT852226 |
| Actiniidae | *Phlyctenactis tuberculosa* | **KY789292** | **KY789326** | **KY789359** | – | – |
| Actiniidae | *Phymactis clematis* | **KY789291** | – | **KY789358** | – | **KY789425** |
| Actiniidae | *Pseudactinia varia* | **KY789294** | **KY789328** | **KY789361** | – | – |
| Actiniidae | *Urticina coriacea* | GU473351.1 | GU473282.1 | KT852114 | KT852265 | – |
| Actiniidae | *Urticina grebelnyi* | KT852318 | KT852034 | KT852098 | – | KT852227 |
| Actinodendridae | *Actinostephanus* sp. | GU473353 | – | EU190762 | – | – |
| Capneidae | *Capnea georgiana* | KJ482990 | – | KJ482951 | KJ483050 | – |
| Haloclavidae | *Haloclava producta* | JF833008 | EU190734 | EU190779 | KJ483097 | **KY789421** |
| Haloclavidae | *Harenactis argentina* | KJ482984 | KJ482926 | KJ482964 | KJ483047 | – |
| Haloclavidae | *Peachia cylindrica* | – | EU190743 | EU190789 | EU190732 | – |
| Haloclavidae | *Stephanthus antarcticus* | KJ482983 | KJ482927 | KJ482960 | KJ483092 | – |
| Liponematidae | *Liponema brevicornis* | KJ483001 | EU190738 | EU190784 | KJ483139 | **KY789423** |
| Liponematidae | *Liponema multiporum* | – | KJ482922 | KJ482962 | – | – |
| Phymanthidae | *Phymanthus loligo* | GU473345 | EU190745 | EU190791 | – | – |
| Preactiniidae | *Dactylanthus antarcticus* | GU473358 | GU473272 | AY345877 | KJ483086 | – |
| Preactiniidae | *Preactis millardae* | KJ482986 | KJ482921 | KJ482957 | KJ483118 | – |
| Stichodactylidae | *Heteractis magnifica* | KJ482988 | EU190732 | EU190777 | KJ483093 | **KY789420** |
| Stichodactylidae | *Stichodactyla gigantea* | **KY789299** | EU190747 | EU190793 | EU190835 | **KY789431** |
| Diadumenidae | *Diadumene leucolena* | JF833006 | JF832957 | JF832977 | KJ483123 | – |
| Metridiidae | *Metridium senile* | KT852309 | KJ482916 | KJ482950 | KJ483113 | KT852219 |

See Fautin (2016) for authorship of species. GenBank accession numbers in bold are new to this analysis. All taxa belong to superfamily Actinioidea except the outgroups, *Diadumene leucolena* and *Metridium senile* (both superfamily Metridioidea)

and cytochrome *c* oxidase III (COIII)) and two nuclear markers (internal transcribed spacer (ITS) and partial 28S rRNA (28S)). We used primers published previously for 12S, 16S, 28S, and COIII (Geller and Walton 2001; Daly et al. 2008; Gusmão and Daly 2010). ITS was amplified using 5′-GGT TTC CGT AGG TGA ACC TGC GGA A-3′ as the forward primer and 5′-GTT CCC GCT TCA TTC GCC ATT AC-3′ as the reverse primer. Samples that could not be readily amplified using standard protocols were amplified with the high-fidelity enzyme Herculase® (Stratagene, La Jolla, CA), using manufacturer supplied protocols. All PCR products were cleaned using AMPure® magnetic bead solution (Agencourt, Beverly, MA) and re-hydrated with de-ionized, double-distilled water. Sequencing reactions used a total of 10 μL of cleaned PCR product, at a concentration of 25 ng/μL of product for every 200 bp of marker length. Cleaned PCR products were sequenced using amplification primers on an ABI 3730XL by staff at the sequencing facilities of Genaissance (New Haven, CT), Cogenics (Houston, TX) or Beckman Coulter (Beverley, MA). Forward and reverse sequences were assembled with Sequencher ver. 4.8 (Gene Codes Corporation, Ann Arbor, MI) or Geneious ver. 7.1.8 (Kearse et al. 2012). Once assembled, the contigs were queried against the nucleotide database of NCBI using BLAST to identify possible contaminants (from a symbiont or other contaminant) or cross contaminants. All sequences have been deposited in GenBank (Table 2).

### Phylogenetic analyses

Sequences for each marker were aligned using the default settings of MUSCLE (Edgar 2004) implemented through Geneious ver. 7.1.8 (Kearse et al. 2012). Sequences were concatenated into mitochondrial (12S, 16S, and COIII) and nuclear (ITS and 28S) data sets; the mitochondrial and nuclear data sets were themselves combined into a single ("combined") data set. All matrices were submitted to a test of the partitioning scheme using PartitionFinder ver. 1.1.1 (Lanfear et al. 2012) under the corrected Akaike Information Criterion (Akaike 1974). Analyses, described below, were conducted on all three data sets: the mitochondrial data set, the nuclear data set, and the combined

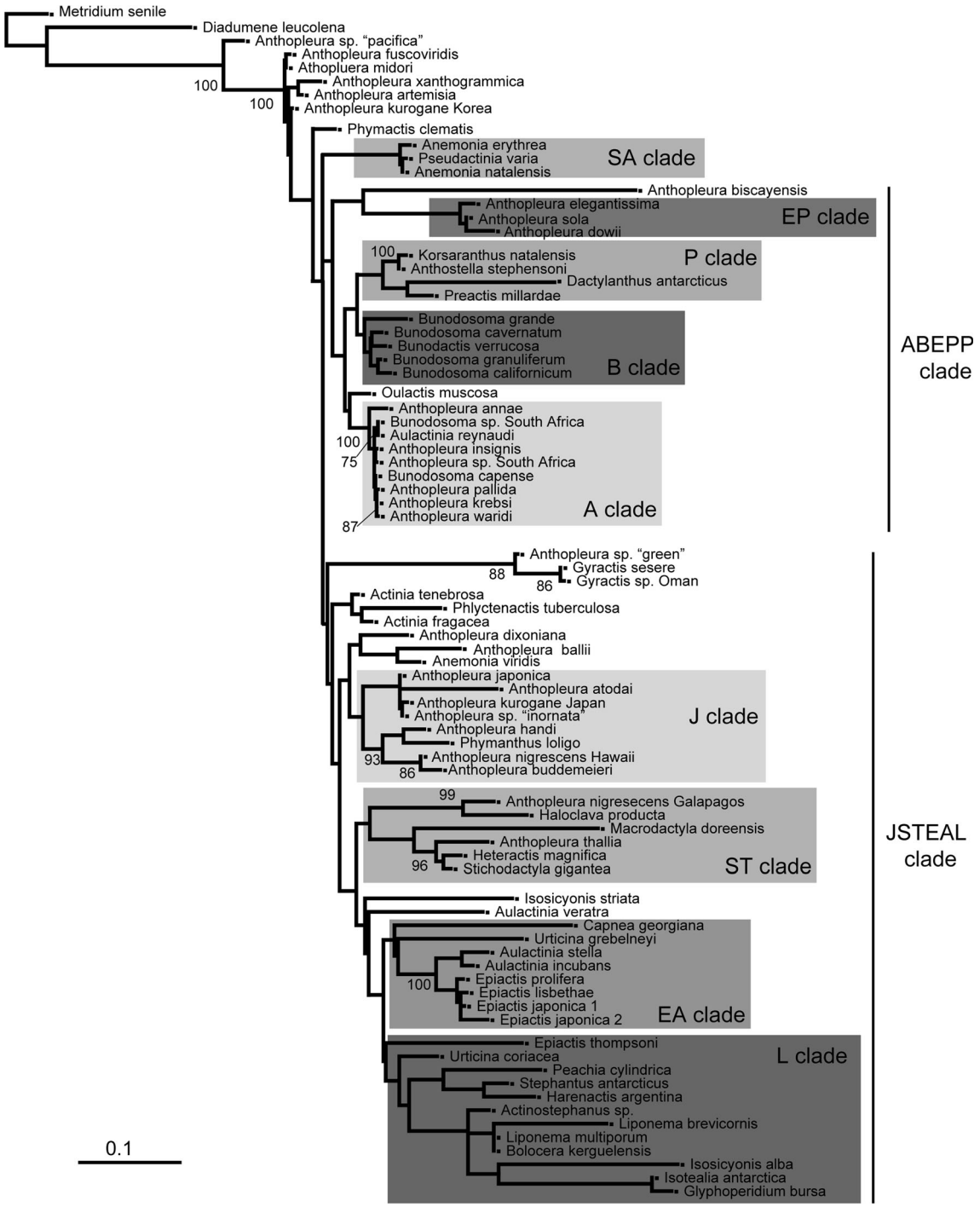



**Fig. 2** Maximum likelihood tree resulting from analysis of combined data set. Branch lengths are to scale. Bootstrap values (1000 replicates) greater than 75% are indicated. Clade labels apply to taxa within each *shaded box*

