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Swierts, T.

Citation

Swierts, T. (2019, December 17). *Diversity in the globally intertwined giant barrel sponge species complex*. Retrieved from <https://hdl.handle.net/1887/81578>

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Author: Swierts, T.

Title: Diversity in the globally intertwined giant barrel sponge species complex

Issue Date: 2019-12-17

2

Globally intertwined evolutionary history of giant barrel sponges

Swierts, T., Peijnenburg, K. T. C. A., de Leeuw, C. A., Breeuwer, J. A.,
Cleary, D. F. R., & de Voogd, N. J.

(2017). *Globally intertwined evolutionary history of giant barrel sponges*. *Coral reefs*, 36(3), 933-945.

ABSTRACT

Three species of giant barrel sponge are currently recognized in two distinct geographic regions, the tropical Atlantic and the Indo-Pacific. In this study, we used molecular techniques to study populations of giant barrel sponges across the globe and assessed whether the genetic structure of these populations agreed with current taxonomic consensus or, in contrast, whether there was evidence of cryptic species. Using molecular data, we assessed whether giant barrel sponges in each oceanic realm represented separate monophyletic lineages. Giant barrel sponges from 17 coral reef systems across the globe were sequenced for mitochondrial (partial CO1 and ATP6 genes) and nuclear (ATPs β intron) DNA markers. In total, we obtained 395 combined sequences of the mitochondrial CO1 and ATP6 markers, which resulted in 17 different haplotypes. We compared a phylogenetic tree constructed from 285 alleles of the nuclear intron ATPs β to the 17 mitochondrial haplotypes. Congruent patterns between mitochondrial and nuclear gene trees of giant barrel sponges provided evidence for the existence of multiple reproductively isolated species, particularly where they occurred in sympatry. The species complexes in the tropical Atlantic and the Indo-Pacific, however, do not form separate monophyletic lineages. This rules out the scenario that one species of giant barrel sponge developed into separate species complexes following geographic separation and instead suggests that multiple species of giant barrel sponges already existed prior to the physical separation of the Indo-Pacific and tropical Atlantic.

INTRODUCTION

There has been much controversy over the processes driving evolution and speciation in marine environments (e.g., Mayr 1942; Rocha and Bowen 2008). Physical barriers are less obvious in seas and oceans than on land, and many marine organisms have long-range dispersal capabilities during early life stages. Taken together, these factors were believed to lead to fewer opportunities for allopatric speciation compared to terrestrial ecosystems (Palumbi 1997; Rocha and Bowen 2008). However, this is inconsistent with the high biodiversity found in coral reefs, which rivals numbers found in tropical rainforests (Reaka-Kudla et al. 1997). Coral reefs are currently among the most vulnerable of ecosystems (Bridge et al. 2013) due to the combined threat of climate change and anthropogenic stressors including pollution and overfishing (Hughes 1994; Pandolfi et al. 2005). It is, therefore, important to study and quantify the diversity of these systems and understand the evolutionary processes that have led to this diversity.

Marine speciation does not fundamentally differ from terrestrial speciation, but ecological partitions among populations are believed to be more important in the former, whereas geographic partitions are more important in the latter (Bowen et al. 2013). An increasing number of examples of non-allopatric speciation along ecological gradients (reviewed in Bowen et al. 2013) illustrate the evolutionary potential of tropical marine environments. Furthermore, numerous phylogenetic studies have provided evidence of cryptic species, i.e., species that are indistinguishable from congeners in morphology and spatial distribution, but that are clearly differentiated genetically (Bickford et al. 2007). In contrast, certain species show strong genetic connectivity at a global scale (Horne et al. 2008; Reece et al. 2011) despite apparent morphological variation (Rocha et al. 2005).

Correct identification of species is a fundamental part of conservation and management, and misidentification of cryptic species may impair conservation efforts (Robinson et al. 2014). Genetic markers have become increasingly important tools to identify divergent cryptic species and have forced the rejection of the long-believed assumption of cosmopolitan distribution of certain species (Boury-Esnault et al. 1992; Knowlton 1993; Klautau et al. 1999). Molecular techniques have also helped to reconstruct the distributional patterns of invasive species, which have become major drivers of ecosystem change due to the increase in global shipping (Concepcion et al. 2010; Teske et al. 2011). Most studies that have focused on the distribution and evolution of marine species cover small spatial scales and become more useful when they are compared to more wide-ranging studies (Briggs and Bowen 2013; Cowman and Bellwood 2013a). A focus on wide-ranging studies within each marine phylum should therefore be a priority for the scientific community.

Sponges (Porifera) are an animal group with a relatively simple morphology and often pronounced morphological plasticity (Knowlton 2000). Hence, they can be notoriously

difficult to identify to species or even to a higher taxonomic level due to the lack of reliable morphological markers (Knowlton 2000). They are considered the oldest multicellular animal lineage (van Soest et al. 2012), having evolved more than 500 million yr ago (Love et al. 2009; Maloof et al. 2010), and are widespread in many aquatic systems. On tropical reefs, sponge diversity and abundance can be higher than that of corals (Diaz and Rützler 2001). Unfortunately, this large and important animal group has long been understudied in coral reef ecology (Diaz and Rützler 2001). Most genetic studies of sponges have indicated the existence of cryptic species and refuted ocean-wide distributions of several taxa (Duran and Rützler 2006; Swierts et al. 2013; Bell et al. 2014; Knapp et al. 2015). However, these studies were done at small spatial scales, and, to the best of our knowledge, there has been no global phylogenetic study of any sponge taxon.

Giant barrel sponges (genus *Xestospongia*, family Petrosiidae, order Haplosclerida) are widely distributed throughout multiple tropical oceans. Giant barrel sponges are large and long-lived and have therefore been nicknamed ‘the redwoods of the reef’ (McMurray et al. 2008). These conspicuous sponges can measure up to a base diameter of more than 2.5 m (Nagelkerken et al. 2000) and can cover up to 9% of some reefs (Zea 1993); one specimen from Curaçao was estimated to be over 2300 yr old (Nagelkerken et al. 2000). Three species have been described, with the species delineation mainly based on geographic distributions. *Xestospongia muta* occurs in the tropical Atlantic, *X. testudinaria* in the Indo-Pacific from the Red Sea to Taiwan and *X. bergquistia* is thought to be confined to inshore environments in northern Australia where it lives in sympatry with *X. testudinaria*. Recent molecular studies have suggested that these species delineations are incorrect and that both *X. muta* and *X. testudinaria* consist of multiple sympatric species that apparently do not interbreed (Swierts et al. 2013; Bell et al. 2014). This has important implications for a number of published studies on the demography and population genetics of giant barrel sponges which assumed a single population of giant barrel sponge (López-Legentil and Pawlik 2009; McMurray et al. 2010; Richards et al. 2016).

