Origin and Evolution of the Sponge Aggregation Factor Gene Family

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Abstract

Although discriminating self from nonself is a cardinal animal trait, metazoan allorecognition genes do not appear to be homologous. Here, we characterize the Aggregation Factor (AF) gene family, which encodes putative allorecognition factors in the demosponge Amphimedon queenslandica, and trace its evolution across 24 sponge (Porifera) species. The AF locus in Amphimedon is comprised of a cluster of five similar genes that encode Calx-beta and Von Willebrand domains and a newly defined Wreath domain, and are highly polymorphic. Further AF variance appears to be generated through individualistic patterns of RNA editing. The AF gene family varies between poriferans, with protein sequences and domains diagnostic of the AF family being present in Amphimedon and other demosponges, but absent from other sponge classes. Within the demosponges, AFs vary widely with no two species having the same AF repertoire or domain organization. The evolution of AFs suggests that their diversification occurs via high allelism, and the continual and rapid gain, loss and shuffling of domains over evolutionary time. Given the marked differences in metazoan allorecognition genes, we propose the rapid evolution of AFs in sponges provides a model for understanding the extensive diversification of self–nonself recognition systems in the animal kingdom.

Key words: aggregation factor, intron phase, allorecognition, polymorphism, Porifera, RNA editing.

Introduction

Self–nonself recognition is central to the multicellular condition, allowing individuals to avoid invasion and parasitism from conspecific neighbors and other organisms. Sophisticated allorecognition systems capable of discriminating between single individuals within a species are found in a wide range of metazoans. Given the apparent conservation of this process, the genes underlying allorecognition should share a common origin, as is the case with other “essential” metazoan genes, such as those employed during development (Srivastava et al. 2010). However, allorecognition genes show no evidence of homology or conservation between invertebrate animals in which they have been best studied, the colonial ascidian Botryllus schlosseri (Scofield et al. 1982; Rinkevich et al. 1995; De Tomaso et al. 2005; Nyholm et al. 2006; McKirrick and De Tomaso 2010; Nydam et al. 2013a, 2013b; Voskoboynik et al. 2013) and the cnidarian Hydractinia symbioticargus (Mokady and Buss 1996; Cadavid et al. 2004; Powell et al. 2007, 2011; Nicotra et al. 2009; Rosa et al. 2010; Gloria-Soria et al. 2012; Karadge et al. 2015). The lack of similarity between such systems supports either multiple independent origins or rapid evolution of animal allorecognition genes. However, as the majority of allorecognition research to date has focused on a small number of representative species with apparently non-homologous allorecognition systems, little is known about the evolutionary processes by which allorecognition novelty is produced.

Here, we explore allorecognition in poriferans or sponges, one of the oldest surviving phyletic lineages (Erwin et al. 2011). Sponges discriminate between self and nonself by allorecognition, with tissue graft acceptance in all analysed species restricted to grafts involving pieces from a single sponge (Moscona 1968; Hildemann et al. 1979; Smith and Hildemann 1986; Fernàndez-Busquets and Burger 1997; Gauthier and Degnan 2008). As first demonstrated by Wilson in 1907, sponges can be dissociated to the cellular level and allowed to reaggregate, a process which occurs with species specificity (Wilson 1907; Humphreys et al. 1960). An extracellular product named “aggregation factor” (AF) is responsible for this reaggregation (Moscona 1968; Müller and Zahn 1973). These extracellular proteoglycans (Henkart et al. 1973) have been characterized structurally and biochemically in multiple sponge species. AFs appear to exist in either a linear form, similar to a classical proteoglycan, or a circular “sunburst"-like form that is currently unknown outside the sponges (Fernàndez-Busquets and Burger 2003) (supplementary fig. S1.1, Supplementary Material online).
In addition to their well-characterized species-specific cell adhesion role, the AFs have also been proposed as conspecific allorecognition molecules (Fernández-Busquets and Burger 1999). Evidence of AF activity in immunologically challenging contexts provides the first link between these molecules and allorecognition. MAFp3 and MAFp4, contiguously transcribed AF genes in the demosponge Clathria (née Microciona) prolifera, are upregulated in both autografted and allografted tissue, compared with normal tissue (Fernández-Busquets and Burger 1997; Fernández-Busquets et al. 1998). The deglycosylated form of the MAFp3 protein, present exclusively in archaocytes, is recruited to the site of allogeneic contact (Fernández-Busquets et al. 1998, 2002). Furthermore, AFs bear hallmarks found in allorecognition molecules, including the ability to detect, interact with and determine whether a biological entity is derived from self or nonself, and promote a downstream response on the basis of this decision (Grice and Degnan 2015a). AFs allow physical interactions between compatible sponge cells by contributing to a molecular “bridge” between cells, which also includes aggregation receptors and associated glycans and glycoproteins (Jumblatt et al. 1980; Misevic and Burger 1990, 1993). MAFp3 and an associated glycoprotein, p210, are highly polymorphic in C. prolifera and there is a perfect correlation between MAFp3 sequence similarity/dissimilarity and graft fusion/rejection (Fernández-Busquets and Burger 1997). Finally, AF-receptor binding is coupled to various downstream signaling and regulatory pathways, which may stimulate an active rejection response upon exposure to nonself (Müller et al. 1976, 1987, 1994; Dunham et al. 1983; Rottmann et al. 1987; Schröder et al. 1988; Pfeifer et al. 1993; Wimmer et al. 1999). Although these observations gathered over many years from a number of sponge species are together consistent with a central role for AFs in sponge allorecognition, experimental demonstration of AFs directly regulating allorecognition is lacking.

Analysis of AF-related gene products in three demosponges, C. prolifera, Geodia cydonium and Suberites domuncula, reveals both similarities and marked differences between species. In C. prolifera, multiple AF isoforms, containing nucleotide, intronic, exonic and length variants, have been identified across individuals (Fernández-Busquets et al. 1996, 1998; Fernández-Busquets and Burger 1997). MAFp3 does not have any characterized domains, while MAFp4 isoforms possess between 3 and 15 Calx-beta domains (Fernández-Busquets and Burger 1997; Fernández-Busquets et al. 1998) (fig. 1). The G. cydonium AF, named GEOCY AF, includes two Sushi domains (Müller et al. 1999), while the S. domuncula AF, named SdSLIP, has one Calx-beta domain (Wiens et al. 2005); both also have regions similar to C. prolifera MAFp3 (fig. 1).

