Zoologica Scripta

Inferring the ancestral sexuality and reproductive condition in sponges (Porifera)

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Submitted: 5 July 2012
Accepted: 3 July 2013
doi:10.1111/zsc.12031

Riesgo, A., Novo, M., Sharma, P.P., Peterson, M., Maldonado, M., Giribet, G. (2014). Inferring the ancestral sexuality and reproductive condition in sponges (Porifera). —*Zoologica Scripta*, *43*, 101–117.

Considerable diversity abounds among sponges with respect to reproductive and developmental biology. Their ancestral sexual mode (gonochorism vs. hermaphroditism) and reproductive condition (oviparity vs. viviparity) however remain unclear, and these traits appear to have undergone correlated evolution in the phylum. To infer ancestral traits and investigate this putative correlation, we used DNA sequence data from two loci (18S ribosomal RNA and cytochrome *c* oxidase subunit I) to explore the phylogenetic relationships of 62 sponges whose reproductive traits have been previously documented. Although the inferred tree topologies, using the limited data available, favoured paraphyly of sponges, we also investigated ancestral character-state reconstruction on a phylogeny with constrained sponge monophyly. Both parsimony- and likelihood-based ancestral state reconstructions indicate that viviparity (brooding) was the likely reproductive mode of the ancestral sponge. Hermaphroditism is favoured over gonochorism as the sexual condition of the sponge ancestor under parsimony, but the reconstruction is ambiguous under likelihood, rendering the ancestry of sexuality unresolved in our study. These results are insensitive to the constraint of sponge monophyly when tracing the reproductive characters using parsimony methods. However, the maximum likelihood analysis of the monophyletic hypothetical tree rendered gonochorism as ancestral for the phylum. A test of trait correlation unambiguously favours the concerted evolution of sexuality and reproductive mode in sponges (hermaphroditism/viviparity, gonochorism/oviparity). Although testing ecological hypotheses for the pattern of sponge reproduction is beyond the scope of our analyses, we postulate that certain physiological constrains might be key causes for the correlation of reproductive characters.

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Introduction

Sexual reproduction counts among the major forces driving eukaryote evolution (Bernstein *et al.* 1981). Whereas in unicellular eukaryotes of the genera *Giardia* and *Trichomonas*, evidence of sex comes from meiotic gene inventories (Ramesh *et al.* 2005; Malik *et al.* 2008), sexuality in multicellular organisms accounts for one of the most thoroughly characterised biological processes. However, one of the conundrums of eukaryote sexuality is whether the ancestral condition was gonochorism or hermaphroditism. Haag (2007) proposed that mating types in unicellular ancestors engendered the gonochoristic condition of multicellular organisms. However, ancestral land plants were likely hermaphrodites (Barrett 2002), and hermaphroditic strategies are also well represented in animals near the root of Metazoa (Ghiselin 1969). A traditional view, thus deeply rooted in the zoological literature, holds that early-branching metazoans were hermaphroditic, separate sexes being acquired progressively through evolution (Ghiselin 1969). But early theories on the evolution of the diverse life histories of metazoans, specifically invertebrates, were developed in the absence of molecular phylogenetic methods (McHugh & Rouse 1998).

Sexually reproducing metazoans can produce offspring either by spawning or depositing unfertilised or fertilised eggs (i.e. oviparity) or by brooding and nourishing the eggs internally (i.e. viviparity). Both reproductive strategies are often observed in marine benthic invertebrates (Vance 1973) and their occurrence justified using a wide array of adaptive explanations (e.g. Ghiselin 1969; Strathmann & Strathmann 1982). Besides the adaptive advantages of each strategy, the link between sexuality and reproductive condition has been formally tested using parametric methods in just a few instances (Heath 1977, 1979; Strathmann & Strathmann 1982; McFadden *et al.* 2001; Kerr *et al.* 2011). For example, hermaphroditism and brooding strategies are demonstrably correlated in cnidarians (Heath 1977, 1979; Strathmann & Strathmann 1982; McFadden *et al.* 2001; Kerr *et al.* 2011), but this relationship is not applicable to other groups, such as molluscs (Heller 1993). To assess the origins and link between both strategies (sexuality and reproductive condition) in ancestral metazoans, it is imperative to investigate other basal lineages in addition to cnidarians, such as ctenophores and sponges.

Sponges are aquatic, sessile, diploblastic animals that lack organs, with reproductive elements occurring throughout the body. While gametogenesis in different sponge groups occurs in a relatively similar fashion (Fell 1974, 1983; Simpson 1984; Maldonado & Riesgo 2008, 2009; Ereskovsky 2010), embryogenesis is very diverse among sponge groups, producing a plethora of larval forms (Maldonado & Bergquist 2002). Regarding sexual condition, sponges can be either gonochoristic or hermaphroditic. All gonochoristic sponges investigated heretofore lack sexual dimorphism. Among hermaphroditic sponges, simultaneous hermaphroditism (homogamy) is more common than sequential hermaphroditism (dichogamy). With respect to reproductive condition, sponges can be either oviparous or viviparous. In oviparous sponges, the gametes are released into the water column, where embryonic development leads to the formation of a free-living larva. In contrast, in viviparous sponges, embryos are brooded in the sponge body until their release as early zygotes or most commonly larvae (see reviews by Fell 1974, 1983; Simpson 1984; Maldonado & Riesgo 2008, 2009; Ereskovsky 2010).

Only recently, molecular data have been used to investigate sexuality and reproductive strategy in basal metazoans (McFadden *et al.* 2001; Borchiellini *et al.* 2004; Kerr *et al.* 2011; Cárdenas *et al.* 2012). In sponges, only the ancestry of the reproductive strategy (i.e. oviparity/viviparity) has been addressed, and solely for 24 taxa belonging to the class Demospongiae (Borchiellini *et al.* 2004; Cárdenas *et al.* 2012). More importantly, these recent reconstructions have not considered species for which the reproductive condition differs from the general pattern observed in the order.

Our objective was to elucidate the evolutionary history of sexual and reproductive modes for sponges, and as a corollary, to assess whether the evolution of the sexual condition and reproductive condition is correlated in sponges. For such purposes, we inferred a phylogeny using molecular data for 62 sponge species, most of them with known reproductive data, following an exemplar approach (*sensu* Prendini 2001), and then mapped reproductive characters onto the tree to analyse ancestry and correlation. As there is much debate about whether the phylum Porifera is a monophyletic or polyphyletic taxon (Borchiellini *et al.* 2001, 2004; Lavrov *et al.* 2008; Philippe *et al.* 2009, 2011; Sperling *et al.* 2009; Pick *et al.* 2010; Nosenko *et al.* 2013), we mapped the reproductive characters on both competing tree topologies and subsequently discussed the agreements and mismatches.

Materials and methods

Sampling methods

Taxon sampling was designed to cover nearly all sponge species for which reproductive information is available (Table 1). A total of 43 species were collected, preserved in 70–96% ethanol and stored at −20 °C for subsequent DNA extraction. Data about species collection are given in Table 1. Also, sequences from nineteen additional sponge species and five putative outgroups—*Capsaspora owzarzacki* (Filasterea), *Monosiga brevicollis* (Choanoflagellata), *Nemato-*

Table 1 Species included in the phylogenetic analysis and information considered in the study. Voucher accession numbers [Museum of Comparative Zoology (MCZ) at Harvard University] are given for the species sequenced here, and GenBank accession numbers for all the genes included in the analysis, being the sequences generated for this study in bold letters

Sponges	MCZ accession	GenBank accession	
		18S	COI
Class Hexactinellida			
Order Hexactinosida			
Aphrocallistes vastus*	DNA105724	**JX945605**	**JX999081**
Heterochone calyx*		AM886405.1	FR848901
Order Lyssacinosida			
Oopsacas minuta*		AF207844.1	FR848932
Lophocalyx profundum		AM886391.1	FR848926
Rossella nodastrella		AM886386.1	FR848921
Class Demospongiae			
Order Dictyoceratida			
Hippospongia lachne*		EU702419.1	NC_010215.1
Rhopaloeides odorabile*	DNA106623	**JX945630**	EU644448.1
Spongia officinalis*		AY348888.1	HQ830364.1
Order Verongida			
Aplysina aerophoba*	DNA105711	**JX945612**	**JX999079**
Aplysina cavernicola*	DNA105710	**JX945613**	**JX999083**
Aplysina fulva	DNA106221	**JX945641**	**JX999063**
Hexadella pruvoti	DNA105706	**JX945636**	**JX999067**
Order Chondrosida			
Chondrilla australiensis*	DNA106225	**JX945640**	**JX999064**
Chondrilla nucula*	DNA106226	EU702413.1	**JX999065**
Chondrosia reniformis*	DNA105709	AY348876.1	**JX999074**
Halisarca dujardini*		EU702418.1	EU237483.1
Order Haplosclerida			
Amphimedon compressa*	DNA106219	**JX945626**	NO
Amphimedon erina*	DNA106220	**JX945627**	NO
Calyx podatypa*	DNA106223	**JX945624**	**JX999086**
Chalinula molitba	DNA106224	**JX945621**	NO
Ephydatia fluviatilis*		AY578146.1	DQ167174.1
Eunapius fragilis*	DNA105725	AF121111.1	**JX999059**
Haliclona elegans*	DNA106275	**JX945607**	**JX999087**
Haliclona oculata*		AY348888.1	EF655743.1
Haliclona sarai	DNA105702	**JX945632**	NO
Haliclona xena*	DNA105733	**JX945622**	**JX999085**
Petrosia ficiformis*	DNA105722	**JX945623**	**JX999088**
Spongilla lacustris		DQ167161.1	DQ167176.1
Siphonochalina siphonella*	DNA106625	**JX945610**	**JX999082**
Xestospongia muta*	DNA106633	**JX945625**	**JX999069**
Order Hadromerida			
Cliona viridis*	DNA105701	**JX945634**	**JX999076**
Placospongia intermedia*	DNA106621	**JX945628**	**JX999089**
Spirastrella cunctatrix*	DNA105704	**JX945608**	NO
Tethya aurantium*	DNA106628	**JX945619**	**JX999070**
Tethya citrina*	DNA106629	**JX945620**	EF558570.1
***Tethya* nov. sp. 1**	DNA106632	**JX945629**	**JX999072**
***Tethya* nov. sp. 2**	DNA106631	**JX945611**	**JX999071**
Suberites domuncula*	DNA105717	AJ620112.1	**JX999078**
Suberites ficus*		AF100947.1	AJ843891.1
Orden Halichondrida			
Acanthella acuta*	DNA105714	**JX945606**	**JX999080**
Axinella damicornis*	DNA105721	**JX945617**	NO
Axinella verrucosa*		GQ466050.1	NO
Dictyonella incisa	DNA105707	AY348880.1	**JX999084**
Dragmacidon lunaecharta	DNA106232	**JX945615**	**JX999077**
Halichondria okadai*		AB511881.1	EF217341.1
Hymeniacidon perlevis*		HM035977.1	HM035985.1
Scopalina ruetzleri*	DNA106624	**JX945609**	**JX999075**
Orden Agelasida			
Agelas clathrodes*		AY769087.1	DQ075720.1
Agelas oroides*	DNA105715	**JX945633**	**JX999060**
Order Astrophorida			
Geodia barretti*	DNA105734	**JX945638**	**JX999061**
Thenea muricata*	DNA105738	**JX945618**	**JX999068**
Order Poecilosclerida			
Asbestopluma occidentalis*	DNA105732	**JX945639**	**JX999062**
Lissodendoryx colombiensis*	DNA105719	**JX945631**	**JX999090**
Crambe crambe*	DNA105712	EF654524.1	**JX999091**
Microciona prolifera*		EF092268.1	AJ843888.1
Mycale laxissima*		**JX945616**	EF519651.1
Negombata magnifica*	DNA106476	**JX945635**	**JX999092**
Tedania ignis*		AY737642.1	DQ133897.1
Class Calcarea			
Clathrina clathrus	DNA106230	**JX945642**	NO
Sycon coactum*	DNA105723	**JX945637**	NO
Class Homoscleromorpha			
Oscarella lobularis*	DNA105708	HM118536.1	**JX999066**
Corticium candelabrum*	DNA105720	**JX945614**	**JX999073**
Outgroups			
Phylum Filasterea			
Capsaspora owczarzaki		AY363957.1	NO
Phylum Choanoflagellata			
Monosiga brevicollis		AF174375.1	NC_004309.1
Phylum Cnidaria			
Hydra vulgaris*		EU876570.1	NO
Nematostella vectensis*		AF254382.1	DQ538494.1
Phylum Ctenophora			
Mertensia ovum*		AF293679.1	NO