The congruent identification of a phylogenetic lineage by multiple unlinked genetic loci indicates that it is genetically isolated from other such lineages, and thus qualifies as a species, because only in separate species will the coalescent histories of the different markers agree (Avice and Ball 1990; Coyne and Orr 2004; Padial et al. 2010). Nuclear DNA (nDNA) evolves independently from mitochondrial DNA (mtDNA); thus, congruent patterns across these markers support the existence of biological species (Goetze 2010; Padial et al. 2010). In sponges, mitochondrial variation is typically low (Wörheide et al. 2005), but previous studies of giant barrel sponges have shown that the combination of the adenosine triphosphate synthase subunit 6 gene (ATP6) with the I3-M11 partition of the cytochrome oxidase 1 gene (CO1) was informative (Rua et al. 2011; Swierts et al. 2013). The nuclear adenine triphosphate synthesis- β intron (ATPs β) is very variable in giant barrel sponges, and because it is unlinked

to the mtDNA, it serves as a good additional marker to identify potential species (Bentlage and Wörheide 2007; Swierts et al. 2013).

Molecular studies on giant barrel sponges using these mtDNA and nDNA markers have revealed some interesting results. For example, some haplotypes of the I3-M11 partition of the CO1 gene are shared between Indonesia (*X. testudinaria*) and Florida (*X. muta*); hence, two individuals from different ocean basins can be more closely related for this slowly evolving gene than two sympatric individuals on the same reef (Swierts et al. 2013; Setiawan et al. 2016a). Giant barrel sponges from the tropical Atlantic and the Indo-Pacific sharing the same CO1 haplotype have both been related to an exterior morphology consisting of digitate structures (López-Legentil and Pawlik 2009; Swierts et al. 2013). These studies imply that the giant barrel sponge is a classic example of a tropical marine animal in which poor identification at the species level has led to an oversimplified taxonomic classification. Due to its conspicuousness, geographic range and available genetic markers, this group of sponges is suitable as a model for global sponge evolution and phylogeography. A better understanding of these species helps in our understanding of the evolutionary history of tropical marine species in general and marine sponges in particular, which is essential to our understanding of marine diversity. In this study, we sequenced giant barrel sponges from reefs across the globe for a combination of the mtDNA genes ATP6 and CO1 and the nDNA intron ATPs β . The first aim of this study was to assess how many species of giant barrel sponge are present globally and how they are distributed. Our second aim was to test whether the giant barrel sponges in the tropical Atlantic and the Indo-Pacific represent two monophyletic lineages. If this is the case, it would suggest that one species of giant barrel sponge in each ocean basin independently developed into different species and/or species complexes. However, if sponges do not form two distinct monophyletic groups in different ocean basins, it suggests that a species complex already existed prior to the ocean basins becoming separated. This information provides insight into genetic divergence among tropical reefs before physical barriers impeded gene flow between the Indo-Pacific and tropical Atlantic.

METHODS

Giant barrel sponges (*Xestospongia* spp.) were collected by SCUBA diving from 17 different locations (Table 2.1; Fig. 2.1). Sponge tissue for DNA extraction was immediately stored in absolute ethanol (98%) in a cool box. After 6–12 h, the ethanol was changed and samples were stored at -20°C . Fifty-four sponge samples from Lembah Island, Indonesia, were previously described in Swierts et al. (2013), but amplification and sequencing were repeated in this study to confirm haplotype assignment.

DNA was extracted from sponge tissue using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's instructions. We sequenced 395 samples for a combination

of the mitochondrial CO1 (Erpenbeck et al. 2002) and ATP6 genes (Rua et al. 2011). For the CO1 gene, we used the primers C1-J2165 (5'-GAAGTTTATATTTAATTTTACCDGG-3') and C1-Npor2760 (5'-TCTAGGTAATCCAGCTAAACC-3'), which amplified a fragment of 544 base pairs (bp). Amplification was performed in a 25 μ L total reaction volume with 15.5 μ L sterile water, 5 μ L dNTPs (2.5 mM), 2.5 μ L coralloid buffer (Qiagen), 0.4 μ L of each primer (10 μ M), 0.25 μ L taq polymerase (Qiagen) and 1 μ L DNA template (20 ng μ L⁻¹). For the ATP6 gene, we used the primers ATP6porF (5'-GTAGTCCAGGATAATTTAGG-3') and ATP6porR (5'-GTTAATAGACAAAATACATAAGCCTG-3'), which amplified a product of 445 bp. Amplification was performed in a 25 μ L total reaction volume with 14 μ L sterile water, 5 μ L dNTPs (2.5 mM), 2.5 μ L coralloid buffer (Qiagen), 1.5 μ L BSA (Promega), 0.4 μ L (10 μ M) of each primer, 0.25 μ L taq polymerase (Qiagen) and 1 μ L DNA template (20 ng μ L⁻¹). For both genes, we used a PCR protocol that consisted of an initial denaturing step (95 °C for 5 min), followed by 35 cycles of denaturing (95 °C for 30 s), annealing (42 °C for 45 s) and extension (68 °C for 1.30 min), and a final extension step (72 °C for 10 min) executed in a T100 thermal cycler (Bio-Rad).

To test for congruent patterns at an independent genetic locus, the ATPs β nuclear intron was amplified for a subset of 211 samples following Jarman et al. (2002). For this gene, we used the primers ATPS β -F (5'-ATGAGATGATCACATCAGGTG-3') and ATPS β -R (5'-GGTTCGTTTCATCTGTCC-3'), which amplified products in the range of 258–279 bp. Amplification was performed in a 25 μ L total reaction volume with 14.55 μ L sterile water, 4.2 μ L dNTPs (2.5 mM), 2.6 μ L buffer (Qiagen), 1.6 μ L BSA (Promega) 0.4 μ L of each primer (10 μ M), 0.25 μ L taq polymerase (Qiagen) and 1 μ L DNA template (20 ng μ L⁻¹). The PCR protocol consisted of an initial denaturing step (95 °C for 5 min), followed by 35 cycles of denaturing (95 °C for 30 s), annealing (45 °C for 30 s) and extension (72 °C for 45 s), and a final extension step (72 °C for 4 min) executed in a T100 thermal cycler from Bio-Rad. All PCR products were sequenced in both directions by BaseClear, Leiden, the Netherlands or MacroGen Europe, Amsterdam, the Netherlands.