Here, we present a comparative analysis of the AF genes from multiple sponge species, permitting for the first time, to our knowledge, a systematic investigation of the origin and evolution of a putative allorecognition gene family across an animal phylum. We first characterize the genomic structure and organization of the AF locus in the demosponge Amphimedon queenslandica, as this genomic perspective has been lacking in previous studies. We demonstrate that A. queenslandica AF genes bear remarkable similarities to those from other animal allorecognition loci, in that they are highly polymorphic, clustered, encode large extracellular proteins with repeated protein domains, and have structural properties consistent with loci that can generate a high number of variants. Using our new insights from A. queenslandica, combined with previously published observations, we then survey the transcriptomes or genomes of 24 representative species spanning all four poriferan classes—Calcarea, Demospongiae, Hexactinellida and Homoscleromorpha—for AF candidate genes. This phylum-wide analysis reveals that the AF gene family evolved in demosponges after they diverged from other sponge lineages, and included the evolution of a novel domain that we have coined the “Wreath” domain. Differences in extant AFs between and within species are consistent with the continual evolution of this gene family, and provide an explanation as to how other allorecognition genes present in the animal kingdom obtain their unique molecular structure and organization.

### Results

#### Domain Architecture of the Amphimedon queenslandica Aggregation Factor Gene Family

Using BLAST similarity searches, we identified five genomically clustered Amphimedon queenslandica genes (AqAFA–AqAFE; fig. 2A) that exhibit significant sequence similarities to aggregation factor (AF) or AF-like sequences from Clathria prolifera, Geodia cydonium and Suberites domuncula (table 1). Overall amino acid sequence similarities between pairs of AqAF predicted proteins or with other known AFs are relatively low (i.e., <40% identity between matching amino acid regions). Membrane topology predictions from translated peptide sequences indicate that all AqAF proteins are secreted, except perhaps AqAFE, which is predicted to occur extracellularly yet lacks a discernable signal peptide (fig. 2B).
The AqAFs are predicted to encode three characterized domains from the Pfam protein family database (fig. 1). As in C. prolifera MAFp4 and S. domuncula SdSLIP, all AqAFs include Calx-beta domains in varying numbers from two in AqAFB to 14 in AqAFA. The Calx-beta domains of the AqAFs share very little sequence identity with each other (average 23% amino acid identity), the Calx-beta domains from MAFp3 (average 30% identity within MAFp3 isoform C) or Calx-beta domains elsewhere in the A. queenslandica genome (average 25% identity). AqAFB and AqAFE also encode von Willebrand type A (VWA) domains (average 31% identity), and AqAFC and AqAFD each have one von Willebrand type D (VWD) domain (23% pairwise identity).

In C. prolifera, MAFp3 self-adheres to form the central ring of the core AF sunburst structure (Jarchow et al. 2000). BLASTp searches revealed that regions exhibiting MAFp3 sequence similarity also exist in SdSLIP, GEOCY AF, and in all AqAFs. Considering the demonstrated functional importance, structural independence, and multi-species distribution of this region, we propose that MAFp3 and homologous sequences be considered to possess a novel protein domain (Richardson 1981). We suggest the name “Wreath domain” due to this protein region’s role in C. prolifera central AF ring formation (Jarchow et al. 2000). A multiple sequence alignment of the Wreath regions from MAFp3, SdSLIP and AqAFC (supplementary fig. S1.5, Supplementary Material online) was used to generate a profile hidden Markov Model (HMM) (supplementary file S5, Supplementary Material online) for this putative novel domain. HMM searches with this new model identified a single Wreath domain in AqAFA to AqAFE. The Wreath domain
was not found in non-AF predicted proteins encoded in the *A. queenslandica* genome, nor in any non-demosponge species.

**Modular Exon Structure of *A. queenslandica* AF Protein Domains**

To investigate the relationship between AqAF domain architecture and genomic structure, we mapped the positions of all AqAF Calx-beta, VWA, VWD and Wreath domains back to the genome (fig. 2B). Each domain type displays remarkably similar exonic coverage patterns across all AqAFs (fig. 2C and supplementary tables S1.1–S1.4, Supplementary Material online). With the exception of AqAFA Calx-beta domain 10, all AqAF Calx-beta domains are encoded by three exons, which on average span the final 27 base pairs (bp) of exon i, the entirety of exon ii (average 145 bp), and the first 126 bp of exon iii. This pattern repeats starting in the final ~27 bp of exon iii. All VWA domains in AqAFB and AqAFE localize to single exons and are flanked by short spacer regions at both ends of the exon (average 22 bp at either end). Similarly, the single VWD domains in AqAFC and AqAFD both map to single exons, with a short spacer sequence at the beginning of the exon, but with the adjacent Wreath domains beginning immediately after the domains’ end. Finally, all AqAF Wreath domains are encoded by the final three coding exons of each gene, commencing partway through the antepenultimate exons of each gene and running to the end of the sequence. The lengths of the first Wreath domain-containing exons vary between sequences, while the other two exons are more consistently sized between genes (average 523 and 336 bp, respectively).

**Genomic Organization of the *A. queenslandica* AF Locus**

The five AqAF genes (AqAFA–AqAFE) cluster together within an 80-kilobase (kb) genomic region (fig. 2A). The AqAF cluster is tightly packed even in comparison to other regions in the highly compacted *A. queenslandica* genome. The median intergenic distance in the AF region is 103 bp (table 1), which is considerably smaller than that observed genome-wide (589 bp) (Fernandez-Valverde et al. 2015). All AqAFs encode a single contiguous sequence equivalent to *C. prolifera* MAFp4 + MAFp3. The AqAFs are large genes (between 9.5 and 17.0 kb in length) with many exons (between 18 and 48 exons per gene; table 1). The average intron lengths of AqAFA (108 bp), AqAFB (181 bp), AqAFC (96 bp) and AqAFE (250 bp) are shorter than the genome-wide average of 327 bp (Fernandez-Valverde et al. 2015), while those from AqAFD (383 bp) are slightly longer (table 1). The AqAF introns are also generally shorter than those observed in *C. prolifera* MAFp3 (300–600 bp; Fernandez-Busquets and Burger 2003).

Two sets of highly similar repeats are present in the genomic regions encoding AqAFC and AqAFE (fig. 2B). In AqAFC, three repeat units span intron 10 to exon 14, intron 14 to exon 18, and intron 26 to exon 30. These AqAF repeats cover regions encoding two Calx-beta domains each, include both introns and exons, and share ~85% total pairwise nucleotide sequence identity to one another. Two repeats are present in AqAFE, in exons 30 and 31 (96% pairwise identity), and each encode a single VWA domain. These repeats do not cover any intronic sequences and do not bear any particular sequence similarity to the AqAFC repeats.