Asterisks indicate that the reproductive information for each species can be found in Table S2. Species with no reproductive information available were collected to balance the information included in the data set.

stella vectensis and *Hydra vulgaris* (Cnidaria), and *Mertensia ovum* (Ctenophora)—were retrieved from NCBI (Table 1).

Reproductive data

Reproductive data for 50 sponge species, two cnidarians and one ctenophore were obtained from the literature or derived from personal observations (Table S1). We collected data regarding (i) sexuality (or sexual mode) or how investment in sex function is partitioned within an organism; and (ii) reproductive condition, which concerns the manner in which reproduction or mating occurs, via

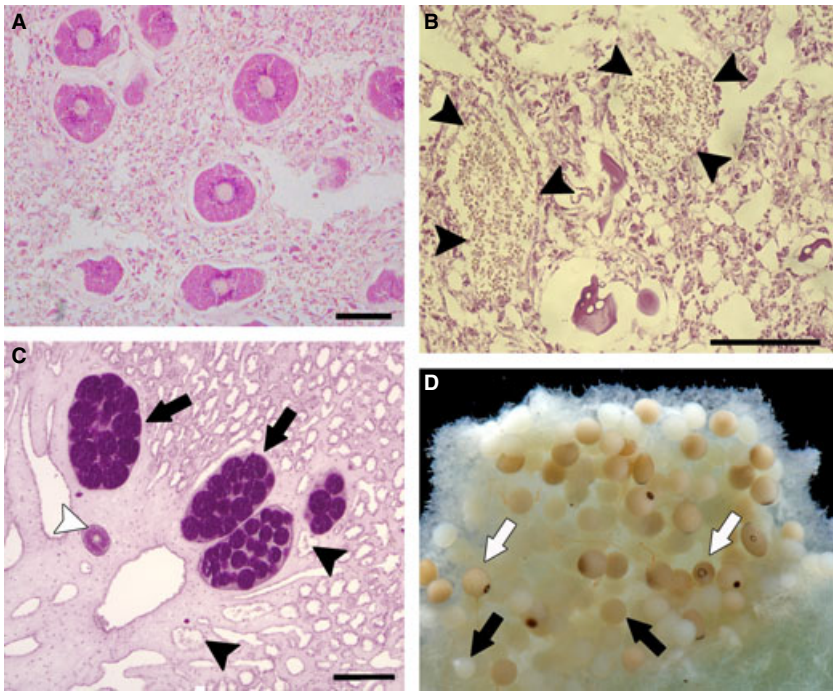

Fig. 1 Reproductive strategies in Porifera: —A–C: sexual mode, —C–D: reproductive condition. —A. Gonochorism in *Raspaciona aculeata*. Oocytes are homogenously distributed throughout the entire mesohyl (scale bar 100 μm). —B. Gonochorism in *Axinella damicornis*. The spermatic cysts (black arrowheads) occupy large areas of the sponge mesohyl (scale bar 100 μm). —C. Hermaphroditism and viviparity in *Corticium candelabrum*. Oocytes (white arrowhead) and spermatic cysts (black arrowheads) co-occur with early-stage embryos (black arrows) (scale bar 500 μm). —D. Brooding (viviparity) in *Niphates erecta*. Embryos (black arrows) and larvae (white arrows) are incubated in brooding chambers.

internal fertilisation, broadcast spawning or brooding (Kerr *et al.* 2011). We defined sexuality as a binary character, gonochorism vs. hermaphroditism (Fig. 1). As the coding of sexuality and reproductive condition as binary characters can be difficult in some instances due to limiting published data, we took into account only long-term studies, in which sex reversals in the sponges were not reported. In some species, when most individuals (even if not all) in the studied population exhibited both sexes, we coded them as hermaphroditic, following Fell (1983). Similarly, when a population is predominantly hermaphroditic with few gonochoric individuals, the latter ones have been suggested to be transitional stages of successive hermaphrodites. The reproductive condition was also coded as binary, oviparity vs. viviparity (Fig. 1). Here, we considered all the broadcasting species, regardless of fertilisation occurring internally or externally, as oviparous and all the brooding species as viviparous, because discerning between ovoviviparity and viviparity proved difficult in many instances.

DNA amplification

Total genomic DNA was extracted from tissue sample using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA), eluting twice with 100 μL of buffer. Molecular markers included a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I (COI) and one nuclear ribosomal gene (complete or partial 18S rRNA). The complete 18S rRNA (ca. 1.8 kb) was amplified in three overlapping fragments of about 950, 900 and 850 bp each, using primer pairs 1F–5R, 3F–18Sbi and 18Sa2.0–9R (primer sequences are listed in Table S2). Additional primer pairs were used when the previous pairs failed to amplify the fragment: 1F–4R internal to 1F–5R, 3F-5R internal to 3F–18Sbi, 4F-18Sbi internal to 3F–18Sbi, 4F–7R internal to 3F–9R and 5F–9R internal to 4F–9R. A fragment of 680 bp of the COI gene was amplified using the primers LCO1490 and HCO2198, developed by Folmer *et al.* (1994).

Polymerase chain reactions (PCRs) of 18S rRNA were performed using AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) and COI with HotMaster Taq (5PRIME, Hamburg, Germany) and a GeneAmp Multicycler Ep gradient (Eppendorf, Hamburg, Germany). PCR programme for 18S rRNA involved an initial denaturation step (5 min at 95 °C) followed by 35 or 40 cycles including denaturation at 95 °C for 30 s, annealing (ranging from 40 °C to 50 °C) for 30 s or 1 min and extension at 72 °C for 1 min, with a final extension step at 72 °C for

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7 min. For COI, the PCR started with an initial denaturation step (3 min at 94 °C) followed by 49 cycles including denaturation at 95 °C for 30 s, annealing (ranging from 40 °C to 45 °C) for 30 s and extension at 65 °C for 3 min, with a final extension step at 65 °C for 7 min. PCR products were purified using 1/3 dilutions of ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and bidirectionally sequenced with the same primer pairs used for amplification and ABI BigDye Terminator (Applied Biosystems). The sequencing reactions involved an initial denaturation step (5 min at 95 °C) and 30 cycles (95 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min). The BigDye-labelled PCR products were cleaned using Performa DTR Plates (Edge Biosystems, Gaithersburg, MD, USA). The sequence reaction products were then analysed using an ABI Prism 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Chromatograms were visualised and sequences were assembled in Sequencher v.4.7 (Gene Codes Corporation, Ann Arbor, MI, USA). These were compared against the GenBank database with the BLAST algorithm (Altschul *et al.* 1997). All new sequences have been deposited in GenBank (see Table 1 for accession numbers).

Phylogenetic analyses

Bayesian inference (BI) and maximum likelihood (ML) analyses were conducted on static alignments. Sequences were aligned using MUSCLE ver. 3.6 (Edgar 2004) with default parameters.

BI analyses were performed using MrBayes ver. 3.1.2 (Huelsenbeck & Ronquist 2005) with a unique GTR model of sequence evolution (Tavaré 1986) with corrections for a discrete gamma distribution and a proportion of invariant sites (GTR + Γ + I; Yang 1996) specified for each partition, as selected in jModelTest ver. 0.1.1 (Posada 2008) under the Akaike Information Criterion (Posada & Buckley 2004). Two runs, each with three hot chains and one cold chain, were conducted in MrBayes for 20 million generations, sampling every 2000th generation, using random starting trees. The analysis was performed twice, and 25% of the runs were discarded as burn-in after checking for stationarity with Tracer v.1.5. (Rambaut & Drummond 2007). The remaining trees were combined to find the maximum *a posteriori* probability estimate of phylogeny.

ML analyses were conducted using RAxML ver. 7.2.7 (Stamatakis 2006) on 24 CPUs of a cluster at Harvard University, FAS Research Computing (odyssey.fas.harvard.edu). For the maximum likelihood searches, a unique GTR model of sequence evolution with corrections for a discrete gamma distribution (GTR+ Γ) was specified for each data partition, and 500 independent searches were conducted. Nodal support was estimated via the rapid bootstrap algorithm (1000 replicates) using the GTR-CAT model (Stamatakis *et al.* 2008). Bootstrap resampling frequencies were thereafter mapped onto the optimal tree from the independent searches.

Ancestral state reconstruction

Given topological similarity between the BI and ML trees, we performed ancestral state reconstructions on the ML topology (henceforth, hypothesis 1) using Mesquite ver. 2.75 (Maddison & Maddison 2011). All ingroup taxa were coded for the two binary reproductive characters: hermaphroditism vs. gonochorism and oviparity vs. viviparity, as discussed above. We then performed an ancestral character-state reconstruction with equally weighted unordered states under parsimony and a maximum likelihood reconstruction using a Markov 2-state one-parameter model (Lewis 2001). The choice of the 2-state one-parameter model reflects our agnostic approach to ancestral state optimisation, insofar, as we do not have any external evidence for differentially weighting directionality or frequency of reproductive character-state changes across poriferan phylogeny. For this reason, we implemented the simplest possible likelihood model for inferring ancestral states in binary characters with a single parameter for symmetric state changes.

Given ongoing debate on the status of poriferan monophyly, we analysed the distribution of reproductive characters not only on the tree topology favoured by our data, but also on a competing topology showing sponge monophyly (henceforth, hypothesis 2), as suggested in other studies (e.g. Pick *et al.* 2010; Wörheide *et al.* 2012; Nosenko *et al.* 2013). For this topology, we ran RAxML ver. 7.2.7 (Stamatakis 2006) with the same parameters as before, but we implemented a constrained monophyletic backbone for Porifera proposed by Nosenko *et al.* (2013): [(Calcarea + Homoscleromorpha) (Demospongiae + Hexactinellida)]. We then coded sexuality and reproductive condition as in the previous analyses, and reconstructed their ancestral states using parsimony and maximum likelihood models with Mesquite ver. 2.75 (Maddison & Maddison 2011), as in the previous analysis.