Sequences were checked using CodonCode Aligner version 3.7.1.2 (CodonCode Corporation). Double peaks were called when the height of the secondary peak was at least 60% of that of the primary peak in both the forward and reverse sequence reads. Samples that contained two nucleotide positions with double peaks were reconstructed using DnaSP v5.10.01 with the PHASE v2.1 algorithm (Stephens et al. 2001). Only reconstructed haplotypes with probabilities >0.9 were used for further analysis. Samples that contained many double peaks may have represented mixtures of multiple sequences and were therefore cloned using the pGEM-T Easy kit (Promega Corporation) or the TOPO-TA cloning kit (Thermo Fisher Scientific), following the manufacturers' protocols. Primer sequences were trimmed of the final sequences, and alignments were obtained using ClustalW (Larkin et al. 2007) in Geneious v9.04 (Kearse et al. 2012) for both the combined mtDNA and single nDNA markers using the default software settings.

Table 2.1. Overview of sampling locations and the number of samples from each region sequenced for mitochondrial DNA (mtDNA) genes CO1 and ATP6, and for the nuclear gene (nDNA) ATPsβ respectively. Abbreviations in region names: C= Central; W=Western.

Location	Abbreviation	Region	mtDNA	nDNA
Derawan Islands - Indonesia	Der	C Indo-Pacific	46	26
Jakarta Bay; Thousand Islands- Indonesia	Jak	C Indo-Pacific	20	11
Lembeh Island - Indonesia	Lem	C Indo-Pacific	54	21
Spermonde Archipelago - Indonesia	Spe	C Indo-Pacific	67	49
Tioman Island - Malaysia	Tio	C Indo-Pacific	9	7
St. John Island - Singapore	Sin	C Indo-Pacific	15	7
Penghu Islands - Taiwan	Tai	C Indo-Pacific	48	7
Pattaya - Thailand	Pat	C Indo-Pacific	14	9
Phuket - Thailand	Phu	C Indo-Pacific	13	5
Koh Tao - Thailand	Koh	C Indo-Pacific	10	4
Halong Bay - Vietnam	HB	C Indo-Pacific	2	1
Phu Quoc - Vietnam	PQ	C Indo-Pacific	10	3
Jeddah - Saudi Arabia	Sau	Red Sea	11	4
Santa Barbara - Curaçao	Cur	Tropical Atlantic	28	22
Sint-Eustatius – the Netherlands	SE	Tropical Atlantic	27	23
Mayotte – France	May	W Indian Ocean	10	5
Dar es Salaam - Tanzania	Tan	W Indian Ocean	11	6
Total			395	210

We made separate statistical parsimony networks for the combined mtDNA sequences (CO1 + ATP6) and the nDNA sequences with TCS v 1.21 (Clement et al. 2000). A maximum likelihood phylogenetic tree was constructed for the ATPsβ -intron in Geneious using the PHYML plugin (Guindon et al. 2010) with the GTR model, which was the best fit model according to jModelTest2 (Guindon and Gascuel 2003; Darriba et al. 2012) based on the Akaike information criterion (Akaike 1974). The number of bootstrap replications was set at 1000. We also calculated Bayesian support values with MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The analysis of every gene consisted of two independent runs of four Metropolis-coupled Markov chains, sampled at every 1,000th generation. Analyses were terminated after the chains converged significantly as indicated by an average standard deviation of split frequencies <0.01. Trees were visualized with FigTree v1.4.2 (Morariu et al. 2009).

Individuals were grouped based on a combination of mtDNA, nDNA and the geographic origin of the sample. Mean genetic distance was calculated between these groups for the mtDNA and nDNA genes in MEGA 7.0.21 (Kumar et al. 2016) using the Tamura–Nei model (Tamura and Nei 1993) with standard settings. We conducted an automated barcoding gap discovery (ABGD) analysis with standard settings to split our sequences into candidate

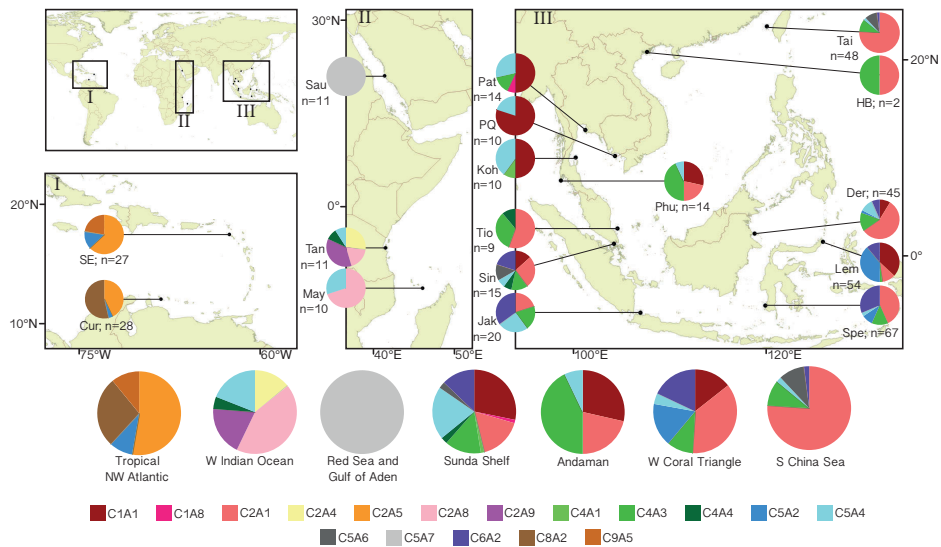


Figure 2.1. Location maps with haplotype frequencies of the mitochondrial DNA genes CO1 and ATP6 of giant barrel sponges. *SE* = Sint-Eustatius, the Netherlands; *Cur* = Santa Barbara, Curaçao; *Sau* = Jeddah, Saudi-Arabia; *Tan* = Dar es Salaam, Tanzania; *May* = Mayotte, France; *Pat* = Pattaya, Thailand; *PQ* = Phu Quoc, Vietnam; *Koh* = Koh Tao, Thailand; *Tio* = Tioman Island, Malaysia; *Sin* = St. John's Island, Singapore; *Jak* = Jakarta Bay and Thousand Islands, Indonesia; *Phu* = Phuket, Thailand; *Tai* = Taiwan; *HB* = Halong Bay, Vietnam; *Der* = Derawan Islands, Indonesia; *Lem* = Lembah Island, Indonesia; *Spe* = Spermonde Archipelago, Indonesia

species and compare those to our identified groups based on congruence between mtDNA, nDNA and geography (Puillandre et al. 2012).

