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Extensive cryptic species diversity and fine-scale endemism in the marine red alga *Portieria* in the Philippines

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We investigated species diversity and distribution patterns of the marine red alga *Portieria* in the Philippine archipelago. Species boundaries were tested based on mitochondrial, plastid and nuclear encoded loci, using a general mixed Yule-coalescent (GMYC) model-based approach and a Bayesian multilocus species delimitation method. The outcome of the GMYC analysis of the mitochondrial encoded *cox*2-3 dataset was highly congruent with the multilocus analysis. In stark contrast with the current morphology-based assumption that the genus includes a single, widely distributed species in the Indo-West Pacific (*Portieria hornemannii*), DNA-based species delimitation resulted in the recognition of 21 species within the Philippines. Species distributions were found to be highly structured with most species restricted to island groups within the archipelago. These extremely narrow species ranges and high levels of intra-archipelagic endemism contrast with the wide-held belief that marine organisms generally have large geographical ranges and that endemism is at most restricted to the archipelagic level. Our results indicate that speciation in the marine environment may occur at spatial scales smaller than 100 km, comparable with some terrestrial systems. Our finding of fine-scale endemism has important consequences for marine conservation and management.

1. Introduction

A traditional view holds that many marine species have large geographical ranges because of their high dispersal potential by pelagic larval stages or propagules, and a lack of apparent dispersal barriers in the sea [1,2]. The reef-rich and diverse Indo-West Pacific (IWP) biogeographic region harbours species with particularly wide ranges and little archipelagic endemism [3]. Most species are characterized by subbasinal distributions, being widespread in either the Indian or Pacific Ocean, but a substantial fraction spans both oceans [4].

However, the view that most marine species have broad ranges is being challenged. Molecular evidence indicates that some marine species comprise several cryptic species (i.e. distinct species that are erroneously classified under a single species due to the lack of clear morphological differences) [2,5,6]. While in some studies cryptic species themselves were found to be wide-ranging [7,8], there is accumulating evidence for the prevalence of geographically restricted cryptic species in allegedly widely distributed marine organisms [9–15]. Studies focusing on marine invertebrates and fish have shown that proportions of range-restricted species are highest in remote peripheral archipelagos [16,17], but archipelagic endemism has also been demonstrated in the central IWP [4,9,18].

The spatial scale at which neutral diversification occurs is a function of dispersal distance and frequency [4,19]. High dispersal or gene-flow will generally prevent diversification and result in large geographical ranges, while ineffective dispersal will result in the absence of species in a given locality. At intermediate levels of dispersal, populations may diverge to form new species.

Consequently, many studies focus on the link between intrinsic dispersal capacity (e.g. pelagic larval duration, behaviour and ecology), geographical range and diversification [20–22]. The observation that the Society Islands and Tuamotu harbour their own endemic gastropod species led Meyer *et al*. [9] to conclude that speciation in the marine environment may act at much more local scales than previously anticipated. Despite indications for archipelagic endemism there are no reports of intra-archipelagic endemism of shallow-water marine organisms, suggesting that dispersal within archipelagos is too high to allow diversification. This contrasts markedly with terrestrial organisms, for which intra-archipelagic endemism is common [23–25].

Compared with marine invertebrates and fish, marine macroalgae are considered poor dispersers [26–30]. The spores or zygotes of most marine macroalgae are typically short-lived and often negatively buoyant [31]. A few species have high dispersal potential by detached and floating reproductive thallus fragments that act as propagules. In the absence of such propagules, the limited dispersal capacity of most macroalgae may reflect strongly on diversity patterns and the spatial scale at which speciation takes place. Several morphologically well-circumscribed and easily recognizable macroalgal species are known to have restricted distributions [31,32]. Moreover, wide distribution ranges of many algae are an artefact of pervasive cryptic diversity [10,33–37].