Statistical analysis of trait evolution

To investigate reproductive trait evolution, we tested for correlation of character states for both reproductive traits (i.e. sexuality and reproductive condition) in our phylogeny using BayesTraits ver. 1.0 (Pagel 2007). We tested two models of character evolution, the discrete independent model and the discrete dependent model, and analysed trait evolution under maximum likelihood. One hundred likelihood optimizations were run for each model.

We also analysed a smaller 54-taxon data set, in which 11 terminals lacking reproductive data were removed

(Table 1) to test the potential effect of missing traits for some taxa. We reanalysed the reduced data set with the same models and optimality criterion in BayesTraits ver. 1.0.

Results

Phylogenetic reconstruction of the phylum Porifera

The total data set contained 62 sponge species and five outgroup taxa. The aligned small ribosomal subunit data set comprised 2222 characters, and the cytochrome *c* oxidase subunit I data set included 675 characters (Table 1). The phylogenetic analyses of the data set (a total of 2897 characters with 1249 invariable sites) analysed using maximum likelihood (ML) and Bayesian inference (BI) provided similar topologies (Fig. 2 and Figs S1 and S2). Using the protozoans *Capsaspora owzcarzaki* and *Monosiga brevicollis* as outgroups, the recovered topologies resulted in metazoan monophyly and poriferan paraphyly (Fig. 2), with the latter divided into four major clades: Hexactinellida, Calcarea, Homoscleromorpha and Demospongiae.

The earliest branching sponge group was Hexactinellida in both ML and BI analyses (Fig. 2), but the relationships among the three other major sponge clades were not recovered with robust support (Fig. 2). In the BI analysis, homoscleromorphs and calcareous sponges were sister groups (Figs S1 and S2).

Within Demospongiae, we recovered the four clades (G1–4) initially proposed by Borchiellini *et al.* (2004) and subsequently named by Cárdenas *et al.* (2012), with high support for both ML and BI (Fig. 2). Clades G1 (Keratosa) and G2 (i.e. subclass Verongimorpha, former Myxospongiae) were sister groups, although the relationship showed low support in both analyses (Fig. 2, Figs S1 and S2). G2 included members of the orders Verongida and Chondrosida. Chondrosida included the former order Halisarcida (see also Ereskovsky *et al.* 2011), with *Chondrosia reniformis* sister to the remaining species (Fig. 2). Clade G3 comprising the former order Haplosclerida (referred to as Haplosclemorpha in Cárdenas *et al.* 2012) was found to be the sister group of clade G4 (referred to as Heteroscleromorpha in Cárdenas *et al.* 2012) (Fig. 2). The latter comprised six monophyletic groups, reflecting only partially the traditional demosponge orders (Agelasida, Astrophorida, Hadromerida, Halichondrida and Poecilosclerida), and

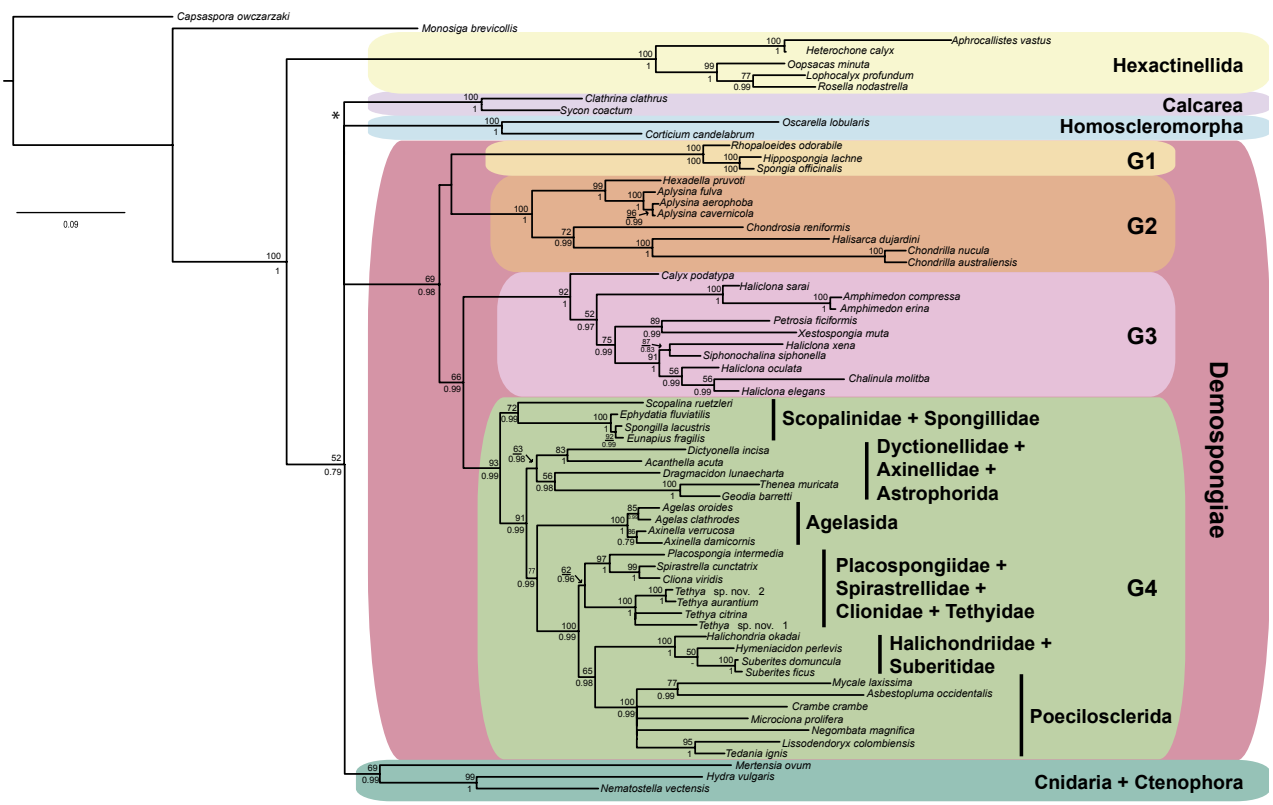

Fig. 2 Strict consensus tree of the maximum likelihood and Bayesian inference phylogenetic analyses using the combined data set (18S rRNA and COI genes). Bootstrap support values are shown over the branches and posterior probabilities below. Asterisk indicates a sister group relationship between Calcarea and Homoscleromorpha recovered with a *pp* = 0.82 with Bayesian inference.

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corresponded to the clades obtained by Morrow *et al.* (2012). The first clade comprised the species *Scopalina ruetzleri* (family Scopalinidae, order Halichondrida) and three species of the family Spongillidae (Fig. 2), which were sister groups (this clade corresponds to clades C13 and C14 in Morrow *et al.* 2012). The following clade contained species of the orders Halichondrida (families Dictyonellidae and Axinellidae) and Astrophorida (families Geodiidae and Pachastrellidae) and corresponds to the clade containing C9, C10 and C11 in Morrow *et al.* (2012). Nevertheless, it should be kept in mind that Halichondrida and Astrophorida are very large and internally diverse taxa, which may have been underestimated in both our sampling and others previously published. The sequence corresponding to the axinellid *Dragmacidon lunaecharta* was found close to Astrophorida (Fig. 2). The order Agelasida clustered with some species that, prior to the molecular phylogenetic inference, had traditionally been considered in the genus *Axinella*, and now all together form a clade different from that of true axinidellids and equivalent to that containing C6 in Morrow *et al.* (2012). The families Tethyidae, Clionidae, Spirastrellidae and Placospongiidae (all belonging to the order Hadromerida, and recovered also in clade C3 in Morrow *et al.* 2012) clustered together in a clade sister to the 'Agelasida+ ex-axinellids' clade (Fig. 2). Finally, two other clades were recovered as sister groups: the first one comprised some species from the traditional orders Halichondrida (*Halichondria okadai* and *Hymeniacidon perlevis*) and Hadromerida (family Suberitidae: *Suberites domuncula* and *S. ficus*); the second consisted entirely of members of the order Poecilosclerida (Fig. 2). These two clades were also equivalent to clades C1 and C5 in Morrow *et al.* (2012), respectively.

Ancestral reconstruction of reproductive traits

Hypothesis 1. As bootstrap support values and posterior probabilities are low for deep nodes (relationships between the four Porifera classes and with the rest of metazoans), there remain grounds for contention over the ancestral stages inferred in the analyses for the sexual and reproductive conditions. According to the parsimony reconstruction, the ancestral states of sexual and reproductive conditions for metazoans were hermaphroditism and viviparity, respectively (Fig. 3). The maximum likelihood analysis was equivocal as to the ancestral state of sexuality for metazoans (Fig. 4), but recovered viviparity as the metazoan ancestral condition. For Porifera, hermaphroditism and viviparity were optimised as the ancestral state in the parsimony analysis (Figs 3 and 5). In the maximum likelihood reconstruction, both gonochorism and hermaphroditism showed equal probabilities as the ancestral sexual condition of sponges; however, regarding reproductive strategy, viviparity was unequivocally ancestral for the phylum (Fig. 4).

For demosponges (clade containing subclades G1–4), the reconstruction of gonochorism vs. hermaphroditism was unequivocal in the parsimony analysis, gonochorism being the ancestral character state (Fig. 3), also showing a slightly higher probability in the maximum likelihood reconstruction (Fig. 4). The analyses show that hermaphroditism was acquired independently by Halisarcidae, the members of Haplosclerida (excluding the petrosiids *P. ficiformis* and *X. muta* and also *Haliclona elegans*) and the subclades Scopalinidae, Clionidae, Suberitidae, and Poecilosclerida (Figs 3 and 4).

Viviparity was the exclusive condition found in Hexactinellida, Calcarea and Homoscleromorpha (Figs 3–5) and was favoured as the ancestral condition for Demospongiae in both maximum likelihood and parsimony analyses, in spite of the lability of this character in the internal nodes of Demospongiae (Figs 3–5). Oviparity was acquired independently in several groups: by clade G2, within clade G3 (here represented by the petrosiids *P. ficiformis* and *X. muta*), and at the base of the clade containing groups Axinellidae, Dyctionellidae, Astrophorida, Agelasida, Tethyidae, Clionidae, Spirastrellidae and Placospongiidae (Figs 2–4). The reproductive condition reverted to viviparity in Halichondriidae and Poecilosclerida (Figs 2–4). The viviparity of the species *Halisarca dujardini* (included in clade G2) was better explained as a reversion to the ancestral condition, whereas the strategy of Suberitidae (clade G4e) most parsimoniously fitted with a reversion to oviparity (Fig. 4).