RESULTS

We obtained a total of 395 combined sequences of partial mitochondrial CO1 and ATP6 genes. In the final alignment of 989 base pairs, we found 13 variable sites: six were located in the CO1 gene and seven in the ATP6 gene, resulting in 17 different haplotypes (Table 2.2). Seven of the nine CO1-haplotypes (C1–C9) previously submitted to GenBank (López-Legentil and Pawlik 2009; Swierts et al. 2013; Setiawan et al. 2016) were present in this dataset. Re-analysis of the sample carrying the C3 haplotype from Lembah Island showed that the sample was C2, and hence, this haplotype was wrongly identified (Swierts et al. 2013). Haplotype C7, described by Setiawan et al. (2016) from one sample from Tanzania, was not found in our dataset, and no new haplotypes were found for the CO1 gene. For the ATP6 gene, only three haplotypes were previously known (A1–A3; Swierts et al. 2013) and six new haplotypes were identified (A4–A9; GenBank accession numbers: KY381287–KY381292). Adding this gene to the CO1 gene expanded the number of haplotypes in our dataset from seven (C1, C2, C4–C6, C8, C9) to seventeen (Table 2.2). As is common in sponges

(Wörheide et al. 2005), mitochondrial variation was low ($\pi = 0.0032$). With one exception, connected haplotypes in the statistical parsimony network were differentiated by a single mutation (Fig. 2.2).

The tropical Atlantic haplotypes C2A5 and C9A5 are located at the opposite end of the network compared to the only other tropical Atlantic haplotype C8A2 and separated by 11 mutational steps. All 11 sequences from the Red Sea were identical and unique for the region (C5A7). In the western Indian Ocean, we found five different haplotypes in 21 sponges that were spread over the haplotype network; three of these haplotypes were only present in this region. Six haplotypes from the Indo-Pacific were represented by more than 25 individuals in our dataset, and the majority of these haplotypes were widespread and occurred at multiple sampling sites. Haplotype C5A2 was found in the central Indo-Pacific, but also in the tropical Atlantic. The four regions in which we sampled (central Indo-Pacific, tropical Atlantic, Red Sea, western Indian Ocean), which are geographically distant from one another, were characterized by different haplotype compositions, and all hosted unique haplotypes (Fig. 2.1).

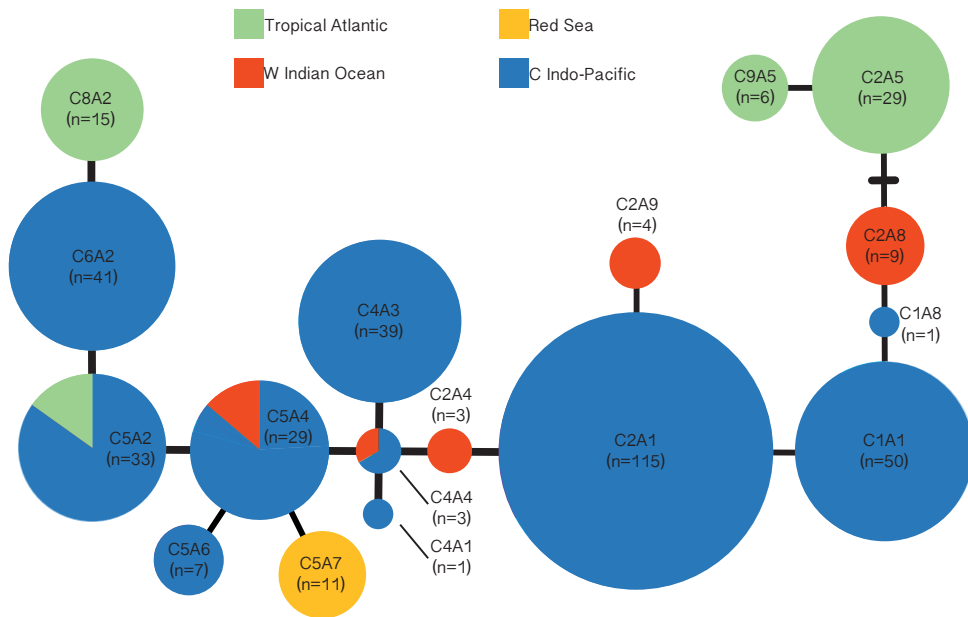


Figure 2.2. Haplotype network of the mitochondrial DNA genes CO1 and ATP6 of giant barrel sponges. *Pie chart size* is relative to the number of individuals with that haplotype. *Colors* indicate regions of origin. *Lines* connecting haplotypes represent one base substitution between two haplotypes; *additional crossbars* indicate an additional base substitution each. *Green* tropical Atlantic; *red* western Indian Ocean; *yellow* Red Sea; *blue* central Indo-Pacific.

2

Table 2.2. Nucleotide differences for mitochondrial markers CO1 and ATP6. Nucleotide differences in mitochondrial markers Cytochrome Oxidase I (CO1) and adenosine triphosphate synthase subunit 6 (ATP6). Seven haplotypes (C1, C2, C4-C6, C8, C9) are found for the CO1 fragment (base pairs 1-544) with a total of six variable sites. Nine haplotypes (A1-A9) are found for the ATP6 fragment (base pairs 545-989) with a total of seven variable sites. Seventeen different haplotypes are found when the CO1 and ATP6 markers combined (e.g. C1A1, base pairs 1-989).

mtDNA	CO1						ATP6						N	
	11	22	28	133	347	463	576	725	749	785	891	902	933	
CO1+ATP6														
C1A1	A	T	C	A	G	T	T	T	G	T	G	G	T	50
C1A8	C	1
C2A1	C	115
C2A4	C	C	3
C2A5	C	.	C	.	.	A	A	.	29
C2A8	C	.	C	9
C2A9	C	.	.	.	C	.	.	.	4
C4A1	.	.	.	G	.	C	1
C4A3	.	.	.	G	.	C	C	C	39
C4A4	.	.	.	G	.	C	C	3
C5A2	.	A	.	G	.	C	C	.	.	C	.	.	.	33
C5A4	.	A	.	G	.	C	C	28
C5A6	.	A	.	G	.	C	C	.	A	7
C5A7	.	A	.	G	.	C	C	C	11
C6A2	G	A	.	G	.	C	C	.	.	C	.	.	.	41
C8A2	G	A	.	G	A	C	C	.	.	C	.	.	.	15
C9A5	.	.	T	.	.	C	.	C	.	.	A	A	.	6
Total														395

We successfully amplified the nuclear intron ATPsβ from 211 individuals. The nuclear intron ATPsβ provided much more genetic variation (157 segregating sites; $\pi = 0.0767$) than the mitochondrial genes (13 segregating sites; $\pi = 0.0032$). A circular phylogenetic tree consisting of 285 alleles (137 homozygotes, 74 heterozygotes) was constructed for the nuclear intron and compared to the 17 different mitochondrial haplotypes (Fig. 2.3); a larger and more detailed rectangular phylogenetic tree is provided in Appendix 2.1. All sequences were submitted to GenBank under accession numbers KY381293–KY381577. The nuclear sequences provided much more information than the mitochondrial markers, but were mostly phylogenetically congruent with the mtDNA. While not all branches in the nDNA tree were statistically supported and some mtDNA haplotypes were shared between regions, we could identify multiple groups that potentially operate as reproductively isolated populations.