**Intron Phase Distribution in the AqAFs and Other Calx-Beta Domain-Encoding Sequences**

We compared intron phase frequencies across the AqAFs and other *A. queenslandica* Calx-beta domain-containing genes to those observed genome-wide for this species. The AqAFs show an extreme bias in intron phase distribution; all AqAF introns except one are in phase 1 (n = 154 of 155; fig. 2B and table 2).
This distribution differs significantly from both the genome-wide (44% phase 0, 36% phase 1, 20% phase 2) and non-AF Calx-beta domain-containing (16% phase 0, 78% phase 1, 6% phase 2) gene sets. For comparison, all introns of MAFp3 and MAFp4 are equipped with phase 0 introns only (Fernández-Busquets and Burger 1999). The distribution of intron phases across all A. queenslandica non-AF Calx-beta domain-containing genes also differs significantly from the genome-wide distribution (table 2), however not to the extreme extent as in the AqAFs.

Polymorphism and RNA–DNA Differences in the AqAFs

As the C. prolifera MAFp3 and MAFp4 genes are highly polymorphic (Fernández-Busquets and Burger 1997; Fernández-Busquets et al. 1998), we determined whether the A. queenslandica AF genes also vary between alleles and individuals. First, we compared Illumina RNA-Seq reads from three sponge adults (supplementary methods, Supplementary Material online). For comparison, all introns of MAFp3 and MAFp4 are equipped with phase 0 introns only (Fernández-Busquets and Burger 1999). The distribution of intron phases across all A. queenslandica non-AF Calx-beta domain-containing genes also differs significantly from the genome-wide distribution (table 2), however not to the extreme extent as in the AqAFs.

We selected three “hotspots” of higher variability for closer analysis—one from each of AqAFB, AqAFC and AqAFE—with each hotspot spanning between ~1,500 and ~3,000 bp of genomic DNA. We also included a ~2,000-bp region of AqAFE (including the two-exon repeat region) that exhibited lower sequence variability in this initial screen (supplementary fig. S1.2, Supplementary Material online). Genomic DNA and complementary DNA (cDNA) of these regions from four unrelated larval individuals (Larvae 3.1, 6, 7 and 9) were amplified and directly Sanger sequenced, which generated individual sequencing reads potentially capturing multiple alleles. These were compared with the A. queenslandica reference genome to identify variable sites in DNA and messenger RNA (mRNA) in each individual (supplementary file S3, Supplementary Material online). Levels of polymorphism were found to differ both spatially (i.e., in different exons, introns and genes) and between individuals within the hotspots (figs. 3 and 4A and B), such that each individual has its own unique AF sequence pattern. All three hotspot regions exhibit higher frequencies of non-synonymous nucleotide substitutions (average 44.9%, $P \leq 0.0001$–0.002 per individual) than is observed genome-wide (e.g., 25.4% in Sponge A). The putatively less variable AqAFE region also showed this trend (average 68%; $P = 0.0001$–0.0167 per individual), although the
number of variants observed here is lower than in the hotspot regions (fig. 4B). One non-synonymous difference in a single individual (“Larva 9”) is predicted to introduce a termination codon halfway through AqAFB exon 17, truncating the final VWA domain (supplementary file S3, Supplementary Material online); no other variants are predicted to introduce frame-shifts, termination codons or signal peptides.

In one of the four individual larvae surveyed (Larva 3.1), we observed 42 AqAFB sites (3.4% exonic region) with nucleotide differences between the genomic and cDNA sequences, which is a potential hallmark of RNA editing (fig. 3 and supplementary file S3, Supplementary Material online). Such RNA–DNA differences (RDDs) were not observed in the other three sequenced regions of Larva 3.1, or in any sequenced regions of Larvae 6, 7 or 9 (fig. 4C). To explore this phenomenon further, we amplified the AqAFB hotspot from an additional 13 individuals: five larvae that were half-sibs of each other and of Larva 3.1 (Larvae 3.2–3.6); and somatic cells isolated from eight unrelated adults (Adults 1–6, 9 and 10). In total, eight individuals (Larvae 3.1, 3.5, 3.6 and Adults 3, 4, 5, 6, 10) possessed between one and 42 AqAFB RDDs (total 56 unique sites; 4.8% of the sequenced exonic area of AqAFB).
(figs. 3 and 4). Only those sites for which single, different base calls were made between the genomic and cDNA sequences within a given individual are included in this total. All individuals show unique RDD patterns. Each RDD site displayed one of seven genomic DNA-to-mRNA nucleotide substitutions (A-to-G, C-to-U, G-to-A, U-to-C, A-to-U, G-to-C or G-to-U) (fig. 4f). The majority of RDDs are transitions (i.e., purine–purine or pyrimidine–pyrimidine substitutions), with a slightly elevated proportion of these being A-to-G differences and the three other transitions occurring at equal rates (fig. 4f). A small number of transversions (purine–pyrimidine or pyrimidine–purine substitutions) were also observed (fig. 4f). About 43% of RDD sites are predicted to change the encoded amino acid sequence (fig. 4); no frameshifts, stop codons or signal peptide-induced methionine residues are introduced by RDDs (supplementary file S3, Supplementary Material online). If a given RDD site was changed in two or more individuals, these individuals all displayed the same specific nucleotide change at that site, as did any other individuals that were otherwise polymorphic at that site. These observations, plus the existence of multiple sequencing replicates per individual, convinced us that the variable sites represent genuine biological variability and not artifacts of the PCR or sequencing processes.

Although we did not observe more than two nucleotide types at any given position in our sequence alignments, our dataset was generated by directly Sanger sequencing PCR products, meaning that each read represented all captured alleles from a given sample. To reduce the possibility that the mRNA variants were from a previously undetected, duplicated AqAFB gene, we amplified this region again from the genomic and cDNA from six individuals (Larvae 3.1, 3.5, 6 and 7; Adults 5 and 9), and Sanger sequenced 10 cloned sequences of each (120 sequences in total). In no case did we find evidence of a second AqAFB locus and indeed five of the six individuals surveyed appear to be homozygous in their genomic DNA sequences at this locus. Comparison of RDDs detected in individual cloned sequences matched those from the original direct-sequencing sequences, except in some positions in Adult 9 (where a previously undetected cDNA allele was identified) (supplementary file S2, Supplementary Material online).