We aim to assess species distribution patterns of marine plants in the Philippine archipelago, focusing on the red macroalgal genus *Portieria*. The genus is a member of the red algal family Rhizophyllidaceae, which, next to *Portieria*, includes the (sub)tropical and species-poor genera *Contarinia*, *Nesophila* and *Ochtodes* [38]. *Portieria* typically forms bushy plants, up to 15 cm high, composed of flattened fronds with a typical branching pattern (figure 1), which makes the genus easily recognizable in the field. Like most red seaweeds, *Portieria* is characterized by a complex, triphasic life cycle, which includes two free-living stages of different ploidy levels—a diploid (tetrasporophyte) stage and a haploid (gametophyte) stage—as well as a diploid carposporophyte stage, which develops on the female gametophyte [38]. The genus is broadly distributed in the Indo-West Pacific and is common on coral reefs in the Philippines where it occurs from the shallow subtidal to 40 m depth. Owing to its secondary metabolic compounds, *Portieria* persists even in areas with extensive grazing by herbivorous fish [39].

There is considerable uncertainty as to the number of species in the genus. Seven species names are currently accepted in AlgaeBase [40]. Wiseman [41], who conducted the only comprehensive morphological study of *Portieria*, recognized only one species, *P. hornemannii*. Other authors have expressed doubts on this highly restricted view and have recognized a varying number of species [42,43]. Here we use multilocus DNA sequence data to test species boundaries and assess patterns of diversity and distribution.

We focus on the Philippine archipelago as a suitable study region. The archipelago is situated in the northern corner of the Coral Triangle, a region that is known as a global centre of marine biodiversity [44]. The Philippines has a complex geometry, with multiple islands, passages and basins that are interconnected by ocean currents and human activities [45,46]. This high connectivity between islands and basins would suggest the potential for high dispersal and homogeneous species distribution patterns of marine species within the Philippines, which contrasts with the exceptional regional endemism observed for terrestrial habitats [47,48].

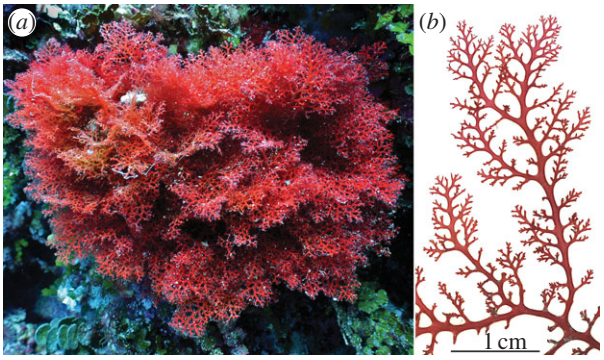

Figure 1. *Portieria hornemannii*. (*a*) Plant growing in the shallow subtidal (image courtesy of David Burdick). (*b*) detail of branches.

2. Material and methods

(a) Sampling

A total of 265 *Portieria* specimens were collected by snorkelling or SCUBA diving from 25 sites (approx. 100 m long stretches of coastline) throughout the Philippines (see the electronic supplementary material, figure S3). Specimens are vouchered in the Ghent University Herbarium (GENT) or the Institute of Environmental and Marine Sciences at Silliman University. A list of specimens with collection data and GenBank accession numbers is provided in the electronic supplementary material, table S1.

(b) DNA sequencing

Total genomic DNA was extracted from ethanol-preserved specimens using a modified CTAB method [35]. We amplified three unlinked loci: the mitochondrial encoded *cox*2 gene (partial) and *cox*2-3 spacer (approx. 320 bp, further referred to as '*cox*2-3'), the chloroplast encoded *rbc*L gene (partial) and *rbc*L-*rbc*S-spacer (approx. 540 bp, further referred to as '*rbc*L') and nuclear elongation factor 2 (EF2) gene (approx. 610 bp; see the electronic supplementary material, table S3). PCR conditions and primer sequences are detailed in the electronic supplementary material, table S2. PCR products were purified using ExoSAP-IT (USB) and sequenced using BigDye v. 3.1 (Applied Biosystems) on an ABI 3100 automated DNA sequencer. Sequences were submitted to EMBL/GenBank under accession numbers HF546576–HF546974. DNA sequences were aligned using ClustalW [49]. Sequence matrix information is given in the electronic supplementary material, table S3.