Sponges were assigned to a separate group when they possessed unique mtDNA haplotypes within one of the geographic regions and also formed a separate cluster of unique nuclear

alleles (Fig. 2.3). Based on these criteria, the individuals from the central Indo-Pacific could be separated into three groups: group 1—haplotypes C1A1, C1A8 and C2A1; group 2—haplotypes C4A3 and C4A4; and group 3—haplotypes C5A2, C5A4 and C6A2. In the tropical Atlantic, the nuclear gene tree also contained three groups that were mostly congruent with mitochondrial haplotypes: group 7—haplotypes C2A5 and C9A5; group 8—haplotype C8A2; and group 9—haplotype C5A2. We found one group in the western Indian Ocean

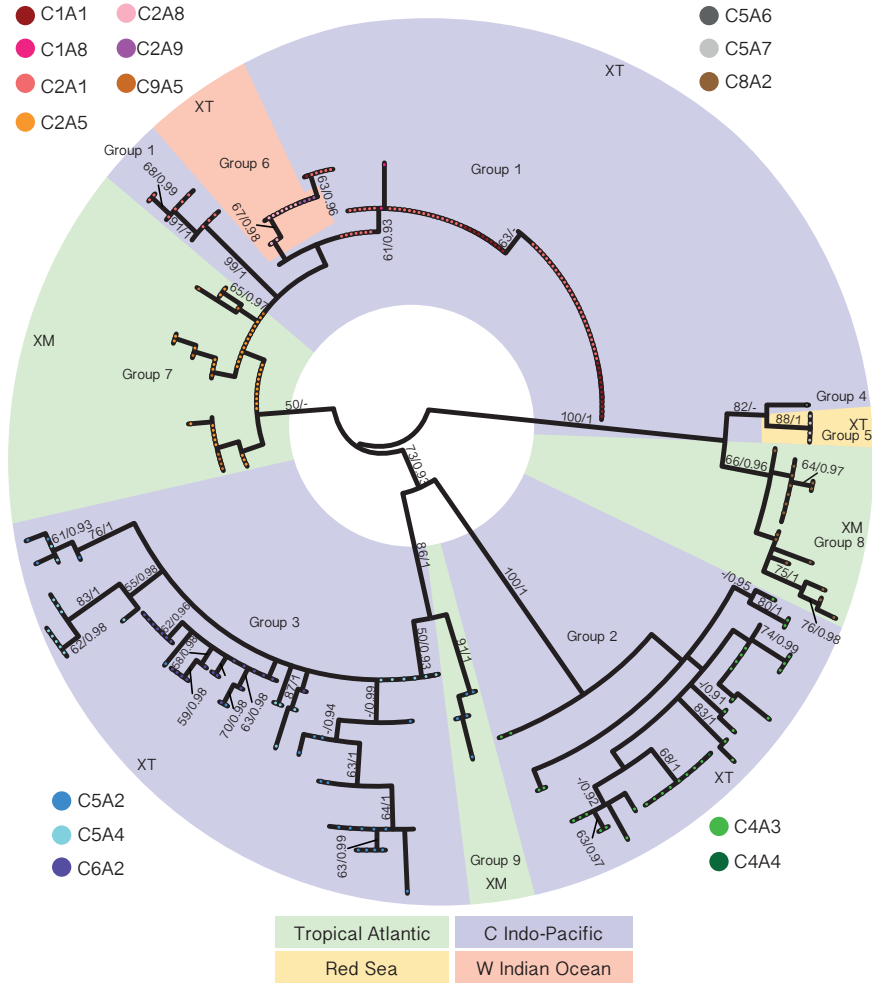


Figure 2.3. Unrooted circular maximum likelihood tree of 285 alleles of the nuclear DNA gene *ATPsβ*. Values on branches indicate bootstrap support (only shown when >50) and Bayesian support value (only shown when >0.90). Dots on the branches indicate the number of individuals with that allele, and the colors of the dots indicate the haplotype of the individual for the mitochondrial CO1 and ATP6 haplotypes. Background colors represent geographic origin of the lineages. Abbreviations in the legend of the background colors indicate the current species consensus (XT, *Xestospongia testudinaria*; XM, *Xestospongia muta*)

with mitochondrial haplotypes C2A8 and C2A9 (group 6), and one group in the Red Sea with mitochondrial haplotype C5A7 (group 5). The only individual with haplotype C5A6 was found in Taiwan; this individual had unique nuclear DNA and did not fit in any of the other three groups of the Indo-Pacific (group 4). Table 2.3 shows the mean genetic distances between these drafted groups. Mean genetic distance for the nDNA was considerably higher than for the mtDNA. An ABGD analysis on our nuclear data supported the groups 2, 3, 4, 5, 8 and 9 with recursive partitions at a prior maximal distance of 0.0046, while groups 1, 6 and 7 were not supported as separate groups (Appendix 2.2). The statistical parsimony network constructed with TCS from our nuclear data resulted in seven unconnected statistical parsimony networks. Group 8 represented one network, group 3 represented three networks, and the remaining three networks consisted of the combinations of groups 2 and 9, groups 1, 6 and 7, and groups 4 and 5, respectively (Appendix 2.3; 2.4).

The groups from the tropical Atlantic (7, 8, 9) and the Indo-Pacific (1, 2, 3) were not monophyletic per region for the nuclear marker, but rather were intertwined in a generally congruent pattern with the mtDNA (Fig. 2.3). They did not share any nuclear alleles, indicating that there has been no recent genetic exchange between giant barrel sponges from these areas. Also, heterozygotes were only found with both alleles within the same nuclear group providing further support that different groups are reproductively isolated (Appendix 2.1). Note that not all groups are monophyletic in the nDNA tree. In particular, groups 1, 6 and 7 are 'mixed' in the tree, but they do not share any alleles. Also, some groups are only partly congruent, for example groups 4 and 5. Both groups show congruent patterns in the sense that they both host unique mtDNA haplotypes and unique nDNA alleles and are closely related to each other for both markers. However, there is an inconsistency in their placement relative to the other groups, since they are most closely related to group 8 in the nDNA phylogenetic tree (Fig. 2.3) but most closely related to group 3 in the haplotype network for the mtDNA (Fig. 2.2).