Hypothesis 2. When we traced the ancestry of both characters on a tree topology enforcing sponge monophyly, hermaphroditism appeared as the ancestral sexual condition for metazoans using parsimony (Fig. 5) and gonochorism using maximum likelihood (Fig. 6). Regarding the ancestral reproductive strategy, the parsimony analysis was equivocal on the reconstruction of either oviparity or viviparity as ancestral (Fig. 5), while the maximum likelihood analysis favoured viviparity as ancestral (Fig. 6). Under parsimony, the ancestor of sponges was inferred to be hermaphroditic (Fig. 5), whereas early in the evolution of demosponges, sexuality switched to gonochorism (Fig. 5). Later in the evolution of demosponges, independent reversals to hermaphroditism occurred (in Poecilosclerida, Halisarcidae, Thoosidae, the halichondriid *Hymeniacidon perlevis*, and some petrosiids within Haplosclerida; Fig. 5). In contrast, under maximum likelihood, the ancestor of sponges was inferred to be gonochoristic, like the ancestor of demosponges (Fig. 6). Hermaphroditism was in turn acquired independently by Poecilosclerida, Halisarcidae, Thoosidae,

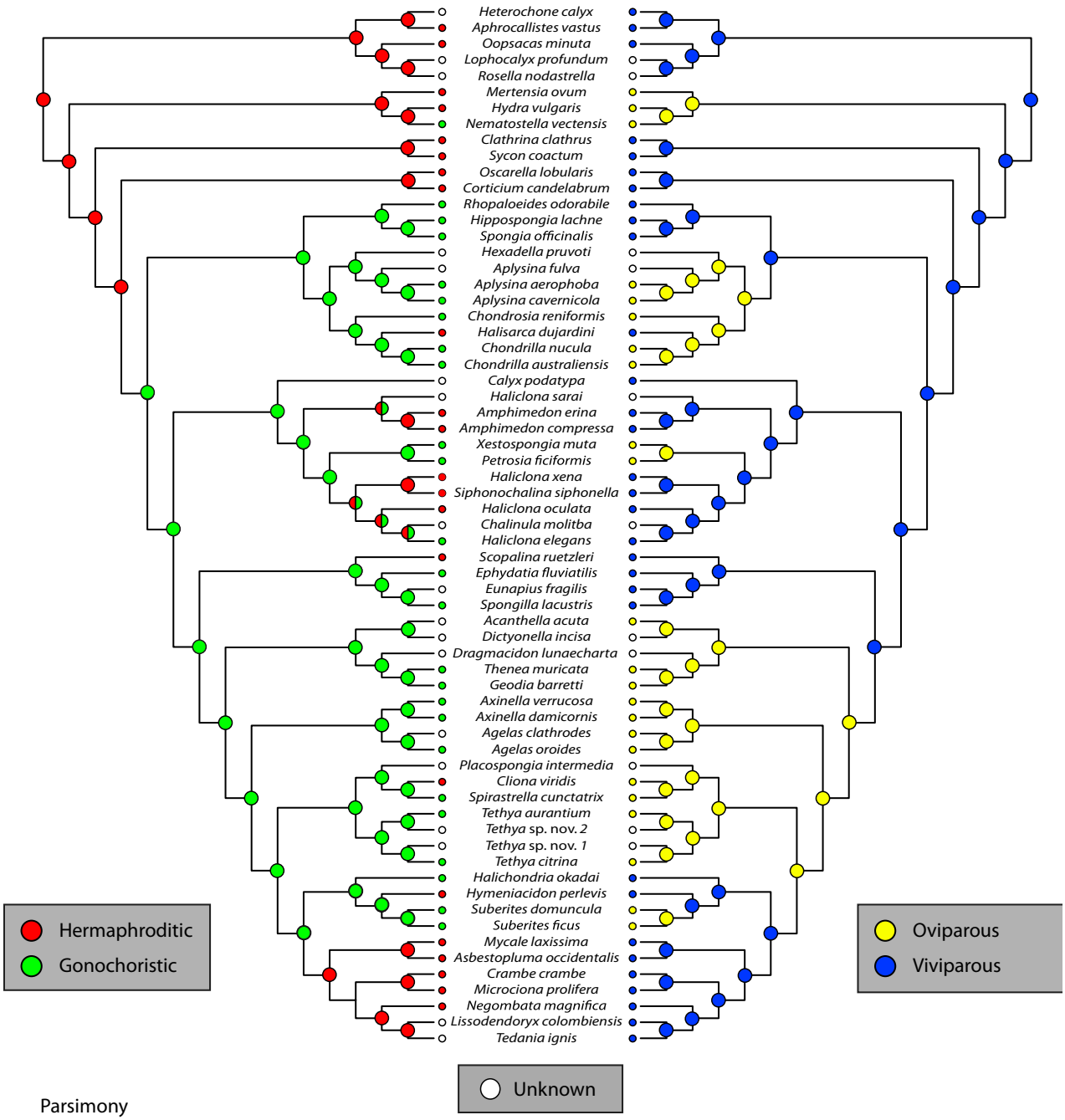

Fig. 3 Parsimony ancestral character-state reconstruction for both sexuality and reproductive condition.

the halichondriid *Hymeniacidon perlevis* and some petrosiids within Haplosclerida (Fig. 6).

Viviparity was reconstructed as the ancestral reproductive condition for sponges in both parsimony and maximum likelihood analyses. Independent acquisitions of oviparity occurred in the clade containing most G4 groups (Fig. 5), except in freshwater sponges, which remained viviparous (i.e. Spongillidae). Also, in clade G2, oviparity characterised two groups, Verongida and Chondrosida, but the analysis still rendered viviparity as the ancestral state (Fig. 5).

Statistical analysis of trait evolution

Under an unconstrained tree topology (Figs 3 and 4), the correlation test recovered significant association of the two

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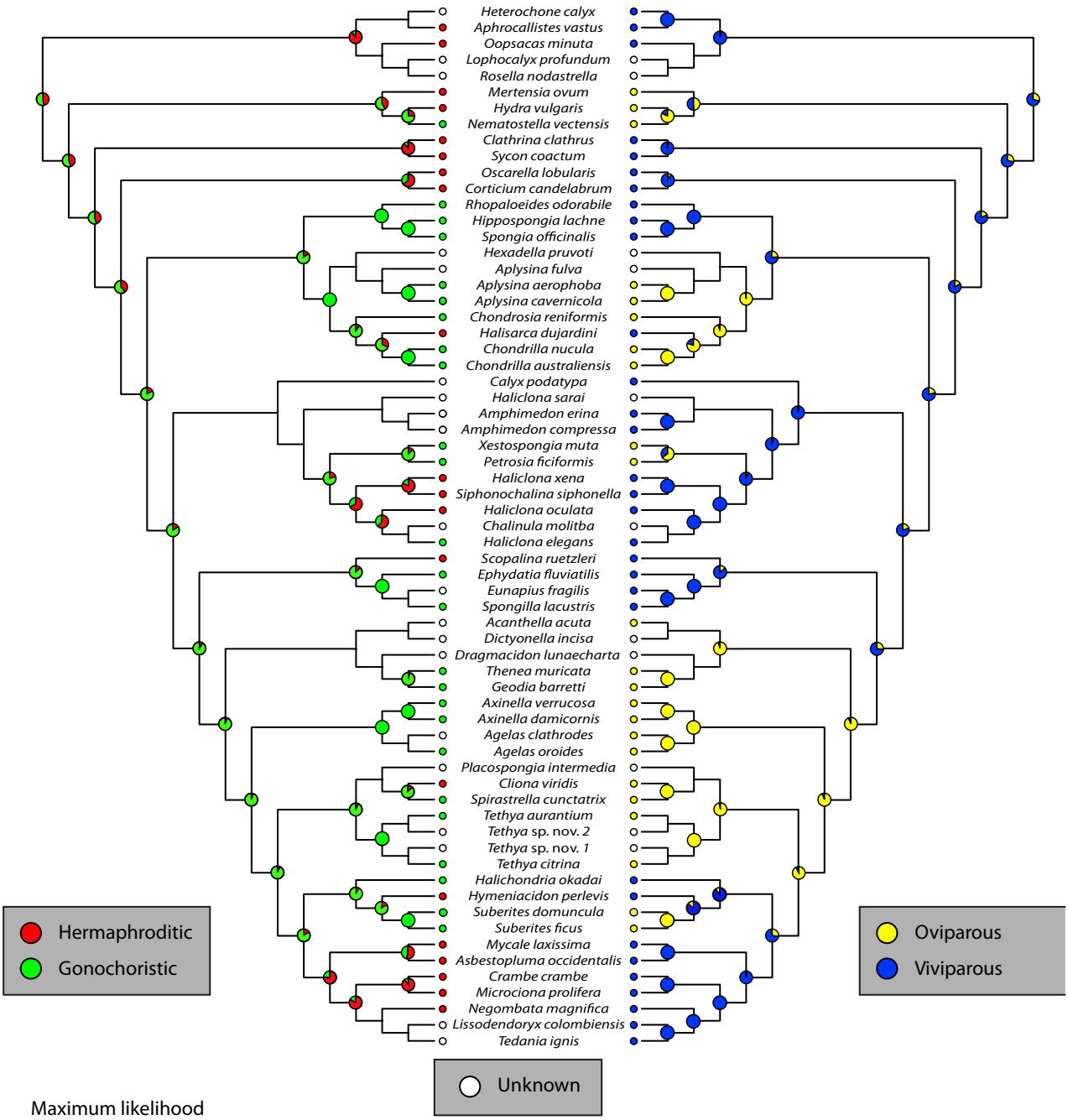

Fig. 4 Maximum likelihood ancestral character-state reconstruction for both sexuality and reproductive condition.

binary reproductive characters, hermaphroditism and viviparity and gonochorism and oviparity (likelihood ratio = 15.332; $P = 0.0040$). The reduced 54-taxon data set wherein reproductive data were available for at least one character in all terminals similarly favoured a significant correlation between the two traits (LR = 14.533; $P = 0.0057$).

The correlation was weakly upheld under an alternative tree topology (Figs 5 and 6) with enforced sponge monophyly (likelihood ratio = 8.741; $P = 0.0678$). However, exclusion of terminals with complete lack of reproductive character data recovered a strongly significant correlation (likelihood ratio = 16.770; $P = 0.0021$).