Identification of AF Candidates in Other Poriferans

We compared the features of the A. queenslandica AFs and other known sponge AF-like sequences, and used this information to design a sequence filtering workflow to allow more efficient identification of novel AF candidates from large transcriptome or genome datasets (fig. 5). We then applied these criteria to identify candidate AFs from the transcriptomes (or genomes, where specified) of 24 sponge species: A. queenslandica (developmental transcriptomes), Aphrocallistes vastus, Chondrilla nucula, C. prolifera, Cliona varians, Corticium candelabrum, Crelia elegans, Ephydatia muelleri, Haliclona amboinensis, Hylanoma populiferum, Ircinia fasiculata, Kirkpatrickia variolosa, Latrunculia apicalis, Niphathidae indet., Oscarella carmela (genome), Petrosia ficiformis, Pseudospongosorites suberitoides, Rossella fibulata, Spongilla lacustris, Sycon ciliatum (genome), Sycon coactum, Sympagella nux, Tethya wilhelma, and Xestospongia testudinaria (genome and transcriptome).

Filtered sequences were considered candidate AFs if they possessed a Wreath domain (Group 1) or showed AF-like domain architecture (fig. 1) and a top BLAST hit to an AqAF, GEOCY AF, MAFp3, MAFp4 or 5dSLIP sequence (Group 2). All remaining sequences (Group 3) were not considered to be candidate AFs for the purposes of this study.

We identified at least one AF candidate in each demosponge transcriptome, but none in any non-demosponge (i.e., calcarean, hexactinellid and homoscleromorph) species surveyed (figs. 6 and 7; supplementary fig. S1.3–S1.4 and supplementary file S4, Supplementary Material online). The majority of identified AF transcripts have the same overall domain composition as seen in the AqAFs: Calx-beta, VWA, VWD and Wreath domains occur in different numbers and combinations. In a small number of species—C. nucula, Niphathidae indet. and P. ficiformis—Calx-beta, VWA and/or VWD, and Wreath domains all co-occur as in AqAFB–AqAFE.

**Fig. 5.** Methodology for aggregation factor candidate sequence identification. Flowchart depicting the filtering process to isolate aggregation factor (AF)-like and candidate AF sequences from genome or transcriptome datasets. Sequences possessing Wreath, Calx-beta, von Willebrand type A (VWA) or D (VWD) domains were identified by searching sequence datasets with hidden Markov model (HMM) profiles. Sequences were eliminated (X) if they encoded only a Wreath domain and this domain did not cover at least 60% of the HMM model. Short or redundant sequences were also removed. The resulting list was divided into three groups, based on domain architecture and sequence similarity. Group 1 sequences possess a Wreath domain, with or without other domain types. Group 2 sequences have a top BLAST hit to a known AF sequence from Amphimedon queenslandica, Clathria prolifera or Suberites domuncula, but do not possess a Wreath domain. Group 3 sequences represent all other filtered sequences, and were not considered AF candidates for the purposes of this study.
When present, Wreath domains are always found at the 3′-most end of the coding sequence. Many \((n = 31)\) demosponge transcripts encode a Wreath domain only. In most cases, it is unknown whether these represent full-length or truncated sequences. However, four such sequences \((C. \text{nucula} \text{ Cn}_{13331.30}; \ C. \text{varians} \text{ Cv}_{16635}, \text{Cv}_{16636}; \ T. \text{wilhelma} \text{ Tw}_{1404})\) include signal peptides, suggesting these do represent full-length transcripts.

Several AF transcripts encode additional domain types not observed in the \(A. \text{queenslandica}\) or \(C. \text{prolifera}\) AFs. Two closely related freshwater haploscleromorph demosponges \(E. \text{muelleri} \text{ Em}_{90236}\) and \(S. \text{lacustris} \text{ SL}_{2436.75}\) both encode proteins equipped with one copy each of Sema \((\text{PF01403})\), PSI \((\text{PF01437})\) and Wreath domains. Immunoglobulin superfamily \((\text{IgSF}; \text{Ig-2 [PF13895]}), \text{V-set [PF07686]}, \text{I-set [PF07679]}, \text{Ig-3 [PF13927]}), \text{fibronectin type III (fn3; PF00041)}, \text{EGF-related (calcium-binding EGF domain, PF07645; human growth factor-like EGF domain, PF12661)}, \text{domains, and a GPS motif (GPCR (G-protein coupled receptor) proteolysis site PF01825)}\) were found in various AF candidates. Finally, Sushi domains \((\text{PF00084})\), as previously documented in the possible \(G. \text{cydonium} \text{ AF GEOCY AF}\), are present in one sequence each from \(I. \text{fasciculata}\) \((\text{IF}_{3013.75}, \text{three domain copies})\) and \(P. \text{suberitoides} \text{ Ps}_{6648.67}\) \(\text{one copy})\).

\(Xenostospongia \text{testudinaria}\) is the only analysed non-\(Ampimedon\) demosponge for which both transcriptome and genomic data are available; analysis of intronic properties is therefore possible for the single candidate AF from this species. The introns of the single \(X. \text{testudinaria} \text{ candidate AF Xt}_{88826}\) \(\text{average size 62 bp}\) are smaller than those of both the \(AqAFs\) \(\text{average size 172 bp}\) and of \(\text{MAFp3 (350–600 bp)}\) \(\text{Fernández-Busquets and Burger 1997). As in the AqAFs, all introns of Xt}_{88826}\) \(\text{are in phase 1; MAFp3 and MAFp4 have phase 0 introns only (Fernández-Busquets and Burger 1999).}\)

**Discussion**

The disparate nature of animal allorecognition means that little is known about how such systems evolve. With the goal of understanding both sponge allorecognition specifically, and the processes driving evolution of animal allorecognition more generally, we undertook a detailed investigation of the aggregation factor (AF) gene family in sponges. We performed a broad-ranging survey for AFs across Porifera, and identified the sponge lineages in which the AFs were present. We then characterized the various domain organizations that candidate AFs took in each species. This allowed us to assess the evolution of a putative allorecognition system in a phylum. We also scrutinized the AFs at a finer scale, exploring the AF locus in the genome of the demosponge \(Ampimedon \text{ queenslandica}\). This provided insight into the genomic background underlying broader-scale evolutionary changes. In addition to revealing high levels of polymorphisms between AqAF alleles, we identified one region of AqAFB that displays extensive RNA–DNA differences (RDDs), suggesting that further AqAF diversification can occur by RNA editing. Overall,
our study reveals that the AF gene family is a demosponge-specific evolutionary novelty, and has undergone continual modification since the demosponge last common ancestor. Amphimedon queenslandica AFs Appear to Be Diversified by High Allelism and RNA Editing Amphimedon queenslandica AF genes exhibit a high degree of between-individual polymorphism, including a greater proportion of non-synonymous nucleotide substitutions than are observed across the genome as a whole. Intriguingly, we also identified a large number of RDDs across the AqAFB sequences of some individuals. We performed additional testing to exclude the possibility that these RDDs could be attributed to the presence of a cryptic AqAFB duplicate gene elsewhere in the A. queenslandica genome. While it is impossible to ensure that any sequencing approach targets the full suite of alleles in any given sample, our consistent observation of no more than two genomic DNA alleles across the six tested individuals suggests that gene duplication is an unlikely explanation for our results.