data set of all markers. Matrix partition files generally separated each marker in each matrix into independent partitions (COIII further subpartitioned into codon positions) but failed to separate 12S and 16S in the mitochondrial matrix.

Twenty maximum likelihood runs were performed on each data matrix in RAxML ver. 8.1.16 (Stamatakis 2014). For all matrices, we used the GTR + G + I substitution model, per the results of jModelTest (Posada 2008). For each analysis, 1000 bootstrap replicates were performed on the best-scoring tree of the 20 maximum likelihood runs.

### Character coding analysis

To understand acrorhagi, pseudoacrorhagi, verrucae, vesicles, and the evolutionary implications of their co-variation, we built a morphological matrix in which these features were coded as two multistate characters: marginal structures [absent; pseudoacrorhagi; acrorhagi] and column structures [absent (= smooth), verrucae, vesicles, solid papillae, or adhesive spots]. This multistate coding strategy approximates the conventional interpretation of the features, with acrorhagi and pseudoacrorhagi as equivalent alternatives to having no marginal swellings and papillae, verrucae, and vesicles as alternatives to a smooth column (Carlgren 1949). Although coding these as alternative states of a single feature implies homology at some level (Freudenstein 2005), the multistate characters propose no relationship among various manifestations of each feature, and we consider the states unordered in the analyses described below.

Although verrucae and vesicles are generally not treated as having underlying similarity beyond being attributes of the column, they are conceptually linked because both verrucae and vesicles are hollow outgrowths of the column (Stephenson 1928; Häussermann 2004), and thus verrucae and vesicles are more alike one another than either is to a smooth column or solid adhesive spots. To recognize this similarity, we also coded the column structures character as a pair of characters: column structures [smooth, solid growths, hollow outgrowths] and hollow outgrowths [cup-like, rounded]. For those taxa having a smooth column or solid outgrowths, the secondary character that describes the nature of the column outgrowth is scored as inapplicable.

To evaluate the pattern of character change, we optimize these characters on the tree of highest likelihood from the combined data set. For each coding strategy, we consider acctran, deltran, and unambiguous optimization strategies using the parsimony criterion as implemented in the "trace characters" function in Mesquite (Maddison and Maddison 2011) and conducted a simple likelihood optimization using Mesquite's embedded stochastic model for character change.

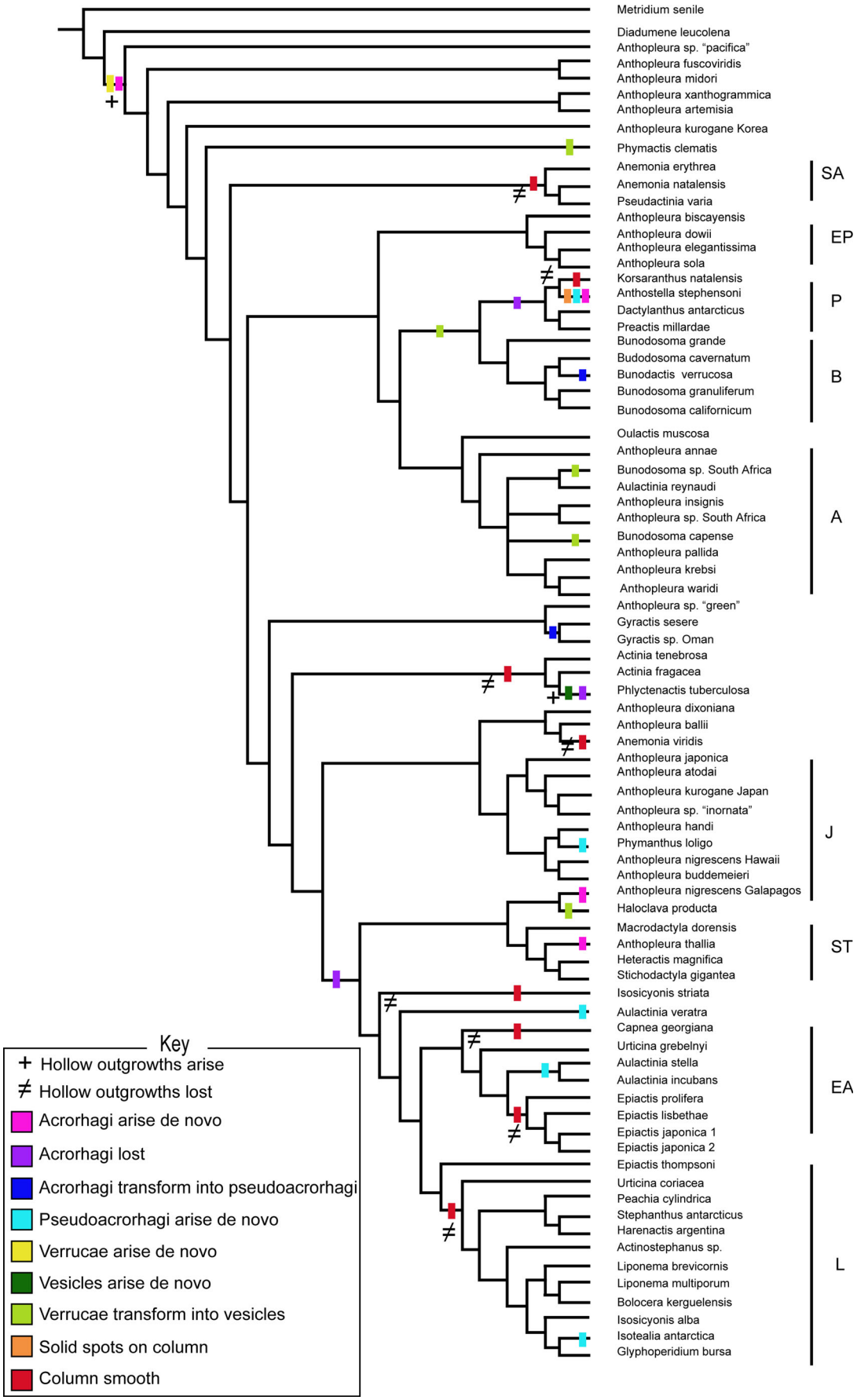
**Fig. 3** Optimization of focal characters onto combined data maximum likelihood tree. *Colors* correspond to the features discussed in the text: acrorhagi are indicated in *pink*, pseudoacrorhagi are indicated in *blue*, verrucae are indicated in *yellow*, vesicles are indicated in *green*, solid spots on the column are indicated in *orange*. The absence of marginal structures is indicated in *purple* and a smooth column is indicated in *red*. Pseudoacrorhagi and vesicles arise either de novo (from the state "absent" or "smooth") or via a transformation of acrorhagi or verrucae, respectively. Transformed versus de novo originations of pseudoacrorhagi and vesicles are differentiated by the *shade of blue* (for pseudoacrorhagi) or *green* (for vesicles). Branch lengths not to scale (color figure online)

## Results

### Interpretations of relationship among Actinioidea

This analysis concurs with broader-scale studies (e.g., Rodríguez et al. 2014) in finding a monophyletic Actinioidea (Figs. 2 and 3). The tree of highest likelihood for the combined data set has generally short internal branches with generally low support (Fig. 2). At the base of the ingroup is a comb-like series of sister group pairs and singleton taxa: *Anthopleura* sp. "*pacifica*", *Anthopleura fuscoviridis* + *Anthopleura midori*, *Anthopleura xanthogrammica* + *Anthopleura artemisia*, *A. kurogane* Korea, and *Phymactis clematis*. These basal branches in the ingroup are not well supported and are generally resolved in other ways in the subset analyses (see Supplemental Figs. 1 and 2), suggesting that the arrangement of species in this part of the tree is unstable. Recognizing these limitations, we focus on the relationships among the major clades, emphasizing those groups that appear in the combined tree.