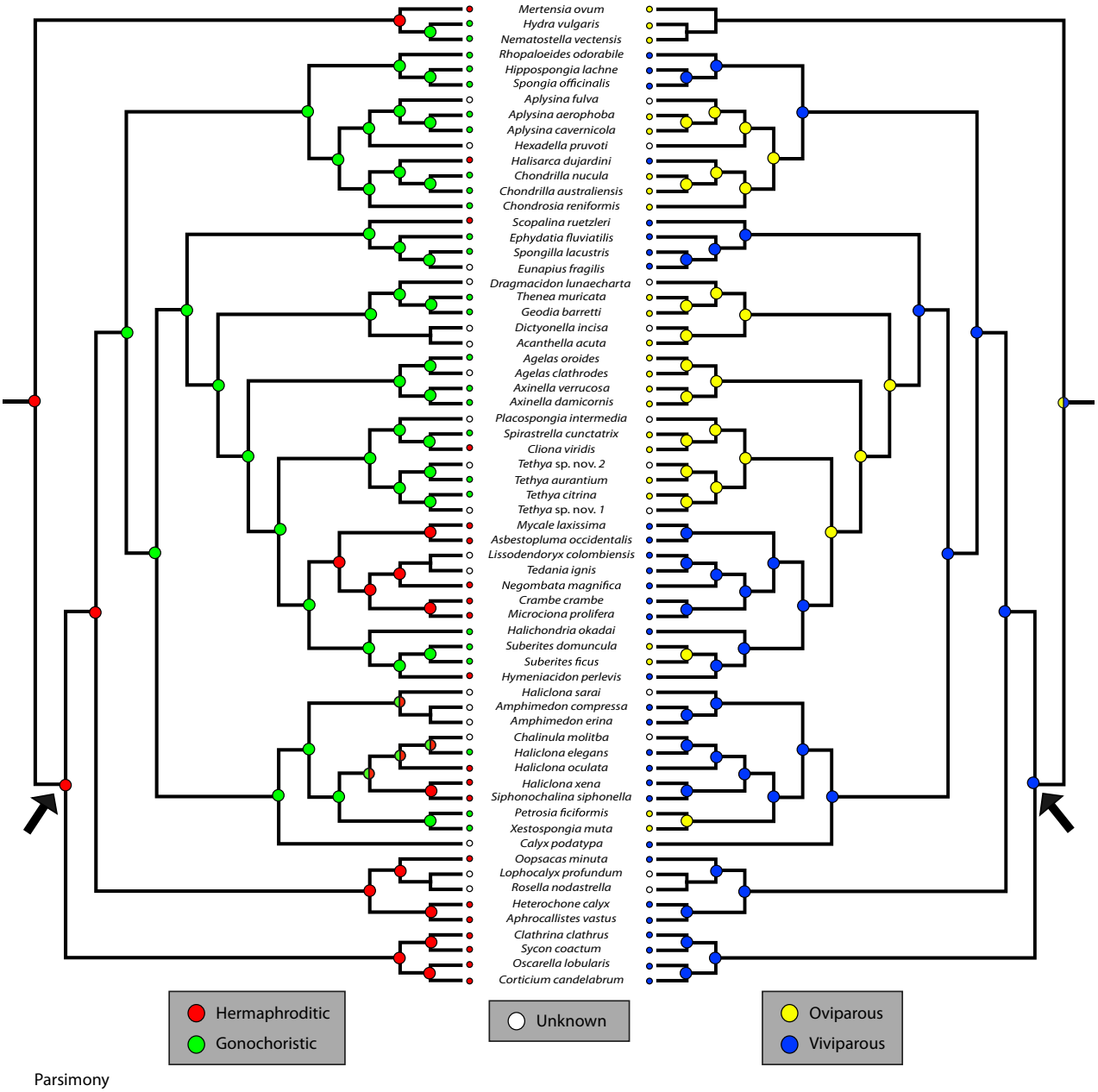

Fig. 5 Parsimony ancestral character-state reconstruction for both sexuality and reproductive condition on the monophyletic hypothesis obtained by constraining the monophyly of sponges (arrow) on the tree topology resulting from our maximum likelihood analysis (Figure S1).

Discussion

Insights into sponge phylogeny

Phylogenetic reconstruction for the phylum Porifera has proven difficult with both molecular and morphological characters (reviewed in Cárdenas *et al.* 2012; Wörheide *et al.* 2012). Both the position of Porifera within Metazoa and the relationships among the four major lineages of sponges are contingent upon different molecular markers and evolutionary models used to infer phylogeny (e.g. Borchiellini *et al.* 2001, 2004; Dunn *et al.* 2008; Lavrov *et al.* 2008; Hejnol *et al.* 2009; Philippe *et al.* 2009, 2011; Sperling *et al.* 2009; Pick *et al.* 2010; Nosenko *et al.* 2013). Sponge paraphyly has commonly been reported when using ribosomal (e.g. Borchiellini *et al.* 2001, 2004), nuclear housekeeping (e.g. Sperling *et al.* 2009) and mitochondrial markers (e.g. Lavrov *et al.* 2008). Sponge monophyly has

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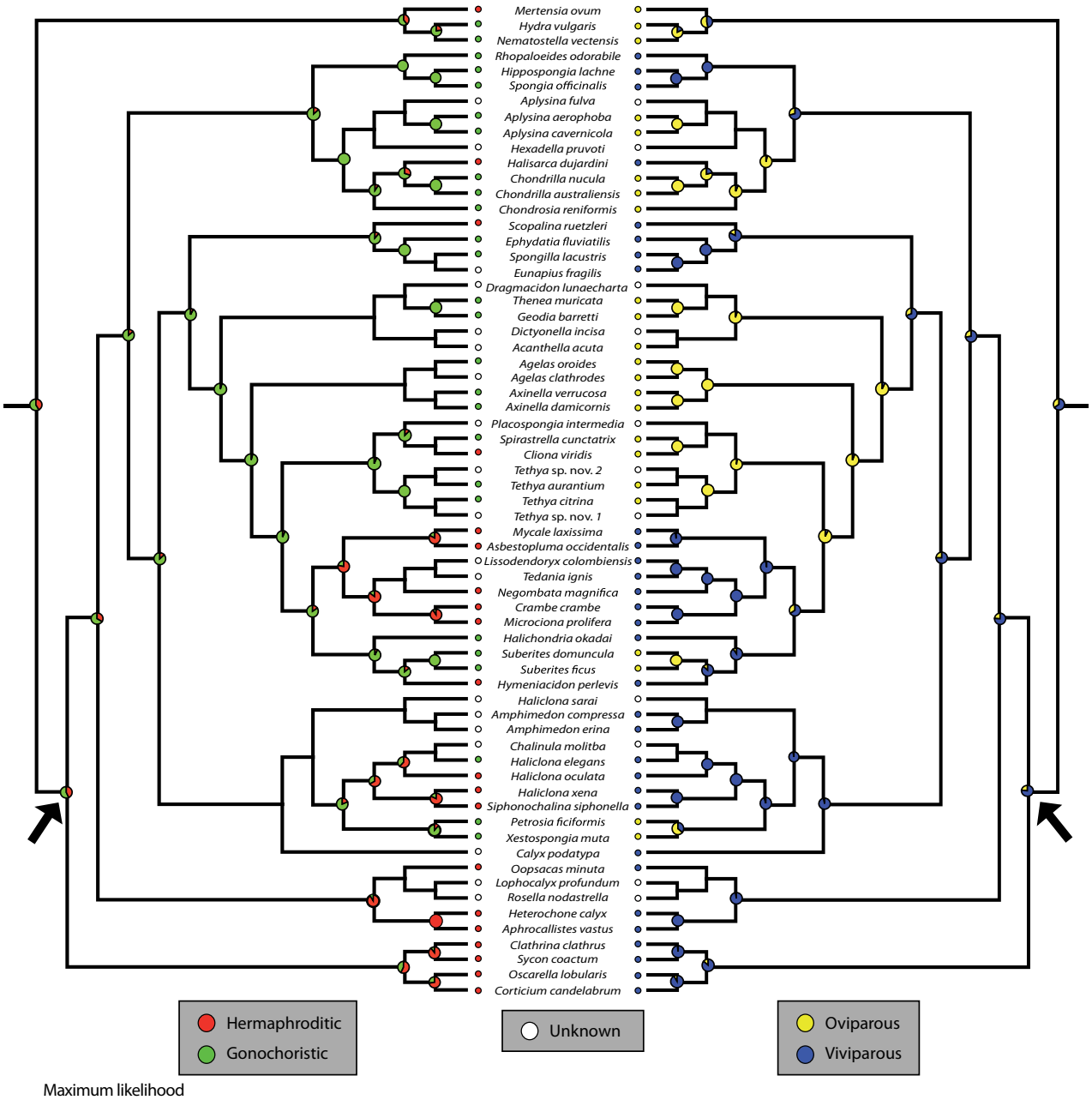

Fig. 6 Maximum likelihood ancestral character-state reconstruction for both sexuality and reproductive condition on the monophyletic hypothesis obtained by constraining the monophyly of sponges (arrow) on the tree topology resulting from our maximum likelihood analysis (Figure S1).

been recovered in recent analyses using protein-encoding genes for a large diversity of sponges (Philippe *et al.* 2009, 2011; Pick *et al.* 2010), but not in others (Dunn *et al.* 2008; Hejnol *et al.* 2009). It has recently been shown that even for the same dense taxon sampling, to conclude monophyly or paraphyly is a matter of a particular selection of genes, a specific choice of outgroup representatives and/or technical limitations for model choice (Nosenko *et al.* 2013). In our combined analysis using the concatenated alignments of 18S rRNA and COI, sponges were paraphyletic (Fig. 2)—as in prior studies exploring the same markers—and divided into four major clades: Hexactinellida, Calcarea, Homoscleromorpha and Demospongiae. It is important to note that this topology might reflect a long-branch attrac-

tion artefact, as neither our taxon sampling nor our selection of molecular markers was suited to resolve whether sponges are a monophyletic or paraphyletic taxon. Our approach was intended to place in a common phylogenetic context those sponge species whose reproductive biology had been investigated to date, to infer evolution of reproductive characters in the phylum and each of its main internal clades. To this end, we investigated character evolution using both an unconstrained topology and an alternative hypothesis whereupon the monophyly of Porifera was enforced (discussed below).

In the unconstrained phylogenetic analyses, Hexactinellida was recovered as the sister group to all other metazoans, including the rest of the sponges (Fig. 2), a result consistent with those by Borchiellini *et al.* (2001) using 18S rRNA and Belinky *et al.* (2012) using ALG11. The basal position of Hexactinellida seems to be driven by the signal obtained from the small ribosomal subunit (18S rRNA), characterised by insertions that form 1 or 2 helices, depending on the taxonomic order, that proved to be phylogenetically informative (Voigt *et al.* 2008). The exclusion of these insertions resulted in the recovery of a clade containing Hexactinellida and Dictyoceratida (data not shown), as also obtained by Belinky *et al.* (2012), a result with no morphological correspondence that has to be interpreted cautiously. Therefore, the position of Hexactinellida might be due to a long-branch attraction artefact caused by the gene selection strategy (see Nosenko *et al.* 2013), which could not be resolved using the data sets obtained for our analyses. The clade Calcarea + Homoscleromorpha was recovered in our BI analysis, but without enough support (pp = 0.82; Figs S1 and S2). A potential synapomorphy for this clade is the presence of two paralogous genes for the protein collagen type IV (Boute *et al.* 1996; Leys & Riesgo 2012), which initially was thought to occur only in eumetazoans.

Within Demospongiae, we recovered the four clades (G1–4) initially proposed by Borchiellini *et al.* (2004) and subsequently reported in several partial reconstructions of the class (Nichols 2005; Sperling *et al.* 2009; Erpenbeck *et al.* 2012b; Morrow *et al.* 2012). In clade G3 (comprising the order Haplosclerida; Borchiellini *et al.* 2004), even though the internal relationships do not correspond to the traditional haplosclerid suborders (see also McCormack *et al.* 2002; Raleigh *et al.* 2007; Redmond *et al.* 2007, 2011; Redmond & McCormack 2008), we found a well-supported internal clade comprised of two species of petrosiids (*Petrosia ficiformis* and *Xestospongia muta;* belonging to the same suborder within Haplosclerida), both gonochoristic and oviparous, whereas the rest of the haplosclerids are viviparous (Fig. 2 and Table 1).