DISCUSSION

Congruent patterns between mtDNA and nDNA markers of giant barrel sponges around the globe point to the existence of multiple genetically isolated taxa and support our hypothesis of the existence of additional species. The genetic differences between the nuclear clades are based on the variation of a single gene and the mitochondrial markers have low variation. Nevertheless, plotting mtDNA haplotypes to the nuclear phylogeny does not result in random distribution. All of the mtDNA haplotypes are confined to one nDNA group within a geographic region, suggesting biological species. Altogether, we identified at least eight potential giant barrel sponge species globally, yet the limitations of the markers make it impossible to exactly determine the number of species with the data presented in this study.

Table 2.3. Mean genetic distances of the mtDNA and the nDNA between identified groups of giant barrel sponges (*Xestospongia* spp.) Mean genetic distances between groups for the mtDNA are shown in the lower left section of the table, and mean genetic distances between groups for the nDNA are shown in the upper right section of the table.

Group	Haplotypes	Region	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
Group 1 ^a	C1A1, C1A8, C2A1	C Indo-Pacific		0.082	0.061	0.065	0.073	0.011	0.015	0.071	0.067
Group 2 ^b	C4A3, C4A4	C Indo-Pacific	0.003		0.085	0.093	0.098	0.079	0.071	0.096	0.078
Group 3 ^c	C5A2, C5A4, C6A2	C Indo-Pacific	0.005	0.003		0.094	0.099	0.058	0.059	0.096	0.036
Group 4 ^d	C5A6	C Indo-Pacific	0.005	0.003	0.002		0.013	0.066	0.059	0.023	0.087
Group 5 ^d	C5A7	Red Sea	0.005	0.003	0.002	0.002		0.070	0.064	0.028	0.092
Group 6 ^a	C2A8, C2A9	W Indian Ocean	0.002	0.003	0.004	0.005	0.005		0.012	0.067	0.065
Group 7 ^a	C2A5, C9A5	Tropical Atlantic	0.004	0.005	0.008	0.008	0.008	0.004		0.062	0.057
Group 8 ^e	C8A2	Tropical Atlantic	0.007	0.005	0.002	0.004	0.004	0.006	0.010		0.090
Group 9 ^c	C5A2	Tropical Atlantic	0.005	0.003	0.001	0.002	0.002	0.004	0.008	0.002	

^a Indicates three groups that are not separated from each other with the automated barcode gap discovery analysis [ABGD analysis; Puillandre et al. (2012)] and that did not form separate haplotype networks in the TCS analysis (Clement et al. 2000).

^b Indicates a group that was separated by the ABGD analysis and formed three haplotype networks in the TCS analysis

^c Indicates two groups that were separated by the ABGD analysis and formed one haplotype networks in the TCS analysis

^d Indicates two groups that are separated from each other by the ABGD analysis and formed one haplotype network in the TCS analysis

^e Indicates a group that was separated by the ABGD analysis and formed a separate network in the TCS analysis

We found three potential giant barrel sponge species in the central Indo-Pacific (groups 1, 2, 3), three in the tropical Atlantic (groups 7, 8, 9), one in the western Indian Ocean (group 6) and one in the Red Sea (group 5). The lack of hybridization between these taxa, especially between those that are sympatric, indicates complete reproductive isolation. A ninth group (group 4) consisted of a single sample from Taiwan. Some genetic groups (e.g., groups 1, 6 and 7) were not statistically supported in the nuclear gene tree, but these could still represent (incipient) species because they occur in different parts of the world's oceans and are thus geographically isolated. Certain other groups were statistically supported, which is, particularly in combination with their sympatric occurrence, a strong indication for speciation. It is important to note that some discrepancies exist in the results from our statistical analyses. Our own interpretation of nine groups differs from the results from the ABGD and TCS analyses, which both find only seven groups, but with partially different compositions. Also, the inconsistencies of the placement of certain groups relative to other groups illustrate that the phylogenetic relationships between the groups cannot be completely resolved with the combination of markers used in this study. The higher variation on the nDNA marker suggests that the evolutionary history of giant barrel sponges is better represented by this marker; however, additional genetic evidence and a thorough morphological analysis are required to delineate and describe distinct groups as species. Nevertheless, our data do indicate that the current taxonomic consensus with *X. muta* occurring in the tropical Atlantic and *X. testudinaria* in the Red Sea, western Indian Ocean and central Indo-Pacific, is incorrect.

Our results suggest the existence of three species in the tropical Atlantic, in line with previous suggestions based on a study of the sterol compositions of giant barrel sponges (Kerr et al. 1991a; 1991b). All CO1 haplotypes of samples from the tropical Atlantic in this analysis were previously described by López-Legentil and Pawlik (2009) from locations in the north (Florida) and west (Belize) of the tropical Atlantic and by de Bakker et al. (2016) from locations in the east (Saba Bank) of the tropical Atlantic. Therefore, these three groups seem to be distributed across the entire tropical Atlantic. Similarly, large geographic ranges can be observed for groups 1, 2 and 3 from the central Indo-Pacific, which have overlapping geographic ranges between locations more than 2000 km apart. Of particular interest is the finding that lineages in a given ocean basin were more closely related to lineages in another ocean basin than to lineages with which they co-occur. For instance, individuals from groups 3 and 9 can share the same mtDNA haplotype and are also closely related in the nuclear gene tree, but are found in the Indo-Pacific and tropical Atlantic, respectively. In other words, different species in each ocean basin do not form separate monophyletic clades. This suggests that distinct species of giant barrel sponges must have existed prior to the most recent physical separation of the tropical Atlantic and the Indo-Pacific.

At present, giant barrel sponges occur in the western Indo-Pacific (including the Red Sea and western Indian Ocean), the central Indo-Pacific and the tropical Atlantic. Giant barrel

sponges have not been found in the eastern Pacific and the eastern tropical Atlantic. Hence, the sponges from the tropical Atlantic and the Indo-Pacific are separated by several barriers that have developed at various times throughout history (Cowman and Bellwood 2013b). The Tethys Seaway may have provided a potential migration route throughout the first half of the Cenozoic era between 60 and 30 million yr ago between the tropical Atlantic and the Indo-Pacific (Vermeij 2001; Harzhauser and Piller 2007). At that time, the western Tethys was the center of global marine biodiversity, but these subsequently shifted eastwards to its present location in the Indo-Australian archipelago (Renema et al. 2008). In the early Miocene, the African-Arabian Plate moved northwards to adjoin the Eurasian Plate (Aitchison et al. 2007), dividing the Tethys realm into a western and an eastern part: the 'Terminal Tethyan Event' (TTE). Previously, the closure of the Isthmus of Panama, approximately 3 million yr ago (Keigwin 1978, was suggested as the final geographic separation between giant barrel sponges from the tropical Atlantic and the Indo-Pacific (Montalvo and Hill 2011; Swierts et al. 2013). However, since giant barrel sponges do not occur east of New Caledonia, the scenario in which the tropical Atlantic and the Indo-Pacific locations became isolated due to the TTE should also be considered. If isolation followed the TTE, the lineages now living in sympatry must have evolved into different species before the TTE occurred. This could be a result of sympatric speciation within the Tethys Sea, or perhaps due to a series of speciation events from periodic geographic isolation as a result of changing sea levels (Haq et al. 1987).