In the combined analysis, the large ingroup clade has three subclades whose interrelationship is not resolved. The smallest of these is the SA clade (Figs. 2 and 3), which consists of three South African species, *Anemonia erythraea*, *Anemonia natalensis*, and *Pseudactinia varia*. This clade is present in the mitochondrial tree, but is disassociated in the nuclear tree, perhaps because *P. varia* is not included in that analysis. The subclade described below and hereafter as ABEPP (Figs. 2 and 3) includes several species of *Anthopleura*, the sampled members of *Bunodosoma*, and the ptychodactiarians *Dactylanthus* and *Preactis*. The third clade, hereafter JSTEAL (Figs. 2 and 3), includes other species of *Anthopleura* and assorted members of Actiniidae, Stichodactylidae, and Liponematidae. The relationships among the SA, ABEPP, and JSTEAL clades are unresolved in the combined analysis and differ in the trees based on either nuclear or mitochondrial sequences alone. For example, the SA clade merges with the ABEPP clade in the tree based on mitochondrial sequences (Supplemental Fig. 1) and its constituents associate with members of this group in the nuclear tree (Supplemental Fig. 2), and in the nuclear tree, the taxa of the JSTEAL clade are paraphyletic with respect to the ABEPP


Metridium senile
Diadumene leucolena
Anthopleura sp. "pacifica"
Anthopleura fuscoviridis
Anthopleura midori
Anthopleura xanthogrammica
Anthopleura artemisia
Anthopleura kurogane Korea
Phymactis clematis
Anemonia erythrea
Anemonia natalensis
Pseudactinia varia
Anthopleura biscayensis
Anthopleura dowii
Anthopleura elegantissima
Anthopleura sola
Korsaranthus natalensis
Anthostella stephensoni
Dactylanthus antarcticus
Preactis millardae
Bunodosoma grande
Budodosoma cavernatum
Bunodactis verrucosa
Bunodosoma granuliferum
Bunodosoma californicum
Oulactis muscosa
Anthopleura annae
Bunodosoma sp. South Africa
Aulactinia reynaudi
Anthopleura insignis
Anthopleura sp. South Africa
Bunodosoma capense
Anthopleura pallida
Anthopleura krebsi
Anthopleura waridi
Anthopleura sp. "green"
Gyractis sesere
Gyractis sp. Oman
Actinia tenebrosa
Actinia fragacea
Phlyctenactis tuberculosa
Anthopleura dixoniana
Anthopleura ballii
Anemonia viridis
Anthopleura japonica
Anthopleura atodai
Anthopleura kurogane Japan
Anthopleura sp. "inornata"
Anthopleura handi
Phymanthus loligo
Anthopleura nigrescens Hawaii
Anthopleura buddemeieri
Anthopleura nigrescens Galapagos
Haloclava producta
Macrodactyla dorensis
Anthopleura thallia
Heteractis magnifica
Stichodactyla gigantea
Isosicyonis striata
Aulactinia veratra
Capnea georgiana
Urticina grebelnyi
Aulactinia stella
Aulactinia incubans
Epiactis prolifera
Epiactis lisbethae
Epiactis japonica 1
Epiactis japonica 2
Epiactis thompsoni
Urticina coriacea
Peachia cylindrica
Stephanthus antarcticus
Harenactis argentina
Actinostephanus sp.
Liponema brevicornis
Liponema multiporum
Bolocera kerguelensis
Isosicyonis alba
Isotealia antarctica
Glyphoperidium bursa
SA
EP
P
B
A
J
ST
EA
L
Key
+ Hollow outgrowths arise
≠ Hollow outgrowths lost
Acrorhagi arise de novo
Acrorhagi lost
Acrorhagi transform into pseudoacrorhagi
Pseudoacrorhagi arise de novo
Verrucae arise de novo
Vesicles arise de novo
Verrucae transform into vesicles
Solid spots on column
Column smooth

clade (Supplemental Fig. 2), whereas in the mitochondrial tree (Supplemental Fig. 1), the taxa of the ABEPP clade nest *within* the JSTEAL clade.

In the combined analysis, the ABEPP clade has four subclades: a clade containing *Anthopleura biscayensis* plus a clade containing *Anthopleura dowii*, *Anthopleura elegantissima*, and *Anthopleura sola* (= EP clade; Fig. 2); a clade that joins the ptychodactiarians and the actiniids *Korsaranthus* and *Anthostella* (= P clade; Fig. 2); a clade containing members of *Bunodosoma* (= B clade; Figs. 2 and 3); and a clade that includes *Oulactis*, plus a clade of several South African species of *Anthopleura* and the type species of the genus *Anthopleura*, *Anthopleura krebsi* (= A clade; Figs. 2 and 3). Within this A clade, there is support for a group consisting of *A. krebsi*, *Anthopleura pallida*, and *Anthopleura waridi*, but other relationships are unresolved. Branches within this clade are very short but are resolved consistently: *A. krebsi*, *A. pallida*, and *A. waridi* are sister taxa to an assemblage of species from the South Atlantic. This South Atlantic assemblage includes *Anthopleura annae*, *Anthopleura insignis*, *Aulactinia reynaudi*, and *Bunodosoma capense*. Although *Oulactis muscosa* is the sister taxon to the rest of the A clade in the combined tree (Figs. 2 and 3) and is part of the ABP clade in all analyses, its position varies across data sets. The B clade (Figs. 2 and 3) includes the new-world *Bunodosoma* (*Bunodosoma californicum*, *Bunodosoma cavernatum*, *Bunodosoma grande*, and *Bunodosoma granuliferum*). In the combined (and nuclear) data sets, the B clade also includes *Bunodactis verrucosa*. The B clade never includes *B. capense*, the only old-world species of the genus included in this analysis, which is instead interpreted as part of the A clade. The B clade is always the sister taxon to the P clade, a finding congruent with that of Rodríguez et al. (2014). The EP clade groups well-known sibling species *A. elegantissima* and *A. sola* (see McFadden et al. 1997; Pearse and Francis 2000; Geller and Walton 2001) with the geographically close *A. dowii*; this novel grouping is consistent across data sets. In contrast, the association of *A. dowii*, *A. elegantissima*, and *A. sola* with *A. biscayensis* is seen only in the combined tree. Relationships of members of the EP clade are sensitive to data set. The P clade (Figs. 2 and 3) includes the two ptychodactiarians (*Dactylanthus antarcticus*, *Preactis millardae*) and the actiniids *Anthostella stephensoni* and *Korsaranthus natalensis*. All of these species are reported only from the Southern Ocean (Fautin 2013).

In the combined analysis, the B, P, and A clades form a clade to the exclusion of the EP clade; within this clade, the B and P clades are sister taxa to the exclusion of the A clade (Figs. 2 and 3). Other than the ptychodactiarians, all members of the ABEPP clade have historically been assigned to Actiniidae. The nuclear tree is consistent with the combined analysis in recovering a clade that encompasses the A, B, and P clades (Supplemental Fig. 2) and in resolving the B and P clades as sister taxa to the exclusion of the A clade. The A, B, and P clades are also present in the mitochondrial tree, and the B and P clades are sister taxa, but the B clade of the mitochondrial analysis does not include *B. verrucosa* and the relationship between the B + P clade and A clade is unlike that in the combined or nuclear tree (Supplemental Fig. 1).