Within clade G4, the basally branching clade containing species from the families Scopalinidae (traditionally within Halichondrida and related to dictyonellids) and Spongillidae (traditionally considered freshwater Haplosclerida) remains puzzling from a morphological point of view, but it has been reported recurrently when ribosomal and mitochondrial data sets are analysed (Nichols 2005; Erpenbeck *et al.* 2007a,b, 2012a,b; Redmond *et al.* 2007; Voigt *et al.* 2008; Morrow *et al.* 2012). Such an intriguing result refutes the traditional view of a relationship between Spongillidae and Haplosclerida, presumed on the basis of skeletal features, a relationship that, on the other side, appears less likely when reproductive features are also considered. The sperm morphology of Spongillidae (e.g. Efremova & Papkovskaya 1980; Paulus & Weissenfels 1986; Paulus 1989) differs greatly from that of the only species of marine Haplosclerida described at the ultrastructural level, *Petrosia ficiformis* (Maldonado and Riesgo, 2009), and presents characters similar to those found in the poecilosclerid *Crambe crambe*, such as the intracellular ciliary channel (Riesgo & Maldonado 2009). However, there are no data on the ultrastructure of the sperm of Scopalinidae, and this character could be of importance to shed light on the true affinity of the group, although the structure of the spermatozoa in many cases reflects adaptive needs and not phylogenetic history (e.g. McHugh & Rouse 1998).

The relationships we found between the remaining subclades of G4 have also been recovered before in several analyses (Chombard & Boury-Esnault 1999; McCormack & Kelly 2002; Erpenbeck *et al.* 2005, 2006, 2012a,b; Nichols 2005; Kober & Nichols 2007; Gazave *et al.* 2010; Morrow *et al.* 2012). Unfortunately, we were not able to include the family Raspailiidae, which clustered with the families Stelligeridae and true axinellids (Axinellidae) in previous analyses (Erpenbeck *et al.* 2007b; Morrow *et al.* 2012), disrupting the monophyly of Poecilosclerida, as recently defined in the Systema Porifera (Hooper & van Soest 2002). This relationship of Raspailiidae and axinellids (*sensu lato*), already vindicated from morphological sponge classifications during most of the 20th century (e.g. Lévi 1973), is also supported from the reproductive point of view. All known poecilosclerids are hermaphroditic and viviparous (except for *Neofibularia nolitangere;* see Hoppe and Reichert, 1987), whereas the few investigated members of Raspailiidae are gonochoristic and oviparous (Riesgo & Maldonado 2008), as are some former axinellids that have recently been transferred to Agelasida by molecular studies

Ancestral state reconstruction of reproductive traits

Hermaphroditism was recovered as the ancestral sexual state of Porifera in the parsimony analyses, irrespective of sponge monophyly or paraphyly. However, the likelihood analysis in the paraphyletic hypothesis was equivocal, ren-

dering the ancestry unresolved, while in the monophyletic hypothesis gonochorism appeared as ancestral. The few reproductive data available for hexactinellids indicate a hermaphroditic and viviparous condition (see Leys *et al.* 2007). Given that hexactinellids predominantly inhabit deep waters, their reproductive dynamics are poorly studied. Both Calcarea and Homoscleromorpha are also hermaphrodites and viviparous, and their reproduction is comparatively better understood than that of hexactinellids (for reviews see Fell 1983; Simpson 1984; Maldonado & Riesgo 2008; Ereskovsky 2010). Nearly, all studies are consistent on the reproductive traits of homoscleromorphs (for conflicting results on the homoscleromorph *Oscarella tuberculata* see Meewis, 1938; Tuzet and Paris, 1964). For demosponges, the analyses agreed in gonochorism being ancestral in the group, under both parsimony and maximum likelihood reconstructions and regardless the topology (paraphyletic/monophyletic sponges) used.

Hermaphroditism has preponderantly been considered the ancestral state for metazoans, and this has been justified using models of evolutionary ecology (see Ghiselin 1969 for details). Among these models, the low-density model, which appears to predict the hermaphroditic condition among sessile animals, invokes the advantage of being able to mate with other members of the population when the probability of encountering a suitable mate is low (Tomlinson 1966; Ghiselin 1969). Testing whether the ancestral metazoan was sessile, and therefore if the low-density model applied to it, is beyond the scope of this study. But the explanation given by Ghiselin fits well with the biology of current sponges, given their lack of motility as adults and concomitant low probability of directly encountering a member of the opposite sex.

In our analysis, sexuality was observed to be homoplastic. Indeed, Simpson (1984) suggested that sex determination in sponges might be a labile character, and most likely not determined genetically but physiologically. This view was mostly driven by the few findings of hermaphroditic individuals among gonochoristic populations of sponges, and also gonochoristic individuals in hermaphroditic species (see Simpson 1984; for a review). In *Halichondria* sp. (Fell and Jacob, 1979), a whole population was gonochoristic 1 year and mostly hermaphroditic the following year. Additionally, *Spongilla lacustris*, a dioecious species, experienced sex reversals when colonising a new habitat (Gilbert and Simpson, 1976), and *Halisarca dujardini* has been described both as hermaphroditic (Chen 1976) and gonochoristic (Ereskovsky 2000). It has thus been conjectured that inconsistencies in sex determination might be caused by adaptation to new habitats during colonisations, and therefore, sexual determination might be influenced by sex ratio (Gilbert and Simpson, 1976).

The reproductive condition only varied among demosponges, and many assumptions regarding the diversity of sexual reproduction strategies in sponges were based on this demosponge variability; however, in Hexactinellida, Calcarea and Homoscleromorpha, viviparity is the only reproductive condition known to date. Given that this character appeared to be more variable among demosponge orders, Lévi (1956) used it, along with other features, to define two demosponge subclasses Ceractinomorpha and Tetractinomorpha (viviparous vs. oviparous, respectively). However, these subclasses are no longer sustainable, for they showing internal inconsistencies in character distribution. In general, it appears that the reproductive mode is a homoplastic character, which disfavours its utility for phylogenetic reconstruction. Current demosponge classification is built upon four major clades (G1–4), all of which were recovered in our phylogenetic analyses. Viviparity was the ancestral condition for demosponges regardless of the hypothesis or analysis method. Oviparity appears to have been acquired independently by the petrosiids in clade G3 and the clades containing the groups Axinellidae, Dyctionellidae, Astrophorida, Agelasida, Tethyidae, Clionidae, Spirastrellidae and Placospongiidae. The reproductive condition would have been reversed again to viviparity in Halichondriidae and Poecilosclerida and in the hadromerid *Stylocordyla borealis* (Sarà et al., 2002). These results are in agreement with previous partial reconstructions of the origin of the reproductive condition in demosponges (Borchiellini *et al.* 2004; Cárdenas *et al.* 2012).

Correlated evolution of sexual mode and reproductive condition

Hermaphroditism and viviparity as well as gonochorism and oviparity were strongly correlated in sponges, as it has been previously demonstrated for cnidarians (Kerr *et al.* 2011). This result was found to be largely insensitive to topological constraint or gaps in reproductive character data. Hypotheses on the evolution of oviparity and viviparity have been postulated for many marine invertebrates (e.g. Thorson 1946; Menge 1975; McHugh & Rouse 1998) and also vertebrates (e.g. Andrews 2002). When investigating the incidence of particular reproductive strategies, it is imperative to consider a complex of interrelated factors, such as nutrient and oxygen availability, morphological traits, temporal and spatial settlement patterns, mortality rates of offspring and interactions between closely related species. The knowledge required for conclusive hypothesis testing of ecological or evolutionary advantages of each reproductive condition is far from complete in sponges. In other animals, ecological advantages of brooding (i.e. viviparity) have been conjectured for small-sized individuals, for example. echinoderms and molluscs (see Menge 1975;

Strathmann & Strathmann 1982; Hart *et al.* 1997). Strathmann (1986) suggested that colonies or animals with high surface areas are preadapted for retention and ventilation of brooded embryos, whereas brooding is less often observed in large solitary animals. Indeed, oxygen is a limiting factor for egg masses and embryos of marine invertebrates. For instance, brooding female crabs adopt embryo ventilation strategies not found in non-brooding species (Fernández *et al.*, 2000). The need to ventilate brooded embryos might explain why thin encrusting sponges, like the poecilosclerid *Crambe crambe*, with large surface area, are more often viviparous. Furthermore, hexactinellid, calcareous, and homoscleromorph sponges, which are usually thin and/or bear a substantial aquiferous system and lax mesohyls—permitting facile oxygenation of embryos—are exclusively viviparous. It is also important to note that other factors, such as phylogenetic history, should be considered as well. Nevertheless, it may be kept in mind that surface area for oxygen exchange is more related to the internal branching structure of the aquiferous system (often unknown) than to the external body surface. For instance, *Spirastrella cunctatrix* is an oviparous species, but its body thickness does not differ from that of viviparous sponges such as *Crambe crambe*. In contrast, very thick mesohyls and/or more limited aquiferous systems, among other factors, might have led to oviparity in some cases, such as in Petrosiidae (here represented by *Xestospongia muta* and *Petrosia ficiformis*), Axinellidae, Dyctionellidae, Astrophorida, Clionidae and Tethyidae, as well as in the orders Chondrosida and Verongida. Also, the poecilosclerid *Neofibularia nolitangere* is reported to be gonochoristic and oviparous (Hoppe & Reichert 1987). But *N. nolitangere* has not always been placed within Poecilosclerida (Hajdu & van Soest 2002), and recent molecular analyses by Erpenbeck *et al.* (2007a) have also placed the genus *Neofibularia* in a non-poecilosclerid clade, along with some clionids (Hadromerida) and the genus *Svenzea* (Halichondrida). Likewise, analyses by Mitchell *et al.* (2011) placed the closely related genera *Neofibularia* and *Biemna* outside of Poecilosclerida and related to the halichondrid lineage. Therefore, the 'exceptional' reproductive pattern of *Neofibularia* within Poecilosclerida may be either a real exception or a taxonomic misplacement. In turn, the appearance of viviparity in sponges possessing a thick mesohyl (like those in the orders Dendroceratida and Dictyoceratida) is conflicting with our hypothesis. However, viviparity in these groups might be explained by the large aquiferous system often observed in their members.

Conclusion

Competing hypotheses of sponge phylogeny both favour character-state reconstructions wherein hermaphroditism and viviparity were ancestral for poriferans, with several independent acquisitions of gonochorism and oviparity in different clades (Figs 3–5), and a few subsequent reversals to the ancestral condition in others. The evolutionary history of the two labile characters is strongly correlated, likely due to comparable ecological pressures on different sponge lineages.

Acknowledgements

We are indebted to Joana Xavier, Micha Ilan, Giuseppe Corriero, Carlo Cerrano, Jane Fromont, Steve Whalan, Joe López, Andia Chaves-Fonnegra, Sally Leys and Alicia R. Pérez-Porro for providing samples for the study; Iosune Uriz, Sergio Taboada, Xavier Turon, Joan Mora and Vanessa González for help in the field; Kylee Jane Clatt, Sónia Andrade, Erin McIntyre and Gisele Kawauchi for laboratory assistance; Albert Barberán for help with statistical analyses. We also acknowledge two anonymous reviewers and the editor for suggestions that helped to improve this article. A.R. was supported by a Marie Curie Outgoing Fellowship (contract number 237219), M.N. by a fellowship from the Fundación Caja Madrid, and M.M. by Grant BFU2008-00227/BMC. Sequencing costs were covered by internal funds from the Museum of Comparative Zoology and the Faculty of Arts and Sciences, Harvard University.