If the observed RDDs are indeed derived from the same genomic locus, this implies that AqAFB RNAs are modified...
post-transcriptionally, and that RNA editing is another possible contributor to AF diversification. RNA editing appears to be possible in A. queenslandica because ADARs, proteins responsible for A-to-I RNA editing, are present in sponge genomes and are likely to be a metazoan-specific innovation (Grice and Degnan 2015b). However, AqAFB RDDs do not seem to favor any particular combination of nucleotide substitutions, with both transitions and transversions observed across 56 unique editing sites; multiple RNA editing profiles were detected between some A. queenslandica individuals. While similar patterns of non-specific substitution have been observed, e.g., in certain dinoflagellate mitochondrial and plastid genes (Lin et al. 2002; Mungpakdee et al. 2014), its occurrence in a metazoan nuclear gene appears to be unusual. Some unknown targeted or motif-guided mode of RNA nucleotide substitution may be at play, although currently it is unclear if the variability of RDD events in AqAFB may be explained by the activity of one or more classes of editing molecules.

The finding of elevated, individual-specific, and often non-synonymous AF polymorphism and editing is consistent with the requirement of an allorecognition gene to display high levels of between-individual variability to in turn allow rejection of nonself contact (Grice and Degnan 2015a). Such an effect has been observed in the sea urchin Strongylocentrotus purpuratus Sp185/333 immune gene family, where polymorphism and RNA editing appears to both diversify encoded Sp185/333 messages and regulate their expression by the introduction of premature stop codons (reviewed by Smith et al. 2010). In A. queenslandica, one edited region of AqAFB encodes a Wreath domain, which in Clathria prolifera contributes to the AF central ring that interacts with other AF rings to form intercellular bridges (Jumblatt et al. 1980; Misivic and Burger 1990, 1993). Sequence variation in this region may affect AF–AF binding and therefore passive “self” recognition, either by directly affecting protein conformation, or altering glycan attachment sites. In addition to these diversifying changes, a large proportion of nucleotide edits are conserved across 56 unique editing sites; multiple RNA editing profiles are likely to be a metazoan-specific innovation. Wreath domains were found to co-occur with other domain types in all demosponges except Tethya wilhelma, but we also observed transcripts (including four with signal peptides) encoding single Wreath domains only. This suggests that the Wreath domain may also play an independent functional role in some species.

All demosponge species express one or more AF candidates equipped with Calx-beta domains. Calx-beta domains contain a small number of residues that are important for binding Ca\(^{2+}\) (Hilge et al. 2006), an ion that is critical for AF stabilization and adhesiveness (Galtsøff 1925; Cauldwell et al. 1973; Fernández-Busquets et al. 2009). Almost all critical residues are conserved in the Calx-beta domains in the A. queenslandica AFs (AqAFs). Outside these conserved residues, the AqAF Calx-beta domains appear to be relatively free to change without disrupting domain functionality.

The AFs are a demosponge-specific evolutionary novelty

To reconstruct the evolutionary origins of the AF gene family, we surveyed for their presence across 24 sponge transcriptomes or genomes. Candidate AFs are present in all demosponges, and AFs are absent from calcarean, hexactinellid and homoscleromorph species included in this study. While failure to detect AF-like sequences in large datasets, particularly those generated from transcriptome libraries, is not definitive evidence of their absence from the sponge species sequenced, it is striking that candidate AFs also were not identified in non-demosponge species with full genome sequences (Oscarella carmela and Sycon ciliatum), where successful sequencing is not context- or expression-dependent. We conclude that the AFs originated in the demosponge common ancestor and evolved rapidly to produce the diversity of AF molecules that exists today.

**Demosponge AFs Are Comprised of Calx-Beta, Wreath, and Other Protein Domains**

In C. prolifera, MAAP3 is responsible for the formation of the AF central ring, and for self–self interactions between AF structures (Jarchow et al. 2000). A novel protein domain, which we have coined the Wreath domain, appears to be responsible for these interactions. The Wreath domain was found in all demosponge species and appears to be a demosponge-specific innovation. Wreath domains were found to co-occur with other domain types in all demosponges except Tethya wilhelma, but we also observed transcripts (including four with signal peptides) encoding single Wreath domains only. This suggests that the Wreath domain may also play an independent functional role in some species.

- **von Willebrand type A (VWA) or D (VWD) domains** were only found in AFs from five species distributed across the demosponges. Elsewhere, VWA domains have been proposed to mediate protein adhesion and aggregation in proteins such as integrins (Whittaker and Hynes 2002). The VWA MIDAS (metal ion-dependent adhesion site) motif has been implicated in divalent cation-dependent (usually Mg\(^{2+}\), but also Ca\(^{2+}\)) ligand binding (Cantí et al. 2005). MIDAS motifs are present within each AqAF VWA domain. As AF functionality is Ca\(^{2+}\)- and/or Mg\(^{2+}\)-dependent (Galtsøff 1925; Humphreys et al. 1960), it is possible that the incorporation of VWA domains into some AFs aids in cation-mediated aggregation. VWD domains lack a MIDAS motif, and the role of these domains in the AFs remains unclear.

- **EGF-related** (fn3, GPS, immunoglobulin-related, PSI), Sema and Sushi domains were present in several demosponge AF candidates, suggesting that novel protein folds may be important in some AFs, and/or that the Wreath domain may be involved in functions beyond AF–AF bridge formation. EGF-related (Campbell and Bork 1993), fn3 (Bork and Doolittle 1992), immunoglobulin-related (Williams and Barclay 1988) and Sushi (Day et al. 1989) domains are all widespread and mediate protein–protein interactions in a range of molecules, including those with cell adhesion, self–nonself recognition or immune functions. Sema and PSI domains, which are...
present in AFs of the freshwater sponges *Ephydatia muelleri* and *Spongilla lacustris*, are best known for their role in semaphorin-mediated axon guidance (Kolodkin et al. 1993), but have also been implicated in cell adhesion and migration processes (reviewed by Casazza et al. 2007). The function of the novel Sema-PSI-Wreath domain combination in these AFs is unknown, although it is possible that the Wreath domains allow these molecules to form circular or linear backbones, while the Sema-PSI region mediates cell–cell or cell–extracellular matrix tethering (Casazza et al. 2007).