The JSTEAL clade (Figs. 2 and 3) contains several members traditionally assigned to families other than Actiniidae: *Actinostephanus* (Actinodendridae); *Capnea georgiana* (Capneidae); *Harenactis* and *Stephanthus* (Haloclavidae); *Liponema* (Liponematidae) and *Stichodactyla gigantea* and *Heteractis magnifica* (Stichodactylidae). *C. georgiana* (Capneidae) is among the members of the larger JSTEAL clade that lack clear affiliation to any named clade. In the results of the combined analysis, the JSTEAL clade contains two subclades at the base: a clade that includes *Phylctenactis tuberculosa* and two species belonging to the genus *Actinia* and a clade that includes specimens of *Gyractis sesere* and an undescribed species of *Anthopleura* (*Anthopleura* sp. Green). The relative position of these clades varies across analyses, with weak support in all cases. These (unnamed) groups are sister taxa to a series of named clades that have more consistency in their membership and resolution.

The J clade (Figs. 2 and 3) includes species of *Anthopleura* from the Pacific; it is sister to a clade that includes *Anemonia viridis*, *Anthopleura ballii*, and *Anthopleura dixoniana*. The J clade has two further subgroups: a clade composed of the Japanese species *Anthopleura atodai*, *Anthopleura inornata*, *A. kurogane* (Japan), and *Anthopleura japonica* and a clade composed of the central to south Pacific species *Anthopleura buddemeieri*, *Anthopleura handi*, *A. nigrescens*, and *Phymanthus loligo*.

The ST clade (Figs. 2 and 3) includes members of Stichodactylidae plus the actiniids *Anthopleura thallia* and *Macrodactyla doreensis*. Although not currently classified among the Stichodactylidae, *Macrodactyla* has historically been considered among the Stichodactylidae (see Dunn 1981), and, as is the case with Stichodactylidae, some of its members harbor clownfish (Dunn 1981). Although we consider the resolution of *M. doreensis* within the ST clade credible (Figs. 2 and 3), in the nuclear analysis (Supplemental Fig. 2), *M. doreensis* is interpreted as the sister to the *A.dowii* + *A. elegantissima* clade rather than as part of the ST clade. The inclusion of *A. thallia* within the ST clade is surprising but consistent across data sets. Despite this consistency, we regard it as suspicious, because *A. thallia* differs from other members of the ST clade in size, ecology, and cnidom (compare e.g., Dunn 1981; Fautin et al. 2008; Daly and Picton 2012).

The EA clade (Figs. 2 and 3) includes the Northern hemisphere members of *Epiactis* (*Epiactis japonica*, *Epiactis lisbethae*, *Epiactis prolifera*), species of *Aulactinia*

(*Aulactinia incubans*, *Aulactinia stella*), the sole included representative of *Capnea*, and *Urticina grebelnyi*. *Aulactinia veratra* is the sister to the union of the EA + L clade rather than part of the EA clade. *U. grebelnyi* groups with the EA clade in the combined and nuclear trees but is part of a polytomy that includes the EA clade in the mitochondrial trees. *C. georgiana* is within the EA clade in the combined analysis but is reconstructed near the base of the ingroup in the mitochondrial tree (Figs. 2 and 3; Supplementary Fig. 1). This taxon is interpreted differently yet in the analyses of Rodríguez et al. (2014): *C. georgiana* lies outside of Actinioidea in that analysis.

The L clade (Figs. 2 and 3) includes more family-level diversity than any of the other well-supported groups, comprising members of Actiniidae (*Bolocera kerguelensis*, *Epiactis thompsoni*, *Glyphoperidium bursa*, *Isosicyonis alba*, *Isotealia antarctica*, *Urticina coriacea*), Haloclavidae (*Peachia cylindrica*, *Harenactis argentina*, *Stephanthus antarcticus*), and Liponematidae (*Liponema brevicornis*, *Liponema multiporum*). *Actinostephanus* sp., a member of Actinodendridae, associates with this clade based on mitochondrial sequences (no nuclear loci sampled for this species). Haloclavids and actinodendrids are burrowers in soft sediments and share aspects of their cnidom, most notably the absence of microbasic *p*-mastigophores (Riemann-Zürneck and Griffiths 1999; Rodríguez and López-González 2003). In the nuclear tree (Supplemental Fig. 2), monophyly of the L clade is disrupted by the association of *G. bursa*, *H. argentina*, and *S. antarcticus* with the basal node of the ingroup. Furthermore, *A. biscayensis* is reconstructed within the L clade, as the sister of *E. thompsoni* (Supplemental Fig. 2), rather than with *A. dowii* and *A. elegantissima* in the EP clade.

We find several taxa that are resolved in dramatically different ways in our analyses. The inferred position of *Haloclava producta* is inconsistent across data sets. These differences in topology have consequences for interpreting evolution, because the different positions for *H. producta* force differences in its inferred branch length: the total branch length (root to tip) is relatively long when *H. producta* is associated with the ST clade (Figs. 2 and 3) and relatively short when it is at the base of the actinioidean clade (Supplemental Fig. 1). In the analysis of Rodríguez et al. (2014), *H. producta* associates with the species that are here within the L clade; that interpretation uses nearly the same genes but a different taxon sample, including more diversity at the ordinal level but less diversity at the subfamilial level. All other haloclavids are part of the L clade in the combined analysis (Figs. 2 and 3), suggesting a relationship between the haloclavids and the other members of the L clade that may be obscured by the peculiarities of sequences for *H. producta*. However, the inclusion of *P. cylindrica* within the L clade is also marker-dependent: although *S. antarcticus* and *H. argentina* remain within the L clade in all analyses, *P. cylindrica* (and *H. producta*) fall to the base of the ingroup in the mitochondrial tree.

*A. dixoniana* is interpreted as the sister to the clade that includes *A. ballii* and *A. viridis* in the combined analysis, but is sister to the clade consisting of *Isosicyonis striata* and *L. brevicornis* in the nuclear tree and associates with *G. sesere* in the mitochondrial tree. *A. biscayensis* is sister to the *A. elegantissima*/*sola*/*dowii* clade in the combined analysis but groups with *H. producta*, *A. nigrescens* Galapagos, and *E. thompsoni* in the nuclear tree and with the *Anthopleura* clade within the ABP clade in the mitochondrial tree.

Although *A. biscayensis* is a relatively long branch (Fig. 2), long branches are not the likely explanation for the differences in resolution. First, not all of the unstable taxa are relatively long branches: *P. clematis* and *H. producta* both vary in position from data set to data set, but neither is associated with a long branch. Second, many of the longer-branched taxa are stable: *A. atodai* is always the sister taxon to *A. kurogane* (Japan) and part of a clade that includes *A. inornata* and *A. japonica*; the resolution of *M. doreensis* is similarly stable within the ST clade despite the length of the branch; *D. antarcticus* is always the sister to *P. millardae* and part of the P clade; and *L. brevicornis* and *I. alba* are both long but stable within the L clade.

## Character analyses

All means of coding and optimizing the focal features on the combined data tree agree that acrorhagi and verrucae are shared primitive features of the ingroup. Both of these characters change across the tree, with the number of transformations depending on the tree used, optimization method, and the coding strategy.

On the combined tree, the most parsimonious interpretation is for acrorhagi to be present ancestrally; the likelihood interpretation of acrorhagi as present in the common ancestor of the focal taxa is 0.975 (probability of pseudoacrorhagi being the ancestral state = 0.01, probability of no spherules = 0.016). Acrorhagi are inferred to have been transformed nine times (Fig. 3): three losses (P clade; *Phlyctenactis*; STEAL clade), three transformations to holotrich-less pseudoacrorhagi (*B. verrucosa*; *P. loligo*, *Gyractis*), and three independent originations (*A. nigrescens* Galapagos; *A. thallia*; *A. stephensoni*). In this scenario, pseudoacrorhagi arise four times de novo: in *A. stephensoni* (which has acrorhagi and pseudoacrorhagi), in *A. veratra*, in the clade composed of *A. incubans* and *A. stella*, and in *I. antarctica*.