References

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped blast and psi--blast: A new generation of protein database search programs. *Nucleic Acids Research*, *25*, 3389–3402.

Andrews, R. M. (2002). Low oxygen: a constraint in the evolution of viviparity in reptiles. *Physiological and Biochemical Zoology*, *75*, 145–154.

Barrett, S. C. H. (2002). The evolution of plant sexual diversity. *Nature Reviews Genetics*, *3*, 274–284.

Belinky, F., Szitenberg, A., Goldfarb, I., Feldstein, T., Wörheide, G., Ilan, M. & Huchon, D. (2012). Alg11 - a new variable DNA marker for sponge phylogeny: Comparison of phylogenetic performances with the 18S and the COI gene. *Molecular Phylogenetics and Evolution*, *63*, 702–713.

Bernstein, H., Byers, G. S. & Michod, R. D. (1981). Evolution of sexual reproduction: importance of DNA repair, complementation, and variation. *The American Naturalist*, *117*, 537–549.

Borchiellini, C., Manuel, M., Alivon, E., Boury-Esnault, N., Vacelet, J. & Le Parco, Y. (2001). Sponge paraphyly and the origin of Metazoa. *Journal of Evolutionary Biology*, *14*, 171–179.

Borchiellini, C., Chombard, C., Manuel, M., Alivon, E., Vacelet, J. & Boury-Esnault, N. (2004). Molecular phylogeny of Demospongiae: Implications for classification and scenarios of character evolution. *Molecular Phylogenetics and Evolution*, *32*, 823–837.

Boute, N., Exposito, J.-Y., Boury-Esnault, N., Vacelet, J., Noro, N., Miyazaki, K., Yoshizato, K. & Garrone, R. (1996). Type IV collagen in sponges, the missing link in basement membrane ubiquity. *Biology of the Cell*, *88*, 37–44.

Cárdenas, P., Pérez, T. & Boury-Esnault, N. (2012). Sponge systematics facing new challenges. *Advances in Marine Biology*, *61*, 79–182.

Chen, T. W. (1976). Reproduction and speciation in *Halisarca*. In F. W. Harrison & R. R. Cowden (Eds) *Aspects of Sponge Biology* (pp. 113–132). New York: Academic Press.

Chombard, C. & Boury-Esnault, N. (1999). Good congruence between morphology and molecular phylogeny of hadromerida, or how to bother sponge taxonomists. *Memoirs of Queensland Museum*, *44*, 100.

Dunn, C. W., Hejnol, A., Matus, D. Q., Pang, K., Browne, W. E., Smith, S. A., Seaver, E. C., Rouse, G. W., Obst, M., Edgecombe, G. D., Sørensen, M. V., Haddock, S. H. D., Schmidt-Rhaesa, A., Okusu, A., Kristensen, R. M., Wheeler, W. C., Martindale, M. Q. & Giribet, G. (2008). Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature*, *452*, 745–749.

Edgar, R. C. (2004). Muscle: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*, 1792–1797.

Efremova, S. & Papkovskaya, M. V. (1980). Spermatogenesis of the sponge *Lubomirskia baicalensis* (Pallas). Ultrastructural investigation. *Arkiv Anatomii, Gistologii i Ehmbriologii*, *12*, 88–95.

Ereskovsky, A. (2000). Reproduction cycles and strategies of the cold-water sponges *Halisarca dujardini* (Demospongiae, Halisarcida), *Myxilla incrustans* and *Iophon piceus* (Demospongiae, Poecilosclerida) from the White Sea. *Biological Bulletin*, *198*, 77–87.

Ereskovsky, A. (2010). *The Comparative Embryology of Sponges*. Dordrecht: Springer.

Ereskovsky, A., Lavrov, D. V., Boury-Esnault, N. & Vacelet, J. (2011). Molecular and morphological description of a new species of *Halisarca* (Demospongiae: Halisarcida) from Mediterranean Sea and a redescription of the type species *Halisarca dujardini*. *Zootaxa*, *2768*, 5–31.

Erpenbeck, D., Breeuwer, J. A. J. & van Soest, R. W. M. (2005). Implications from a 28S rRNA genes fragment for the phylogenetic relationships of halichondrid sponges (Porifera: Demospongiae). *Journal of Zoological Systematics*, *43*, 93–99.

Erpenbeck, D., Breeuwer, J. A. J., Parra-Velandia, F. J. & van Soest, R. W. M. (2006). Speculation with spiculation?—three independent gene fragments and biochemical characters versus morphology in demosponge higher classification. *Molecular Phylogenetics and Evolution*, *38*, 293–305.

Erpenbeck, D., Duran, S., Rützler, K., Paul, V., Hooper, J. N. A. & Wörheide, G. (2007a). Towards a DNA taxonomy of Caribbean demosponges: A gene tree reconstructed from partial mitochondrial CO1 gene sequences supports previous rDNA phylogenies and provides a new perspective on the systematics of Demospongiae. *Journal of the Marine Biological Association of the United Kingdom*, *87*, 1563–1570.

Erpenbeck, D., List-Armitage, S., Alvarez, B., Degnan, B. M., Wörheide, G. & Hooper, J. N. A. (2007b). The systematics of Raspailiidae (Demospongiae: Poecilosclerida: Microcionina) re-analysed with a ribosomal marker. *Journal of the Marine Biological Association of the United Kingdom*, *87*, 1571–1576.

Erpenbeck, D., Hall, K., Alvarez, B., Büttner, G., Sacher, K., Schätzle, S., Schuster, A., Vargas, S., Hooper, J. N. A. & Wörheide, G. (2012a). The phylogeny of halichondrid demosponges: Past and present re-visited with DNA-barcoding data. *Organisms, Diversity, and Evolution*, *12*, 57–70.

Erpenbeck, D., Sutcliffe, P., Cook, S. d. C., Dietzel, A., Maldonado, M., van Soest, R. W. M., Hooper, J. N. A. & Wörheide, G. (2012b). Horny sponges and their affairs: On the phylogenetic relationships of keratose sponges. *Molecular Phylogenetics and Evolution*, *63*, 809–816.

Fell, P. E. (1974). Porifera. In A. C. Giese & J. S. Pearse (Eds) *Reproduction of Marine Invertebrates: Acoelomate and Pseudocoelomate Metazoans* (pp. 51–132). New York: Academic Press.

Fell, P. E. (1983). Porifera. In K. G. Adiyodi & R. G. Adiyodi (Eds) *Reproductive Biology of Invertebrates i: Oogenesis, Oviposition and Oosorption* (pp. 1–29). Chichester: John Wiley and Sons.

Fell, P. E. & Jacob, W. F. (1979). Reproduction and development of *Halichondria* sp. in the Mystic estuary, Connecticut. *Biological Bulletin*, *156*, 62–75.

Fernández, M., Bock, C. & Pörtner, H.-O. (2000). The cost of being a caring mother: the ignored factor in the reproduction of marine invertebrates. *Ecology Letters*, *3*, 487–494.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*, 294–299.

Gazave, E., Carteron, S., Chenuil, A., Richelle-Maurer, E., Boury-Esnault, N. & Borchiellini, C. (2010). Polyphyly of the genus *Axinella* and of the family Axinellidae (Porifera: Demospongiae). *Molecular Phylogenetics and Evolution*, *57*, 35–47.

Ghiselin, M. T. (1969). The evolution of hermaphroditism among animals. *Quarterly Review of Biology*, *44*, 189–208.

Gilbert, J. J. & Simpson, T. L. (1976). Sex reversal in a freshwater sponge. *Journal of Experimental Zoology*, *195*, 145–151.

Haag, E. S. (2007). Why two sexes? Sex determination in multicellular organisms and protistan mating types *Seminars in Cell & Developmental Biology*, *18*, 348–349.

Hajdu, E. & van Soest, R. W. M. (2002). Family Desmacellidae Ridley and Dendy, 1886. In J. N. A. Hooper & R. W. M. van Soest (Eds) *Systema Porifera* (pp. 642–650). Kluwer Academic/Plenum Publishers, New York.

Hart, M. W., Byrne, M. & Smith, M. J. (1997). Molecular phylogenetic analysis of life-history evolution in asterinid starfish. *Evolution*, *51*, 1848–1861.

Heath, D. J. (1977). Simultaneous hermaphroditism; cost and benefit. *Journal of Theoretical Biology*, *64*, 363–373.

Heath, D. J. (1979). Brooding and the evolution of hermaphroditism. *Journal of Theoretical Biology*, *81*, 151–155.

Hejnol, A., Obst, M., Stamatakis, A., Ott, M., Rouse, G. W., Edgecombe, G. D., Martinez, P., Baguñà, J., Bailly, X., Jondelius, U., Wiens, M., Müller, W. E. G., Seaver, E., Wheeler, W. C., Martindale, M. Q., Giribet, G. & Dunn, C. W. (2009). Assessing the root of bilaterian animals with scalable phylogenomic methods. *Proceedings of the Royal Society B: Biological Sciences*, *276*, 4261–4270.

Heller, J. (1993). Hermaphroditism in molluscs. *Biological Journal of the Linnean Society*, *48*, 19–42.

Hooper, J. N. A. & van Soest, R. W. M. (2002). *Systema Porifera*. New York: Kluwer Academic/Plenum Publishers.

Hoppe, W. F. & Reichert, J. M. (1987). Predictable annual mass release of gametes by the coral reef sponge *Neofibularia nolitangere* (Porifera: Demospongiae). *Marine Biology*, *94*, 277–285.

Huelsenbeck, J. P. & Ronquist, F. (2005). Bayesian analysis of molecular evolution using mrbayes. In *Statistical Methods in Molecular Evolution* (pp. 183–226). New York: Springer.

Kerr, A. M., Baird, A. H. & Hughes, T. P. (2011). Correlated evolution of sex and reproductive mode in corals (Anthozoa: Scleractinia). *Proceedings of the Royal Society B: Biological Sciences*, *278*, 75–81.

Kober, M. K. & Nichols, S. A. (2007). On the phylogenetic relationships of hadromerid and poecilosclerid sponges. *Journal of the Marine Biological Association of the United Kingdom*, *87*, 1585–1598.

Lavrov, D. V., Wang, X. & Kelly, M. (2008). Reconstructing ordinal relationships in the demospongiae using mitochondrial genomic data. *Molecular Phylogenetics and Evolution*, *49*, 111–124.

Lévi, C. (1956). Étude des *Halisarca* de roscoff. Embryologie et systématique des démosponges. *Archives de zoologie expérimentale et générale*, *93*, 1–181.

Lévi, C. (1973). Systématique de la classe des demospongiaria (démosponges). In P. P. Grassé (Ed.) *Traité de Zoologie. Anatomie, Systématique, Biologie. Tome iii. Fascicule i. Spongiaires* (pp. 133–461). Paris: Masson et Cie.

Lewis, M. E. (2001). A probabilistic analysis of bias optimality in unchain Markov decision processes. *IEEE Transactions on Automatic Control*, *46*, 96–100.

Leys, S. P. & Riesgo, A. (2012). Epithelia, an evolutionary novelty of metazoans. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *38*, 438–447.

Leys, S. P., Mackie, G. O. & Reiswig, H. M. (2007). The biology of glass sponges. *Advances in Marine Biology*, *52*, 1–145.