**Exon Shuffling and the Evolution of Demosponge AFs**

The diversity of protein domain content and organization across the AFs indicates that these molecules evolved, in part, by domain cooption, gain and loss. This notion is further supported by the high level of structural constraint observed across the AF loci of *A. queenslandica* and other species, which is probably a signature of prior exon shuffling. First, despite the low sequence similarity observed between *A. queenslandica* AF protein domains of all types, these domains conform to precise boundaries within their encoding exon/s. These boundaries may be remnants of an ancestral domain module structure. While VWA and VWD domains are bound within single exons, most *A. queenslandica* AF Calx-beta and Wreath domains conform to repeated three-exon structures, with flanking and internal phase 1 introns. This suggests either that Calx-beta and Wreath domains inserted into the AFs as multi-exon modules, or that their phase 1 introns allowed the domains to be “mixed and matched” from pools of single-exon building blocks.

Another diagnostic feature of a shuffled gene is bias towards a particular intron phase (Pathy 1987, 1988). While the precise intron phase distributions differ between species (i.e., phase 1 in *A. queenslandica* and *X. testudinaria*, and phase 0 in the more distantly related *C. prolifera*; Fernández-Busquets and Burger 1999), AF exons in *A. queenslandica*, *C. prolifera* and *X. testudinaria* are all flanked by introns in the same phase, suggesting prior expansion and diversification of the AF gene family by exon shuffling. Despite this striking commonality, intron–exon and domain architectures of these AFs are unique in each species. Thus, although the AFs in these species show hallmarks of evolutionary relatedness, it is difficult to discern the ancestral condition of their common AF ancestor and the precise evolutionary processes leading to the extant AF repertoires in these and other species.

**Non-Demosponges Lack an AF Gene Family**

Candidate AFs were not identified in any analysed hexactinellid (*Hyalonema populiferum*, *Sympagella nux*, *Rosella fibulata* or *Aphrocallistes vastus*), homoscleromorph (*O. carmela* or *Corticium candebalbrum*) or calcareous (*Sycon coactum* and *S. ciliatum*) sponges, suggesting that the AF gene family, at least in the form best known from *C. prolifera*, is an innovation restricted to demosponges. This implies either that non-demosponge poriferans do not possess the ability to discriminate self from nonself by allorecognition—implausible given functional evidence to the contrary (discussed below) and the importance of self–nonself recognition—or that reaggregation and discrimination in these poriferan classes relies on a non-AF-based allorecognition systems. This latter inference is consistent with rapid evolution of allorecognition occurring in sponges, and probably in other taxa.

Calcareous sponges are capable of discriminating between self and nonself at the tissue level (Amano 1990), however reaggregation of dissociated cells in this sponge class does not appear to be enabled by a soluble aggregation factor (Müller 1982). This observation is consistent with our inability to identify MAFp3/4-like AFs in calcareans, and suggests that the mechanisms underlying aggregation and self–nonself discrimination are different between demosponges and calcareous sponges. Allorecognition also exists in hexactinellids, with *Rhabdocalyptus dawsoni* able to discriminate between self and nonself in mixed tissue/aggregate graft experiments (Leys et al. 1999). Again, however, a different molecular self–nonself recognition system seems to be operating in this class of sponges; a C-type lectin appears to be responsible for cell reaggregation in fellow hexactinellid *A. vastus*, although it is unclear if it can promote species-specific differentiation (Müller et al. 1984; Gundacker et al. 2001). Finally, we did not detect AFs in homoscleromorphs. Although cellular reaggregation or allorecognition phenomena have not been investigated in this class of sponge, the presence of a circular molecule closely resembling the circular core AF has been found in *Oscarella tuberculata*; it is currently unknown whether this represents a true AF (Humbert-David and Garrone 1993). Overall, these observations, coupled with our inability to identify AF-like sequences from any non-demosponge species, suggest that one or more unknown allorecognition mechanisms await discovery in these other sponge lineages.

**Conclusions**

Although metazoan allorecognition does not appear to be under the control of conserved genes, similar molecular and genomic properties can be observed between these unrelated systems; the demosponge AFs also display these properties. First, allorecognition molecules are often encoded by genes that are clustered in the genome, a phenomenon that supports further molecular diversification (Grice and Degnan 2015a). In *A. queenslandica*, the five AF genes are clustered within an 80-kb genomic locus. Second, allorecognition molecules are often large extracellular or membrane-bound proteins with numerous repeated domains, facilitating the physical interactions between self and/or nonself cells (Grice and Degnan 2015a). In *A. queenslandica*, the five AFs are large, and most demosponge AFs are predicted to occur extracellularly—either secreted or membrane-bound—and have domains involved in cell-cell interaction or adhesion. Finally, allorecognition molecules often exhibit high degrees of sequence polymorphism, enabling the high level of specificity required of a molecule responsible for discriminating between conspecific individuals (Grice and Degnan 2015a). *C. prolifera* AF isoforms have been previously demonstrated to be highly polymorphic between and within individuals.
(Fernández-Busquets and Burger 1997; Fernández-Busquets et al. 1998), and here we have demonstrated that the same applies in A. queenslandica (fig. 3). Indeed, our identification of RDDs in AqAFB in some A. queenslandica individuals—an indicator of RNA editing—implies the contribution of an additional level of complexity to the diversification of these molecules. The existence of these commonalities between unrelated metazoan allorecognition systems suggests there are similar selective pressures driving the evolution and function of these divergent systems, regardless of the fact that allorecognition molecules vary markedly between taxa.