When verrucae are as part of a single multistate character (Fig. 3), they are inferred to be primitively present, with verrucae transforming into vesicles five times (*P. clematis*; BP clade; *Bunodosoma* sp. South Africa; *B. capense*; *H. producta*) and being wholly lost five times (SA clade; *Korsaranthus*, *Actinia* + *Phylctenactis*; *A. viridis*; within

EAL clade). In this coding scheme, vesicles are lost twice (*Korsaranthus*, *I. striata*), transform into solid papillae once (*Anthostella*), and arise de novo (*Phylctenactis*, *U. grebelnyi*) from a smooth column or transform from verrucae (see above), but never give rise to verrucae.

When verrucae are broken into two independent characters, hollow outgrowths on the column are interpreted to have been lost seven times (Fig. 3: in the SA clade; *Korsaranthus* + *Anthostella*; *A. viridis*; *Actinia* + *Phylctenactis*; within the L clade; within the EA clade) and gained once (*Phylctenactis*). In the coding scheme in which verrucae and vesicles are alternate states of the character "hollow outgrowth," outgrowths are present at the base of the tree (Fig. 3) and verrucae (adhesive, cup-like) are the initial state, with transformations to vesicle (rounded, non adhesive) in *P. clematis*, the BP clade, *Bunodosoma* sp. South Africa, *B. capense*, and *H. producta* (Fig. 3). These are all branches in which verrucae are inferred to transform into vesicles in the single character coding scheme.

The probability of a particular ancestral state as inferred by likelihood reconstruction differs slightly depending on the atomization of the column features. The likelihood value for verrucae at the ingroup node when verrucae are part of a single multistate character is >0.9 (probability of vesicle being the ancestral state >0.01, probability of smooth column >0.01). When the column feature is atomized into two characters, the probability increases slightly: at the ingroup node, hollow column outgrowths have a probability of 0.988 (probability of smooth column >0.01). However, this increase in support obscures ambiguity at another level, as the probability of these hollow outgrowths being cup-like (and thus equivalent to verrucae) is 0.82, a value slightly lower than the inferred probability of verrucae being present at this node in the single-character analysis.

## Discussion

### Phylogeny of Actinioidea and Actiniidae

Our trees concur with most broad-scale analyses of Actiniaria (e.g., Daly et al. 2008; Rodríguez et al. 2012; but see Rodríguez et al. 2014) in finding a monophyletic Actinioidea. One notable difference is that *E. lisbethae* was not recovered within Actinioidea by Rodríguez et al. (2014); our denser sampling of *Epiactis* specifically and Actinioidea effectively resolves what Rodríguez et al. (2014) considered a spurious placement. Despite consistent and relatively strong support for ingroup monophyly, our analyses do not effectively resolve relationships among the major groups within Actinioidea. The internal branches at the deepest nodes are short, their resolution is not well supported, and the differences among the data sets largely reflect alternative interpretations of the basal branching order.

We find that Actiniidae is polyphyletic with respect to other families of Actinioidea: the node at which all taxa currently within Actiniidae are monophyletic is the node that includes all members of Actinioidea. Despite failing to recover a monophyletic Actiniidae, the combined, mitochondrial, and nuclear trees agree on many clades and recover several groups consistently or with high support, and thus provide a starting point for circumscribing groups within Actinioidea. Our taxon sampling precludes taxonomic revision for most groups because of its incompleteness at the genus- and family-level and because almost all of the clades we identify include members of multiple genera, if not multiple families. Furthermore, at present, none of these groups are clearly definable based on features typically used for classification. Defining the boundaries and membership of these and identifying the features by which these can be recognized is beyond the scope of the present study, but will be facilitated by the delimitation of these broad groupings.

The combined tree and the single-data set analyses differ in their interpretation of the primary split within Actinioidea. In both the mitochondrial and nuclear trees, species that are part of the EA and L clades form a grade sister to the majority of ingroup species (Supplemental Figs. 1 and 2), whereas the combined data tree recovers the EA and L as sister clades within the large ingroup clade (Fig. 2). Although Larson and Daly (2016) studied relatively fewer lineages in Actiniidae, their analyses included relatively denser sampling of what here constitutes the EA group, and they found support for EA and L as a grade outside of a clade that included species of e.g., *Actinia*, *Anthopleura*, *Bunodactis*, *Bunodosoma*, *Gyractis*, and *Oulactis*. The ecology, bathymetric distribution, and natural history of species in the EA and L groups are more varied than that of the species in the rest of the ingroup. The different interpretations of the relative position of the taxa in the EA and L groups are important for interpreting the evolution of features like acrorhagi (discussed below) and for understanding the evolution of photosymbiosis or asexual reproduction. Especially in light of their diversity in biology, the EA and L lineages are probably under sampled relative to the rest of the ingroup in our analyses, but this is necessary, given our primary interest in *Anthopleura* and its monophyly. We anticipate that denser sampling of the groups represented in the EA and L clades will resolve this instability.

All of our analyses find a sister-group relationship between the ptychodactiarians and the South African species *K. natalensis* and *A. stephensoni*. In their description of *Korsaranthus*, Riemann-Zürneck and Griffiths (1999) note the similarity of *K. natalensis* to *Dactylanthus* and other ptychodactiarians with respect to the anatomy of the actinopharynx and cnidom. However, they interpret these as convergent similarities based on similar diet and mode

of locomotion, an interpretation accepted by Cappola and Fautin (2000). Although several attributes that have been interpreted to support their separation as a higher taxon (reviewed by Cappola and Fautin 2000), the affiliation between ptychodactiarians and actiniids has been recovered in several molecular phylogenetic analyses (Daly et al. 2003, 2008; Rodríguez et al. 2012, 2014) and the sister group relationship between the clade containing the ptychodactiarians *Dactylanthus* and *Preactis* and members of the B clade is consistent across data sets.

Our results have clear implications for a handful of relatively minor taxonomic issues. We never find monophyly of the included species of *Anemonia*, *Aulactinia*, *Epiactis*, or *Urticina* (Fig. 2). Members of *Bunodosoma* are divided between the B and A clades within the ABEPP clade in groupings that are both consistent across analyses and well supported in the combined analysis. The placement of *A. biscayensis* outside of *Bunodosoma* (and sister to other nominal *Anthopleura*) supports the reassignment of this species proposed by Daly (2004b). *A. viridis* and *A. ballii* are recovered as sister taxa in all analyses. Although assigned to different genera and distinct in the anatomy of the column (smooth in *A. viridis*, verrucose in *A. ballii*), these northern hemisphere species are similar in having weakly muscled columns and nonretractile tentacles and are also similar in coloration (see Stephenson 1935). The other species of *Anemonia* included in this analysis, *A. erythraea* and *A. natalensis*, are southern hemisphere species that group with *P. varia* in the highly supported SA clade. The close association we find between *Aulactinia* and the Northern hemisphere species of *Epiactis* was also found in a more narrowly focused analysis (Larson and Daly 2016) but is not obvious in terms of anatomy: the included species of *Aulactinia* have a verrucose column whereas that of *Epiactis* is smooth (Tables 2 and 3). All of these polyphyletic genera (*Anemonia*, *Aulactinia*, *Bunodosoma*, *Epiactis*, *Urticina*) require revision. In addition, we find that *Actinia*, *Isosicyonis*, and *Liponema* are paraphyletic, but acknowledge that support for their paraphyly is weak and these lineages have not been the focus of our sampling.