Reproductive mechanisms can explain how different species of *Xestospongia* are maintained in sympatry. Sympatric populations of *X. testudinaria* and *X. bergquistia* spawn at different times of the year near Australia, possibly triggered by water temperature (Fromont and Bergquist 1994). This is comparable to corals of the *Montastrea annularis* species complex (van Veghel et al. 1996) in the tropical Atlantic, which are unlikely to interbreed due to a combination of temporal differences in spawning, sperm aging, gamete dispersal and dilution, and gametic incompatibility (Levitan et al. 2004). In the tropical Atlantic, *X. muta* has been observed to spawn and recruit twice a year, in spring and in late summer (Ritson-Williams et al. 2005; McMurray et al. 2008). However, it is possible that all giant barrel sponges spawn just once a year, and these different times in fact correspond to different mass spawning events of distinct species. Spawning events of giant barrel sponges have also been reported at different times of the year in the Indo-Pacific (Röthing and Voolstra 2016; Swierts et al. 2013).

Some of the previously assumed 'morphological plasticity' of giant barrel sponges might actually be morphological differentiation between species. For instance, sponges with haplotype C5 for the CO1 gene are associated with a digitate outer morphology in both the tropical Atlantic and the Indo-Pacific (López-Legentil and Pawlik 2009; Swierts et al. 2013). Other morphological characteristics might also differentiate between 'cryptic' species. For example, the spicules of *X. muta* have a broad size range (length 290–435 µm, width 8.3–23 µm) and vary between geographic locations (van Soest 1980). With possibly three

species present in the tropical Atlantic, we have to consider that such a broad size range may be the result of lumping three different spicule size ranges and an uneven distribution of the various cryptic species among locations. Also in the tropical Atlantic, McMurray et al. (2014) found large differences in spongocoel morphology that were independent of sponge size and strongly influenced excurrent seawater velocity. Given that only three species have been described globally so far, scientific priority should be given to resolving this species complex further and differentiating between their potentially distinct ecological roles.

To our knowledge, the intertwined evolutionary history of tropical Atlantic and Indo-Pacific taxa we found for giant barrel sponges has never been found in other benthic reef animals. Usually, there is a general conformity between phylogeography and biogeography in marine animal groups, which suggests that geographic isolation is a starting point for divergences between species (Teske et al. 2011; Bowen et al. 2016). Most global marine species complexes are so-called 'sibling species complexes' that developed simultaneously in different ocean basins, potentially followed by cryptic invasions (Schwaninger 2008; Geller et al. 2010). The importance of geographic isolation, possibly related to sea currents, was suggested as a driving force in sponge speciation (DeBiasse et al. 2016). It may also be a driving force for giant barrel sponges, especially considering the groups that are confined to the Red Sea and the western Indian Ocean, which represent distinct biogeographic provinces (Briggs and Bowen 2012; Bowen et al. 2016). However, present-day ocean currents and geographic barriers cannot explain why giant barrel sponges in the Tropical Atlantic and the Indo-Pacific do not form monophyletic lineages, and why in each ocean basin multiple genetically isolated lineages exist in sympatry. Trumpet fish represent a so-called 'global ring species complex', in which different lineages have come into contact after three to four million years of isolation and appear to be merging (Bowen et al. 2001). Four highly divergent lineages have been identified for vase tunicates; these four were originally limited to a certain geographic range, but two are now considered invasive with global distributions (Zhan et al. 2010). Sponge larvae, however, are generally considered to have low survival under environmental stress, and their transport in the ballast water of ships is unlikely and has not yet been reported (Klautau et al. 1999). Our data do not support any recent invasions of giant barrel sponges from one ocean basin to another and none of the candidate species has a global distribution. Instead, our results show a unique evolutionary history, suggesting intertwined species complexes in different ocean basins. This pattern may, however, be found in other tropical marine species, especially those with a long evolutionary history such as other globally distributed sponge groups. Resolving accurate species boundaries in such groups is important for the conservation of tropical marine ecosystems.

ACKNOWLEDGEMENTS

We thank Friso Dekker, Maarten van Gemert, Christine Hörnlein, Yusheng Huang, Laurie van Reemst, Ee Crovetto, Ana-Rita Polonia, Rossana Freitas, Anne Bialecki, Mike Berumen, Jaaziel Garcia Hernandez, Sumaitt Putchakarn, Chad Scott, Nguyen Khac Bat, Swee Cheng Lim, Zarinah Waheed, Yosephine Tuti, Betsie Voetdijk, Peter Kuperus and Marcel Eurlings for logistic support, collecting and laboratory assistance. The feedback and constructive suggestions made during the review process are much appreciated and significantly improved this manuscript.

APPENDIX 2.1

Unrooted circular maximum likelihood tree of 285 alleles of the nuclear DNA gene ATPs β . *Values* on branches indicate bootstrap support (only shown when >50) and Bayesian support value (only shown when >0.90). *Dots* on the branches indicate the number of individuals with that allele, and the *colors* of the dots indicate the haplotype of the individual for the mitochondrial CO1 and ATP6 haplotypes. *Background colors* represent geographic origin of the lineages. Abbreviations in the legend of the background colors indicate the current species consensus (XT, *Xestospongia testudinaria*; XM, *Xestospongia muta*)

APPENDIX 2.2

<https://link.springer.com/article/10.1007/s00338-017-1585-6#SupplementaryMaterial>

APPENDIX 2.3

https://link.springer.com/article/10.1007/MediaObjects/338_2017_1585_MOESM2_ESM.graph.log

APPENDIX 2.4

List of hypothetical species of giant barrel sponge species (*Xestospongia spp.*) in which all individuals are grouped according to an Automatic Barcode Gap Discovery (ABGD) analysis (Puillandre et al. 2012). Settings of the analysis: Partition with prior maximal distance $P=7.74e-03$ Distance JC69 Jukes-Cantor MinSlope=1.500000