We found Wreath and Calx-beta domain-equipped AF candidates in all analysed demosponges. The ubiquity of these domains suggests that the ancestral AF probably encoded one Wreath domain sequence—itself a demosponge-specific novelty—or precursor, and one or more Calx-beta domains. Prior to demosponge cladogenesis, AFs appear to have acquired a self–nonself recognition role, possibly as a result of increasing specificity of a more ancient role in cell-cell interaction or adhesion activity (Grice and Degnan 2015a). The AF gene family most likely expanded via multiple duplication events, as inferred from the clustered A. queenslandica AF locus. Diversification of the demosponge AFs occurred via the inclusion of additional domain types and their subsequent gain and loss. Many of the domains present in extant AFs have been implicated elsewhere in cell adhesion and interaction processes; these domains likely play similar roles within the AFs. Finally, beyond the between-species diversification discussed above, a fundamental requirement of an allorecognition molecule is to be sufficiently variable within a species, such that every individual possesses its own “self marker”. Sponge allorecognition has been proposed to operate via self-recognition, whereby binding of matching self markers is required to prevent rejection between adjacent cells (Sabella et al. 2007). Generation of this required variation could be achieved potentially in several ways, including high allelic variance as observed in the A. queenslandica and C. prolifera AFs (Fernández-Busquets and Burger 1997; Fernández-Busquets et al. 1998), alternative splicing (aided, perhaps, by frameshift-minimizing symmetrical exons as present in the A. queenslandica, C. prolifera and X. testudinaria AFs), post-transcriptional or post-translational modification, the former of which appears to occur in the A. queenslandica AFs, somatic recombination and variation in associated non-protein molecules such as glycans.

To the best of our knowledge, this study represents the first instance of a phylum wide survey of putative allorecognition genes. The AFs appear to have evolved rapidly by mechanisms including exon shuffling and nucleotide mutation, and continue to generate within-species variation required for self–nonself recognition by the means listed above. Reconstruction of the evolution of AFs in sponges exposes the evolutionary processes underpinning the disparity of allorecognition factors in the animal kingdom, and similar evolutionary mechanisms to those seen in sponges are likely operational across the Metazoa. Therefore, understanding of the sponge AF gene family sheds light on one of the central features of being an animal, self–nonself recognition.

Materials and Methods

Identification of Aggregation Factors from the Amphimedon queenslandica Genome

Amphimedon queenslandica AF (AqAF) sequences were identified using BLASTP and TBLASTN searches with Clathria prolifera MAfP3 and MAfP4 isoforms (Genbank: AAB71890, AAB71891, AAC33162, AAC33163, CAA65098), Geodia cydonium GEOCY AF (Müller et al. 1999), and Suberites domuncula SdSLIP (Genbank: CAL68017.1) used as queries. Searches were performed against A. queenslandica genomic traces and assemblies, expressed sequence tags (Srivastava et al. 2010), and subsequently with Aqu2.1 gene model predictions (Fernandez-Valverde et al. 2015). Aqu2.1 annotations were derived from new transcriptomic data combined with the original genome assembly (Srivastava et al. 2010; Fernandez-Valverde et al. 2015) and can be accessed at http://amphimedon.qcloud.qcif.edu.au/index.html (last accessed December 16, 2015).

Protein Domain and Topology Predictions

Domain architectures of the AqAF proteins were predicted using Pfam with default parameters (Finn et al. 2014), and verified using hmmscan, available in the HMMER 3.0 software package (Eddy 1998). Domain hits were counted if they returned an expect (e)-value $\leq 10^{-4}$. For each sequence, signal peptides and transmembrane domains were also predicted using Phobius (Käll et al. 2004).

Generation of a Wreath Domain Hidden Markov Model

NCBI BLAST searches (http://blast.ncbi.nlm.nih.gov/; last accessed November 9, 2015) between the MAfP3 region and the publicly available translated genomes of various model metazoan species (Acropora digitifera, A. queenslandica, Arabidopsis thaliana, Branchiostoma floridae, Caenorhabditis elegans, Capitella teleta, Capsaspora owczarzaki, Ciona intestinalis, Dictyostelium discoideum, Drosophila melanogaster, Helobdella robusta, Homo sapiens, Hydra magnipapillata, Lottia gigantea, Mnemiopsis leidyi, Monosiga brevicollis, Nemastostella vectensis, Neurospora tetrasperma, Pleurobrachia bachei, Salpingoeca rosetta, Strangylomacentrus purpuratus, or Trichoplax adhaerens) indicated that this region is not present outside sponges. We propose that the MAfP3 region encodes a sponge-specific domain we coin the “Wreath” domain. A multiple sequence alignment of MAfP3 isoform C, SdSLIP and AqAFC (supplementary fig. S1.5 and supplementary methods, Supplementary Material online) was used to generate a profile hidden Markov model (HMM) (supplementary methods and supplementary file S5, Supplementary Material online) for the MAfP3-equivalent region (i.e., “Wreath domain”). The model was tested using hmmsrch as above.

Calculation of Intron Phase Distribution Frequencies

Amphimedon queenslandica intron phase values were derived by modification of a publicly available Aqu2.1 GFF3 file (http://amphimedon.qcloud.qcif.edu.au/index.html;
were simultaneously extracted from individuals using TRIzol preserved in RNA Later (Ambion). RNA and genomic DNA were obtained as previously described (Leys et al. 2008) and slices of tissue from eight adult A. queenslandica online).

### Analysis of RDDs and Gene Duplication in AqAFB

We sought to determine the number of AqAFB alleles in Larvae 3.1, 3.5, 6, 7 and 9 and Adults 5 and 9. We amplified the AqAFB hotspot region from the genomic and cDNA of each individual as described above and cloned the amplicons into pGEM-T Easy Vectors (Promega). Clones were screened for inserts and plasmid inserts were Sanger sequenced. Raw sequencing chromatograms were trimmed using Geneious Pro. Matched pairs of forward and reverse sequences from individual clones were aligned to one another using the De Novo Assemble tool. Consensus sequences were extracted for each alignment, and were manually annotated to flag read mismatches or low-quality base calls. Genetic and cDNA sequences from each individual were assembled to the AqAFB gene model, and were manually inspected to identify polymorphic sites.

### Sanger Sequencing-Based Analysis of Polymorphism

Three AqAF polymorphism "hotspots" (within AqAFB, AqAFC and AqAFE) and one less variable region (within AqAFE) were selected for in-depth analysis of polymorphism (fig. 3), based on identification of regions of the AqAF locus exhibiting high numbers of variants (supplementary fig. S1.2 and supplementary methods, Supplementary Material online).