Maddison, W. P. & Maddison, D. R. (2011). *Mesquite: A Modular System for Evolutionary Analysis. Version 2.75*. Available via http://mesquiteproject. org/mesquite/download/download.html

Maldonado, M. & Bergquist, P. R. (2002). Phylum Porifera. In C. M. Young, M. A. Sewell & M. E. Rice (Eds) *Atlas of Marine Larvae* (pp. 21–50). San Diego: Academic Press.

Maldonado, M. & Riesgo, A. (2008). Reproduction in the phylum Porifera: A synoptic overview. *Treballs de la Societat Catalana de Biologia*, *59*, 29–49.

Maldonado, M. & Riesgo, A. (2009). Gametogenesis, embryogenesis, and larval features of the oviparous sponge *Petrosia ficiformis* (Haplosclerida, Demospongiae). *Marine Biology*, *156*, 2181–2197.

Malik, S.-B., Pightling, A. W., Stefaniak, L. M., Schurko, A. M. & Logsdon, J. M. Jr (2008). An expanded inventory of conserved meiotic genes provides evidence for sex in *Trichomonas vaginalis*. *PLoS ONE*, *3*, e2879.

McCormack, G. P. & Kelly, M. (2002). New indications of the phylogenetic affinity of *Spongosorites suberitoides* Diaz et al., 1993 (Porifera, Demospongiae) as revealed by 28S ribosomal DNA. *Journal of Natural History*, *36*, 1009–1021.

McCormack, G. P., Erpenbeck, D. & van Soest, R. W. M. (2002). Major discrepancy between phylogenetic hypotheses based on molecular data and morphological criteria within the order Haplosclerida (phylum Porifera: class Demospongiae). *Journal of Zoology Systematics and Evolutionary Research*, *40*, 237–240.

McFadden, C. S., Donahue, R., Hadland, B. K. & Weston, R. (2001). A molecular phylogenetic analysis of reproductive trait evolution in the soft coral genus *Alcyonium*. *Evolution*, *55*, 54–67.

McHugh, D. & Rouse, G. W. (1998). Life history evolution of marine invertebrates: New views from phylogenetic systematics. *Trends in Ecology & Evolution*, *13*, 182–186.

Meewis, H. (1938). Contribution à l'étude de l'embryogenèse des Myxospongiae: *Halisarca lobularis* (Schmidt). *Archives du Biologie de Liége*, *59*, 1–66.

Menge, B. A. (1975). Brood or broadcast? The adaptive significance of different reproductive strategies in the two intertidal sea stars Leptasterias hexactis and Pisaster ochraceus. *Marine Biology*, *31*, 87–100.

Mitchell, K. D., Hall, K. A. & Hooper, J. N. A. (2011). A new species of *Sigmaxinella* Dendy, 1897 (Demospongiae, Poecilosclerida, Desmacellidae) from the Tasman Sea. *Zootaxa*, *2901*, 19–34.

Morrow, C. C., Picton, B. E., Erpenbeck, D., Boury-Esnault, N., Maggs, C. A. & Allcock, A. L. (2012). Congruence between nuclear and mitochondrial genes in Demospongiae: A new hypothesis for relationships within the G4 clade. *Molecular Phylogenetics and Evolution*, *62*, 174–190.

Nichols, S. A. (2005). An evaluation of support for the order-level monophyly and interrelationships within the class Demospongiae using partial data from the large subunit rDNA and cytochrome oxidase subunit I. *Molecular Phylogenetics and Evolution*, *34*, 81–96.

Nosenko, T., Schreiber, F., Adamska, M., Adamski, M., Eitel, M., Hammel, J., Maldonado, M., Muller, W. E. G., Nickel, M., Schierwater, B., Vacelet, J., Wiens, M. & Worheide, G. (2013). Deep metazoan phylogeny: When different genes tell different stories. *Molecular Phylogenetics and Evolution*, *67*, 223–233.

Pagel, M. (2007). User's manual for continuous in Bayestraits. http://www.evolution.rdg.ac.uk/Files/ContinuousManual.

Paulus, W. (1989). Ultrastructural investigation of spermatogenesis in *Spongilla lacustris* and *Ephydatia fluviatilis* (Porifera, Spongillidae). *Zoomorphology*, *109*, 123–130.

Paulus, W. & Weissenfels, N. (1986). The spermatogenesis of *Ephydatia fluviatilis* (Porifera). *Zoomorphology*, *106*, 155–162.

Philippe, H., Derelle, R., Lopez, P., Pick, K., Borchiellini, C., Boury-Esnault, N., Vacelet, J., Renard, E., Houliston, E., Quélnnec, E., Da Silva, C., Wincker, P., Le Guyader, H., Leys, S., Jackson, D. L., Schreiber, F., Erpenbeck, D., Morgenstern, B., Wörheide, G. & Manuel, M. (2009). Phylogenomics revives traditional views on deep animal relationships. *Current Biology*, *19*, 706–712.

Philippe, H., Brinkmann, H., Lavrov, D. V., Littlewood, D. T. J., Manuel, M., Wörheide, G. & Baurain, D. (2011). Resolving difficult phylogenetic questions: Why more sequences are not enough. *PLoS Biology*, *9*, e1000602.

Pick, K. S., Philippe, H., Schreiber, F., Erpenbeck, D., Jackson, D. J., Wrede, P., Wiens, M., Alié, A., Morgenstern, B., Manuel, M. & Wörheide, G. (2010). Improved phylogenomic taxon sampling noticeably affects nonbilaterian relationships. *Molecular Biology and Evolution*, *27*, 1983–1987.

Posada, D. (2008). jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution*, *25*, 1253–1256.

Posada, D. & Buckley, T. R. (2004). Model selection and model averaging in phylogenetics: Advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. *Systematic Biology*, *53*, 793–808.

Prendini, L. (2001). Species or supraspecific taxa as terminals in cladistic analysis? Groundplans versus exemplars revisited *Systematic Biology*, *50*, 290–300.

Raleigh, J., Redmond, N. E., Delahan, E., Torpey, S., van Soest, R. W. M., Kelly, M. & McCormack, G. (2007). Mitochondrial cytochrome oxidase 1 phylogeny supports alternative taxonomic scheme for the marine Haplosclerida. *Journal of the Marine Biological Association of the United Kingdom*, *87*, 1577–1584.

Rambaut, A. & Drummond, A. J. (2007) Tracer v1.4. Available via http://beast.bio.ed.ac.uk/Tracer

Ramesh, M. A., Malik, S.-B. & Logsdon, J. M. Jr (2005). A phylogenomic inventory of meiotic genes: Evidence for sex in *Giardia* and an early eukaryotic origin of meiosis. *Current Biology*, *15*, 185–191.

Redmond, N. E. & McCormack, G. P. (2008). Large expansion segments in 18S rDNA support a new sponge clade (class Demospongiae, order Haplosclerida). *Molecular Phylogenetics and Evolution*, *47*, 1090–1099.

Redmond, N. E., van Soest, R. W. M., Kelly, M., Raleigh, J., Travers, S. A. A. & McCormack, G. P. (2007). Reassessment of the classification of the order Haplosclerida (class Demospongiae, phylum Porifera) using 18s rRNA sequence data. *Molecular Phylogenetics and Evolution*, *43*, 344–352.

Redmond, N. E., Raleigh, J., van Soest, R. W. M., Kelly, M., Travers, S. A. A., Bradshaw, B., Vartia, S., Stephens, K. M. & McCormack, G. (2011). Phylogenetic relationships of the marine Haplosclerida (phylum Porifera) employing ribosomal (28S rRNA) and mitochondrial (*cox1*, *nad1*) gene sequence data. *PLoS ONE*, *6*, e24344.

Riesgo, A. & Maldonado, M. (2008). Differences in reproductive timing among sponges sharing habitat and thermal regime. *Invertebrate Biology*, *127*, 357–367.

Riesgo, A. & Maldonado, M. (2009). An unexpectedly sophisticated, V-shaped spermatozoon in Demospongiae (Porifera): reproductive and evolutionary implications. *Biological Journal of the Linnean Society*, *97*, 413–426.

Sarà, A., Cerrano, C. & Sarà, M. (2002). Viviparous development in the Antarctic sponge *Stylocordyla borealis* Loven, 1868. *Polar Biology*, *25*, 425–431.

Simpson, T. L. (1984). Gamete, embryo, larval development. In *The Cell Biology of Sponges* (pp. 341–413). Berlin: Springer Verlag.

Sperling, E. A., Peterson, K. J. & Pisani, D. (2009). Phylogenetic-signal dissection of nuclear housekeeping genes supports the paraphyly of sponges and the monophyly of Eumetazoa. *Molecular Biology and Evolution*, *26*, 2261–2274.

Stamatakis, A. (2006). Raxml-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, *22*, 2688–2690.

Stamatakis, A., Hoover, P. & Rougemont, J. (2008). A rapid bootstrap algorithm for the RAxML Web servers. *Systematic Biology*, *57*, 758–771.

Strathmann, R. R. (1986). What controls the type of larval development? Summary statement for the evolution session *Bulletin of Marine Science*, *39*, 616–622.

Strathmann, R. R. & Strathmann, M. F. (1982). The relationship between adult size and brooding in marine invertebrates. *The American Naturalist*, *119*, 91–101.

Tavaré, S. (1986). Some probabilistic and statistical problems in the analysis of DNA sequences. *Lectures on Mathematics in the Life Sciences*, *17*, 57–67.

Thorson, G. (1946). Reproductive and larval development of Danish marine bottom invertebrates, with special reference to planktonic larvae in the sound (Úresund). *Meddelelser Komm Danmarks Fiskeri Havundersõgelser*, *Serie Plankton*, *4*, 1–523.

Tomlinson, J. (1966). The advantages of hermaphroditism and parthenogenesis. *Journal of Theoretica Biology*, *11*, 54–58.

Tuzet, O. & Paris, J. (1964). La spermatogenèse, l'ovogenése, la fécondation et les premiers stades du développement chez. *Octavella galangaui Vie et Milieu*, *15*, 309–327.

Vance, R. R. (1973). On reproductive strategies in marine benthic invertebrates. *The American Naturalist*, *107*, 339–352.

Voigt, O., Erpenbeck, D. & Wörheide, G. (2008). Molecular evolution of rDNA in early diverging Metazoa: First comparative analysis and phylogenetic application of complete SSU rRNA secondary structures in Porifera. *BMC Evolutionary Biology*, *8*, 69–85.

Wörheide, G., Dohrmann, M., Erpenbeck, D., Larroux, C., Maldonado, M., Borchiellini, C. & Lavrov, D. V. (2012). Deep phylogeny and evolution of sponges (phylum Porifera). *Advances in Marine Biology*, *61*, 1–62.

Yang, Z. (1996). Among-site rate variation and its impact on phylogenetic analyses. *Trends in Ecology & Evolution*, *11*, 367–372.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic tree resulting from the maximum likelihood analysis of the combined data set (18S rRNA and COI genes).

Fig. S1. Phylogenetic tree resulting from the Bayesian inference analysis using the combined data set (18S rRNA and COI genes).

Table S1. Species included in the phylogenetic analysis and information considered in the study.

Table S2. Primer sequences used in the study.