Dunn et al. (1980) conditionally proposed a synonymy between *Bunodactis* and *Aulactinia*. Although this proposal was limited in the scope of the synonymy and advocated further study, it has been generally adopted (without the recommended specimen-level studies), and most authors now apply *Aulactinia* as the valid name for the species originally described as *Actinia verrucosa* (see Fautin 2016), although this is not uniformly the case (Spano et al. 2013, Garese et al. 2014). Because we never find a close relationship between *B. verrucosa* and species of *Aulactinia*, we reject the proposition that *Bunodactis* is wholly in synonymy with *Aulactinia* and

**Table 3** Characters and character states for features discussed in the text

| Species | Characters | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| *Actinia fragacea* | 2 | 0 | 0 | ? |
| *Actinia tenebrosa* | 2 | 0 | 0 | ? |
| *Actinostephanus* sp. | 0 | 0 | 0 | ? |
| *Anthopleura annae* | 2 | 1 | 1 | 0 |
| *Anthopleura artemisia* | 2 | 1 | 1 | 1 |
| *Anthopleura atodai* | 2 | 1 | 1 | 1 |
| *Anthopleura ballii* | 2 | 1 | 1 | 1 |
| *Anthopleura biscayensis* | 2 | 1 | 1 | 1 |
| *Anthopleura buddemeieri* | 2 | 1 | 1 | 1 |
| *Anthopleura dixoniana* | 2 | 1 | 1 | 1 |
| *Anthopleura dowii* | 2 | 1 | 1 | 1 |
| *Anthopleura elegantissima* | 2 | 1 | 1 | 1 |
| *Anthopleura fuscoviridis* | 2 | 1 | 1 | 1 |
| *Anthopleura handi* | 2 | 1 | 1 | 1 |
| *Anthopleura insignis* | 2 | 1 | 1 | 1 |
| *Anthopleura japonica* | 2 | 1 | 1 | 1 |
| *Anthopleura krebsi* | 2 | 1 | 1 | 1 |
| *Anthopleura kurogane* Japan | 2 | 1 | 1 | 1 |
| *Anthopleura kurogane* Korea | 2 | 1 | 1 | 1 |
| *Anthopleura midori* | 2 | 1 | 1 | 1 |
| *Anthopleura nigrescens* Galapagos | 2 | 1 | 1 | 1 |
| *Anthopleura nigrescens* Hawaii | 2 | 1 | 1 | 1 |
| *Anthopleura pacifica* | 2 | 1 | 1 | 1 |
| *Anthopleura pallida* | 2 | 1 | 1 | 1 |
| *Anthopleura sola* | 2 | 1 | 1 | 1 |
| *Anthopleura thallia* | 2 | 1 | 1 | 1 |
| *Anthopleura waridi* | 2 | 1 | 1 | 1 |
| *Anthopleura xanthogrammica* | 2 | 1 | 1 | 1 |
| *Anemonia erythraea* | 2 | 0 | 0 | ? |
| *Anemonia natalensis* | 2 | 0 | 0 | ? |
| *Anemonia viridis* | 2 | 0 | 0 | ? |
| *Anthopleura* sp. inornata | 2 | 1 | 1 | 1 |
| *Anthopleura* sp. South Africa | 2 | 1 | 1 | 1 |
| *Anthostella stephensoni* | 1 | 3 | 2 | ? |
| *Aulactinia incubans* | 1 | 1 | 1 | 1 |
| *Aulactinia reynaudi* | 1 | 1 | 1 | 1 |
| *Aulactinia stella* | 1 | 1 | 1 | 1 |
| *Aulactinia veratra* | 1 | 1 | 1 | 1 |
| *Bunodactis verrucosa* | 1 | 1 | 1 | 1 |
| *Bunodosoma californicum* | 2 | 2 | 1 | 0 |
| *Bunodosoma capense* | 2 | 2 | 1 | 0 |
| *Bunodosoma cavernatum* | 2 | 2 | 1 | 0 |
| *Bunodosoma grande* | 2 | 2 | 1 | 0 |
| *Bunodosoma granuliferum* | 2 | 2 | 1 | 0 |
| *Bunodosoma sp.* South Africa | 2 | 2 | 1 | 1 |
| *Bolocera kerguelensis* | 0 | 0 | 0 | ? |
| *Capnea georgiana* | 0 | 0 | 0 | ? |
| *Dactylanthus antarcticus* | 0 | 2 | 1 | 1 |
| *Diadumene leucolena* | 0 | 0 | 0 | ? |
| *Epiactis japonica* 1 | 0 | 0 | 0 | ? |
| *Epiactis japonica* 2 | 0 | 0 | 0 | ? |
| *Epiactis lisbethae* | 0 | 0 | 0 | ? |
| *Epiactis prolifera* | 0 | 0 | 0 | ? |
| *Epiactis thompsoni* | 0 | 0 | 0 | ? |
| *Glyphoperidium bursa* | 0 | 0 | 0 | ? |
| *Gyractis sesere* | 1 | 1 | 1 | 1 |
| *Gyractis* sp. Oman | 1 | 1 | 1 | 1 |
| *Haloclava producta* | 0 | 2 | 1 | 0 |
| *Harenactis argentina* | 0 | 0 | 0 | ? |
| *Heteractis magnifica* | 0 | 1 | 1 | 1 |
| *Isosicyonis alba* | 0 | 0 | 0 | ? |

**Table 3** (continued)

| Species | Characters | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| *Isosicyonis striata* | 0 | 0 | 0 | ? |
| *Isotealia antarctica* | 1 | 0 | 0 | ? |
| *Korsaranthus natalensis* | 0 | 0 | 0 | ? |
| *Liponema brevicornis* | 0 | 0 | 0 | ? |
| *Liponema multiporum* | 0 | 0 | 0 | ? |
| *Macrodactyla doreensis* | 0 | 1 | 1 | 1 |
| *Metridium senile* | 0 | 0 | 0 | ? |
| *Oulactis muscosa* | 2 | 1 | 1 | 1 |
| *Peachia cylindrica* | 0 | 0 | 0 | ? |
| *Phymactis clematis* | 2 | 2 | 1 | 0 |
| *Phlyctenactis tuberculosa* | 0 | 2 | 1 | 0 |
| *Phymanthus loligo* | 1 | 1 | 1 | 1 |
| *Preactis millardae* | 0 | 2 | 1 | 1 |
| *Pseudactinia varia* | 1 | 2 | 1 | 0 |
| *Stephanthus antarcticus* | 0 | 3 | 0 | ? |
| *Stichodactyla gigantea* | 0 | 1 | 1 | 1 |
| *Urticina coriacea* | 0 | 0 | 0 | ? |
| *Urticina grebelnyi* | 0 | 2 | 1 | 0 |

Multistate (characters 1–2) and atomized (characters 3–4) coding strategies have been presented side by side for ease of comparison. Character 1: marginal structures (0 = absent; 1 = pseudoacrorhagi; 2 = acrorhagi); character 2: column structures (0 = absent (smooth); 1 = verrucae; 2 = vesicles; 3 = solid papillae); character 3: column structures (0 = absent (smooth); 1 = hollow; 2 = solid); character 4: hollow column structures (0 = cup-like; 1 = rounded;? = inapplicable)

consider *Bunodactis* the appropriate generic epithet for the species originally described as *A. verrucosa*. The limits of our taxon sampling for *Aulactinia* and the non-monophyly of its included species (which remains non-monophyletic even if we ignore *B. verrucosa*) prevent us from making broader taxonomic recommendations for *Aulactinia* or *Bunodactis*.

### Phylogeny of *Anthopleura*

We find no evidence for monophyly of *Anthopleura*: its members are scattered across the tree, associated with members of other actiniid genera and with members of other actinioidean families (Fig. 2; Supplemental Figs. 1 and 2). Genera that have been proposed as closely related to *Anthopleura*, such as *Anthostella*, *Aulactinia*, *Bunodactis*, *Bunodosoma*, and *Gyractis*, are closely related to it in the sense that they group with nominal species of *Anthopleura*. The type of *Anthopleura*, *A. krebsi*, lies within the A clade. The interrelationships among nominal *Anthopleura* are difficult to determine given the paraphyly of the genus and the relatively low support for internal branches. Of the well-supported and consistent clades, only the A and ST clades contain members of *Anthopleura*; the majority of included species of *Anthopleura* are part of poorly supported clades. Because we have over-sampled *Anthopleura* relative to other genera, this may reflect sampling depth rather than something more significant about *Anthopleura* relative to other actinioideans: for example, the support for the L, EA, or P clades may be inflated because many members of those clades have not been included and so the support is not divided among the same number of nodes or subjected to the same number of possible resolutions as clades of *Anthopleura*.