Nine larval individuals (0-h post-emergence) and small slices of tissue from eight adult A. queenslandica individuals were obtained as previously described (Leys et al. 2008) and preserved in RNA Later (Ambion). RNA and genomic DNA were simultaneously extracted from individuals using TRIZol (Thermo Fisher Scientific) following manufacturer’s guidelines. Briefly, tissue was homogenized in TRIZol, phase separated with 1-bromo-3-chloropropane (BCP), and RNA precipitated from the aqueous layers using glycogen and isopropanol. Genomic DNA was back-extracted from the interphase and organic layers using 4 M guanidine thiocyanate, 50 mM sodium citrate and 1 M Tris (pH 8) and precipitated from the resulting aqueous layer using glycogen and isopropanol. After DNase I treatment (Invitrogen), cDNA was synthesized from RNA using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT)15 and random pentadecamer primers (Stangegaard et al. 2006), according to manufacturer’s guidelines. AqAF gene-specific primers were designed using Primer3 (Untergasser et al. 2012) to cover < 2 kb of genomic and cDNA sequences (supplementary table S1.8, Supplementary Material online). Primer specificity was checked via BLAT search on an in-house UCSC genome browser and via BLAST search on the NCBI nucleotide database. AqAF fragments were amplified from genomic and cDNA from each individual using Phusion high-fidelity DNA polymerase (New England Biolabs) and touch-down PCR cycling. Size-correct amplicons were gel purified using the QIAquick gel extraction kit (Qiagen) and sequenced using the Big Dye Terminator 3.1 Cycle Sequencing kit (Applied Biosystems) at the Australian Genome Research Facility (AGRF). Internal and overlapping primers were used to obtain double sequence coverage of the entire fragment to ensure precision of base calling and eliminate ambiguity.

Raw sequencing chromatograms were trimmed using Geneious Pro v6.1.8 (Kearse et al. 2012). Genomic and cDNA sequences from each individual were assembled to the AqAFB gene model, and sites exhibiting read mismatches or low-quality base calls were flagged. Sites displaying putative polymorphism, heterozygosity or an RDD were identified by manual inspection. RDD calls were only made for sites where a single allele was identified in both the genomic and cDNA.

Statistical comparisons of nucleotide or amino acid change frequencies are described in supplementary methods, Supplementary Material online.
Sequence Data Used for AF Identification
The sponge referred to here as “Niphatidae indet.” has tentatively been identified as belonging to the demosponge family Niphatidae based on spicule morphology, and its transcriptome has been sequenced and assembled (Gaiti F, Kocot KM, Degnan BM, unpublished data). Transcripts were assembled as per Whelan et al. (2015) and best open reading frame (ORF) predictions were generated using TransDecoder (http://transdecoder.github.io/; last accessed September 28, 2015).

Ephydatia muelleri translated messenger RNA sequences (‘T-PEP’) were downloaded from Compagen (http://www.compgen.org; last accessed September 5, 2013) (Hemmrich and Bosch 2008). Gene models for Oscarella carmela were prepared as described by Grice and Degnan (2015b). Translated peptide sequences from the Sycon ciliatum genome (Fortunato et al. 2015) were provided by M. Adamska and M. Adamski.

For A. queenslandica (developmental transcriptomes) (Fernandez-Valverde et al. 2015). Aphrocallistes vastus, Chondrilla nucula, C. prolifera (Fernandez-Valverde SL, Degnan BM, unpublished data), Corticium candelabrum, Crella elegans, Ircinia fasciculata, Petrosia ficiformis, Spongilla lacustris, Pseudopetrosia suberitoides and Sycon coactum (Riesgo et al. 2012), we determined the longest ORF between stop codons for each assembled transcript using the program getorf from the EMBOSS 6.5.7 software package (Rice et al. 2000). For A. queenslandica and Cr. elegans, sequences from all available developmental stages per species were pooled prior to further analysis.

Best ORF predictions for Hyalonema populiferum, Kirkpatrickia variolosa, Latrunculia apicalis, Rossella fibulata, Sympagella nux, Tethya wilhelma (Whelan et al. 2015) were computed using TransDecoder (Kocot KM, personal communication). Cliona varians (Riesgo et al. 2014), Haliclona amboinensis (SRR1619429) and X. testudinaria (SRR1738101) transcriptomes were assembled as per Whelan et al. (2015) and ORF predictions were determined using TransDecoder (Kocot KM, personal communication).

Identification of AF-Like Sponge Sequences
Sequences of at least 100 amino acids in length from the translated transcriptomes or genomes listed in the previous section were filtered to generate a list of AF-like sequences using criteria described in figure 5. An A. queenslandica developmental transcriptome dataset was included as a point of comparison between genomic and transcriptomic sequences. Sequences encoding Calx-beta (Pfam PF03160), von Willebrand type A (VWA; Pfam PF00092), von Willebrand type D (VWD; Pfam PF00094) or Wreath domains (supplementary file SS, Supplementary Material online) were identified using hmmsearch. Sushi domains, as seen in the putative G. cyclonium AF, GEOCY AF, were not included as search criteria, as this domain combination has only been observed in the AFs of one species to date and has not been well characterized. To remove redundancies, sequences within each species were clustered into groups sharing at least 90% amino acid sequence identity, using the default parameters of the cd-hit tool (Li and Godzik 2006), run using the CD-HIT Suite server (Huang et al. 2010) or, in the case of the A. queenslandica transcriptomes, the cd-hit v4.6.1 command line application. Only the representative sequence from each cluster (as determined by cd-hit; equivalent to the longest sequence) was passed through for further analysis.

Overall domain architecture for each sequence was determined using HMMER 3.0 (Eddy 1998) within the DoMosaics environment (Moore et al. 2014). Batch BLASTp searches were performed using BLAST+ v2.2.28+ and a local nr database, to identify the top hit with a maximum e-value of $10^{-4}$. Sequences were assigned to one of three groups based on these results (fig. 5), as follows. Group 1 sequences are those possessing one or more Wreath domains (which, for sequences not predicted to encode additional domain types or sequence features such as transmembrane domains or signal peptides, had to span 60% or more of the Wreath HMM model). Sequences possessing either three or more Calx-beta domains or at least one each of a Calx-beta and VWA or VWD domain were sorted into Group 2 if they also showed a top BLAST hit to an AqAF, GEOCY AF, MAFp3, MAFp4 or SdSLIP sequence, or into Group 3 if they did not. Only sequences in Groups 1 or 2 were considered candidate AFs and passed onto further analysis.

Presence and absence of AF candidates were mapped to a phylogenetic tree showing relationships between analysed sponge species, with tree topology based on analyses by Thacker et al. (2013), Whelan et al. (2015) and Kocot KM (personal communication).

Supplementary Material
Supplementary data and methods are available at Molecular Biology and Evolution online.

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