Group[1] n: 16 ;id: CUR_XMC01_HETA CUR_XMC01_HETB CUR_XMC07_HETA CUR_XMC07_HETB CUR_XMC12_HETA CUR_XMC12_HETB CUR_XMC13_HOM CUR_XMC14_HETA CUR_XMC14_HETB CUR_XMC25_HETA CUR_XMC25_HETB CUR_XMC35_HETA CUR_XMC35_HETB CUR_XMC71_HOM CUR_XMC85_HETA CUR_XMC85_HETB
Group[2] n: 158 ;id: CUR_XMC05_HOM CUR_XMC18_HOM CUR_XMC22_HETA CUR_XMC22_HETB CUR_XMC32_HOM CUR_XMC33_HETA CUR_XMC33_HETB CUR_XMC45_HOM CUR_XMC54_HOM CUR_XMC55_HOM CUR_XMC77_HOM CUR_XMC78_HETA CUR_XMC78_HETB CUR_XMC81_HOM CUR_XMC83_HETA CUR_XMC83_HETB DER_583A_HETA DER_583A_HETB DER_584_HOM DER_585_HOM DER_586_HOM DER_587_HETA DER_587_HETB DER_588_HOM DER_589_HOM DER_592_HOM DER_593_HETA DER_593_HETB DER_594_HOM DER_595_HETA DER_595_HETB DER_628_HETA DER_628_HETB DER_630_HOM DER_634_HOM JAK_PS355_HETA JAK_PS355_HETB JAK_PS388_HOM KOH_PES200_HOM KOH_THA244_HOM KOH_THA257_HOM KOH_THA258_HOM LEM_XT014_HOM LEM_XT020_HOM LEM_XT021_HOM LEM_XT029_HOM LEM_XT084_HOM LEM_XT123_HOM LEM_XT157_HOM LEM_XT163_HETA LEM_XT163_HETB LEM_XT172_HOM LEM_XT175_HOM LEM_XT178_HOM May083_HETA May083_HETB May137_HETA May137_HETB May142_HOM May154_HOM May162_HOM PAT_THA030_HOM PAT_THA052_HOM PAT_THA054_HETA

PAT_THA054_HETB PES200_HOM PES471_HOM PES482_HOM PES530_HOM PHU_THA112_HOM PHU_THA113_HOM PHU_THA114_HOM PHU_THA140_HOM SE02_HETA SE02_HETB SE03_HOM SE04_HETA SE04_HETB SE07_HOM SE08_HETA SE08_HETB SE09_HOM SE10_HETA SE10_HETB SE11_HOM SE12_HOM SE13_HOM SE14_HETA SE14_HETB SE15_HETA SE15_HETB SE17_HETA SE17_HETB SE18_HETA SE18_HETB SE21_HOM SE23_HETA SE23_HETB SE24_HETA SE24_HETB SE27_HOM SE28_HETA SE28_HETB SE29_HETA SE29_HETB SIN021_HOM SIN026_HOM SIN030_HOM SPER_501_HOM SPER_502_HOM SPER_522_HOM SPER_526_HOM SPER_529_HOM SPER_530A_HETA SPER_530A_HETB SPER_531_HETA SPER_531_HETB SPER_534_HOM SPER_536_HETA SPER_536_HETB SPER_539_HETA SPER_539_HETB SPER_545_HETA SPER_545_HETB SPER_547_HETA SPER_547_HETB SPER_548_HOM SPER_549_HETA SPER_549_HETB SPER_550_HETA SPER_550_HETB SPER_551_HOM SPER_555_HOM SPER_562_HETA SPER_562_HETB SPER_563_HOM SPER_564_HOM SPER_567_HETA SPER_567_HETB SPER_568_HOM SPER_574_HOM SPER_576_HOM SPER_577A_HOM SPER_578_HOM TAN04_HOM TAN05_HOM TAN07_HOM TAN08_HOM TAN12_HOM TAN13_HOM TIO030_HOM TIO032_HOM TIO036_HOM VIE_PQ09_HOM VIE_PQ10_HETA VIE_PQ10_HETB VIE_PQ11_HETA VIE_PQ11_HETB
Group[3] n: 5 ;id: CUR_XMC11_HOM SE05_HOM SE19_HETA SE19_HETB SE20_HOM
Group[4]n:65;id: DER_598A_HOM DER_599_HETA DER_599_HETB DER_621_HETA DER_621_HETB DER_623_HETA DER_623_HETB DER_659_HOM DER_662_HOM JAK_PS197_HOM JAK_PS218_HOM JAK_PS219_HETA JAK_PS219_HETB JAK_PS225_HOM JAK_PS233_HETA JAK_PS233_HETB JAK_PS302_HETA JAK_PS302_HETB JAK_PS324_HOM JAK_PS336_HOM JAK_PS394_HOM LEM_XT005_HETA LEM_XT005_HETB LEM_XT007_HOM LEM_XT009_HETA LEM_XT009_HETB LEM_XT040_HETA LEM_XT040_HETB LEM_XT071_HETA LEM_XT071_HETB LEM_XT126_HETA LEM_XT126_HETB LEM_XT152_HETA LEM_XT152_HETB LEM_XT166_HETA LEM_XT166_HETB LEM_XT176_HOM PAT_THA031_HOM PAT_THA036_HOM PAT_THA070_HOM PAT_THA071_HOM SIN018_HOM SIN029_HOM SIN032_HOM SPER_503_HOM SPER_506_HOM SPER_507_HOM SPER_508_HETA SPER_508_HETB SPER_512_HOM SPER_521_HETA SPER_521_HETB SPER_524_HOM SPER_540A_HOM SPER_552_HOM SPER_554_HOM SPER_570_HOM SPER_572_HETA SPER_572_HETB SPER_573_HETA SPER_573_HETB SPER_575_HOM SPER_579_HOM SPER_580_HOM SPER_581_HOM
Group[5] n: 36 ;id: DER_598B_HETA DER_598B_HETB DER_600_HOM DER_601_HETA DER_601_HETB DER_602_HOM DER_609_HETA DER_609_HETB DER_624_HOM DER_627_HETA DER_627_HETB JAK_PS310_HOM LEM_XT003_HETA LEM_XT003_HETB PAT_THA040_HETA PAT_THA040_HETB PAT_THA053_HETA PAT_THA053_HETB PES473_HOM PES480_HOM PES485_HOM PHU_THA085_HOM SIN023_HETA SIN023_HETB SPER_537_HOM SPER_538_HOM SPER_541_HOM SPER_560_HETA SPER_560_HETB SPER_561_HOM TIO037_HOM TIO038_HETA TIO038_HETB TIO039_HOM VIENORTH_VIE034_HETA VIENORTH_VIE034_HETB
Group[6] n: 4 ;id: JED100_HOM JED135_HOM JED178_HOM JED188_HOM
Group[7] n: 1 ;id: PES481_HOM