Previous studies of relationships among species of *Anthopleura* (Geller and Walton 2001; Daly 2004a) have not included as many nominal species or as many taxa from outside of *Anthopleura*. In light of the broad polyphyly of *Anthopleura*, this difference makes comparing trees difficult. However, we see points of congruence with the results of Geller and Walton (2001), including finding a close relationship between *A. handi* and *A. nigrescens* and between these and a clade of *Anthopleura* from Japan. Geller and Walton (2001) and Daly (2004a) both find that *Anthopleura* is not monophyletic with respect to *Bunodosoma*; the present results extend this finding to include several other genera, but concur in finding a close relationship between *Bunodosoma* and several species of *Anthopleura* (Figs. 2 and 3; Supplemental Figs. 1 and 2). Similarity between the present results and those of Geller and Walton (2001) is not surprising given that their focal markers are among those we analyze here. The tree in Daly (2004a) is based on morphological data and is generally less congruent with these new results than is the tree of Geller and Walton (2001); other than the close association between *Bunodosoma* and *Anthopleura*, only the proposed relationship between *A. dowii* and *A. elegantissima* is seen in our trees and in those of Daly (2004a).

Although the frequency with which species of *Anthopleura* occur in sympatry is explained in part by the broad polyphyly of the genus, geography provides a means of interpreting and synthesizing the diversity of *Anthopleura* and its actinioidean relatives, if only because several of the well-supported or consistently recovered subunits have some geographic signature. Of course, because we have not sampled all members of *Anthopleura* and because the relationships of a few species of *Anthopleura* are highly labile across data sets, these groups and inferences about them are provisional.

In terms of the biogeography of the North Pacific, based on our results, there are at least two colonization events each in the East and West Pacific (Fig. 4). Species of *Anthopleura* from the North East Pacific span two groups: the clade that includes *A. dowii*, *A. elegantissima*, and *A. sola* and the clade containing *A. xanthogrammica* and *A. artemisia*. These two groups are never siblings. *A. xanthogrammica* and *A. artemisia* are generally associated with one another but are not siblings in all analyses. The included species from the Southern and Central West Pacific (*A. handi*, *A. buddemeieri*, *A. nigrescens*) are sister to a clade from the North West Pacific (*A. atodai*, *A. inornata*, *A. kurogane* (Japan), *A. japonica*), together forming the J clade (Fig. 2). Of these, there seem to be

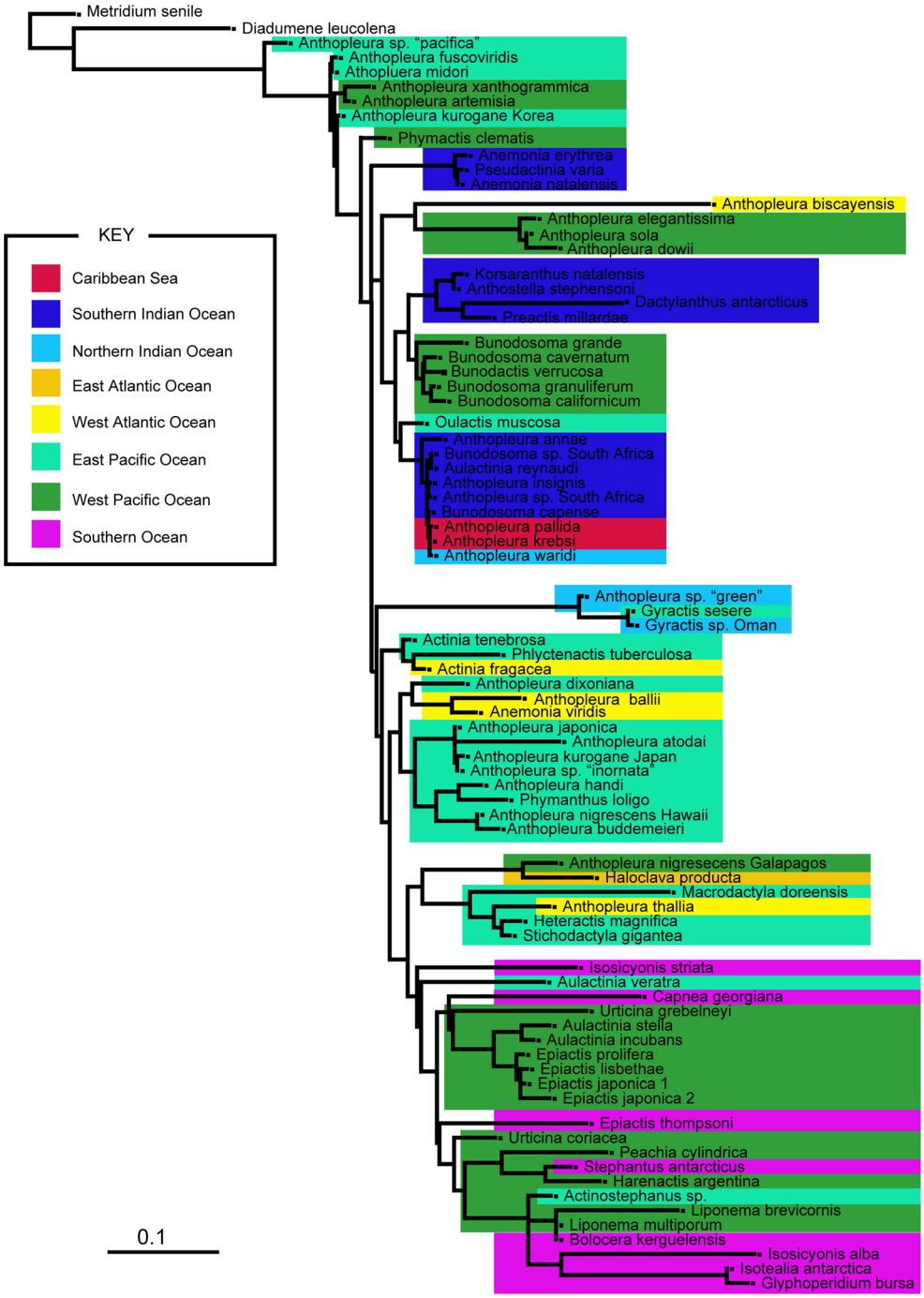



**Fig. 4** Recorded occurrences of focal species mapped onto combined data maximum likelihood tree. Distributional data from Fautin (2013). Branch lengths are proportional to change

two in situ radiations: the *dowii*/*elegantissima*/*sola* group has undergone an in situ radiation on the North American coast and the *atodai*/*inornata*/*kurogane*/*japonica* group has undergone and in situ radiation in Asia. Our finding of multiple origins for the East and West Pacific *Anthopleura* was previously predicted by Geller and Walton (2001).

Geography is more predictive of relationship than taxonomy, for species from the Atlantic (Fig. 4). *Anthopleura* from the Atlantic belong to three clades: *A. balli* is sister to *A. viridis* (also an Atlantic species); *A. thallia* is part of the ST clade; and the remaining species (*A. annae*, *A. insignis*, *A. krebsi*, *A. pallida*, *Anthopleura* sp. South Africa) are part of the A clade, which also contains the Red Sea-Northern Indian Ocean species *A. waridi*. *A. waridi* is reported from the Red Sea, Persian Gulf, and the coasts of East Africa and India (Fautin 2013) and is most closely related to two species from the North Atlantic (*A. krebsi*, *A. pallida*). The taxa within the B and P clades, which are sister to the A clade, are also primarily Atlantic in their distribution. The

distribution of the members of the ABEPP clade suggests an Atlantic (or possibly Tethyan) origin for the group, with *B. californicum* dispersing into the Pacific and *A. waridi* dispersing into the Indian Ocean.

The North American and Northern European costs were under ice during the Quaternary glaciation, as were parts of Argentina and Chile. In light of their current restriction to the shallow subtidal and intertidal zones, it is probable that *Anthopleura* species currently inhabiting these regions are migrants from warmer waters rather than from deepwater refugia.

### Evolution of acrorhagi and verrucae

Our results suggest that acrorhagi are a shared, inherited feature in the majority of taxa that bear them. The combined analysis interprets them as ancestral for the Actinioidea (Fig. 3). The nuclear tree moves the optimization of acrorhagi higher into the tree: because the members of this assemblage at the base of the ingroup (the clade that includes *Stephanthus*, *Peachia*, *Glyphoperidium*, and *A. viridis*, among other taxa) and of the EA clade generally lack acrorhagi, acrorhagi are interpreted as originating in parallel in *A. viridis* + *A. ballii* and in the clade that contains the ABP, ST, and EP clades (see asterisks, Supplemental Fig. 2). The mitochondrial tree also posits that acrorhagi arose relatively later in actinioidean history (see asterisks, Supplemental Fig. 1) and are convergent; the purported instance of convergence is in *Anthopleura* sp. pacifica, rather than *A. viridis* + *A. ballii* as in the nuclear tree. These differences between the mitochondrial, nuclear, and combined trees relate to the relative position of the EA and L clades because members of these groups lack acrorhagi.

Holotrich-bearing acrorhagi are interpreted to have been lost several times. In *B. verrucosa*, *P. loligo*, and *Gyractis*, this loss of acrorhagi creates pseudoacrorhagi; in all other cases, pseudoacrorhagi either arise in the absence of acrorhagi (*A. veratra*, *A. stella* + *A. incubans*, *I. antarctica*) or arise concomitant with the de novo origination of acrorhagi (*Anthostella*) (Fig. 3). This pattern of character evolution means that acrorhagi and pseudoacrorhagi are homologous only in the case of *B. verrucosa*, *P. loligo*, and *Gyractis* (and possibly in the case of *Anthostella*). However, the inducibility of acrorhagi (reviewed by Daly 2003) and the relatively poor state of anatomical descriptions for some of the species inferred to have lost acrorhagi means that some of these instances of loss may be mistaken. Careful re-evaluation of pseudoacrorhagi and of species inferred to have lost acrorhagi are needed to verify the absence of acrorhagi and to document the structure of pseudoacrorhagi, which, because they are convergent in many of the taxa that bear them, are likely more diverse in structure than currently appreciated.

Although we interpret acrorhagi as primitive for the majority of taxa that bear them, the phylogenetic tree we reconstruct here also supports the inference of convergence for acrorhagi. The inferred instances of re-evolution of acrorhagi within the ingroup in the combined tree are generally also required by the single-locus analyses. Acrorhagi are inferred to re-evolve in *Anthostella*; this interpretation is compelling because the resolution of this species is well supported and consistent across data sets, and thus unlikely to change. In contrast, the inferred re-evolution of acrorhagi in *A. nigrescens* and in *A. thallia* is less clear, as these species are each weakly supported within or associated with of the ST clade (in all analyses), and denser sampling of stichodactyline taxa might resolve these two convergences as a single event or align *A. nigrescens* and *A. thallia* with other lineages in ways that eliminate the convergence. In the combined tree, optimization at the base of the node that includes *Anthopleura* sp. Green is ambiguous.

In all of the cases in which acrorhagi are interpreted as re-evolving, holotrichs have been reported from the sister species (see Carlgren 1938; Dunn 1981; Cappola and Fautin 2000; González-Muñoz et al. 2012; Laird 2013; Rodríguez and López-González 2013), which makes the re-acquisition of acrorhagi more plausible. Although acrorhagi are inferred to have been lost at the base of the P clade, *A. stephensoni*, *D. antarcticus*, and *P. millardae* all have holotrichs in their cnidom (see Dunn 1983; England and Robson 1984). Although holotrichs have not been reported in *Korsaranthus* (see Riemann-Zürneck and Griffiths 1999), previous accounts of cnidae are cursory in terms of the tissues examined and number of capsules reported; we have found a few capsules in the specimens sequenced here. Holotrichs are absent from most members of the EA and L clades (e.g., Larson and Daly 2016), although they have been reported from *E. prolifera* and *E. lisbethae* (see Fautin and Chia 1986), *E. japonica* (see Sanamyan and Sanamyan 1998), and *A. incubans* (Dunn et al. 1980). This pattern of character distribution suggests that the loss of the nematocyst is distinct from the loss of the structure or behavior in which it is deployed and argues for a more atomized and finer-scale study of the constituent elements of acrorhagi in the clades in which they show convergent evolution or loss.

Coding verrucae and vesicles as a set of characters rather than a single multistate character reduces the number of inferred character transformations, but does not change the overall inference that verrucae are not necessarily homologous across those Actiniidae that have them. The phylogenetic results suggest that the verrucae of *A. annae*, for example, and the vesicles of *B. capense* are homologous (albeit modified in *B. capense*) but that the verrucae of *A. annae* and e.g., *A. veratra* are not. Detailed functional and anatomical studies are necessary to determine if verrucae show previously unrecognized diversity as a result of their independent origins or whether they represent a convergence that can only be recognized through phylogenetic investigation. Although there are small differences in the interpretation of

verrucae depending on the way in which they are coded, at least one major point is consistent across coding strategies (Fig. 3): cup-like verrucae are never interpreted to have arisen from rounded vesicles, although verrucae give rise to vesicles in several instances. The apparent irreversibility of the cup-like morphology, which is inferred to make the structures adhesive (Häussermann 2003), is interesting and suggests that the microanatomy that differentiates these structures may be more complex than previously appreciated.

Although they frequently co-occur, are symplesiomorphic for Actinioidea in this analysis, and both represent modifications of column tissue, verrucae and acrorhagi are functionally, structurally, and logically distinct. Other than their co-occurrence at the base of the ingroup and in a few clades, we see no evidence for correlation in the evolution of verrucae and acrorhagi. Verrucae are lost in taxa that retain acrorhagi (and vice versa). However, we do see a link between verrucae and pseudoacrorhagi: all species that have been described as bearing pseudoacrorhagi (Tables 1 and 2) have verrucae. This raises the possibility that in some cases, what have been described as pseudoacrorhagi are distal marginal projections bearing verrucae, caused either by differential growth of the column or by distortion of the column by the distension of the verrucae.

In light of their frequency of loss or modification to a functionally distinct state, the adaptive value of acrorhagi and verrucae may be over interpreted. We see no ecological differences between those species with verrucae and those with vesicles or a smooth column. For example, *A. annae*, *A. natalensis*, and *B. capense* co-occur despite differing in their column morphology and *B. verrucosa* is abundant and successful in the same localities as *A. thallia*, despite lacking acrorhagi. Although verrucae have been shown to retard desiccation and UV exposure (e.g., Hart and Crowe 1977; Dykens and Shick 1984) by holding debris to the column, they may serve additional functions. For example, because they are hollow, verrucae increase the surface area of the polyp, which might be valuable for animals that rely on diffusion for many of their physiological processes (Shick 1991); this function would be conserved in vesicles.

Although all major lineages of Actiniaria have shallow water members, Actinioidea is the lineage with the greatest proportion of shallow-water members. The interpretation of verrucae and acrorhagi as symplesiomorphic for Actinioidea could be construed as support for a shallow-water origin for Actinioidea in light of the association of both of these traits with functions that confer advantages in shallow water. However, because *Anthopleura* is a genus whose members are restricted to waters shallower than about 60 m, our sampling strategy emphasized shallow-water species, and this emphasis may contribute to both the inference of symplesiomorphy and to the inference of shallow water as the ancestral habitat for Actinioidea.

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