(c) DNA-based species delimitation and phylogeny

Species boundaries were tested using analyses of single- and multilocus genetic data. The single-locus DNA trees were based on separate analyses of 67 *cox*2-3, *rbc*L and EF2 sequence alignments and were analysed using a GMYC model approach [50,51]. This likelihood-based method aims to detect species boundaries by optimizing the transition from interspecific branching (Yule model) to intraspecific branching (neutral coalescent model) on an ultrametric tree. Ultrametric trees were obtained by Bayesian analyses in Beast v. 1.6.1 [52], under a GTR + I + G model with divergence times estimated under an uncorrelated lognormal relaxed molecular clock model [53] and the constant population size coalescent as the tree prior. Markov chain Monte Carlo (MCMC) analyses were run for 50 million generations, sampling every 10 000 generations. The output was diagnosed for convergence using Tracer v. 1.5 [54], and summary statistics and trees were generated using

the last 40 million generations with TreeAnnotator v. 1.5.3. GMYC analyses were performed on the consensus trees under the single-threshold model, using the SPLITS package [51] in R [55].

Recent studies have raised concerns about the accuracy of defining species boundaries based on single-locus data because of problems related to incomplete lineage sorting, resulting in gene tree–species tree incongruence [56–59]. Therefore, we tested species boundaries using multilocus data, including the *cox*2-3, *rbc*L and EF2 datasets, which contained the same 67 specimens initially used for the GMYC analyses. Individual gene trees were constructed using Bayesian inference (BI) and maximum likelihood (ML). Bayesian trees were estimated using MrBayes v. 3.1.2 under a GTR + I + G model [60]. Two parallel runs, each consisting of four incrementally heated chains, were run for five million generations, sampling every 1000th generation. Convergence of log-likelihoods and parameter values was assessed in Tracer v. 1.4 [54]. A burn-in sample of 1000 trees was removed before constructing the majority rule consensus tree. ML trees and associated rapid bootstrap support were obtained using the GTR + CAT model in the program RAxML v. 7.2.8 [61].

Individual gene trees were visually inspected to identify reciprocal monophyletic groups that were concordantly supported by the three loci as evidence for species boundaries in a genealogical concordance approach [62]. Genealogical concordance of unlinked loci is expected to be present among well-diverged lineages. However, the criteria of reciprocal monophyly and strict congruence will probably fail to detect boundaries between recently diverging species [63]. Therefore, we used a recently developed Bayesian method, BP&P, which aims to detect signals of species divergence in multiple gene trees, even in the absence of monophyly, based on models combining species phylogeny and the ancestral coalescent process, and assuming no admixture following the speciation event [64]. The method calculates posterior probabilities of potential species delimitations given a user-supplied species tree and multilocus sequence data. A species tree was estimated using *BEAST [65], a Bayesian method that coestimates multiple gene trees embedded in a shared species tree using multispecies coalescent estimates. For the species tree estimation, specimens were *a priori* assigned to species based on the results of the *cox*2-3 GMYC results. *BEAST analysis was performed with unlinked models for the three loci: GTR + I + G substitution model, uncorrelated log-normal relaxed molecular clock model and Yule species tree model. Two independent MCMC analyses were run for 20 million generations. Convergence of the runs was assessed by visual examination of parameter traces and marginal densities using Tracer, and the posterior distribution of trees was summarized from the MCMC output excluding the first 10 per cent as burn-in. BP&P v. 2.0 was run using 'algorithm 0', fine-tuning parameter $\varepsilon = 5$, and with each species delimitation model assigned equal prior probability. Because the prior distributions on the ancestral population size (Θ) and root age (τ_0) can affect the posterior probabilities for models, with large values for Θ and small values for τ_0 favouring conservative models containing fewer species [64], we ran the analyses with three different combinations of prior, as proposed by Leaché & Fujita [66]. Two independent reversible jump MCMC analyses were run for 100 000 generations. In order to verify whether the GMYC analysis of the *cox*2-3 dataset might underestimate species diversity, we reran the BP&P analyses with the GMYC clusters of clade V1 and clade B subdivided into multiple entities.

Divergence times were estimated based on the *cox*2-3 calibration reported by Zuccarello & West [67], being 0.25–0.3 per cent sequence divergence per Myr. Although this calibration has been tested in other red algal groups [68], the estimated divergence times have to be interpreted with care because of possible rate variations across red algal lineages and uncertainties regarding the calibration point used (Panama Isthmus). Several recent studies have found that divergence times between trans-isthmian species pairs are often much older than 3 Ma, indicating that in these cases the final closure of the Panama Isthmus may not have been the initial cause of divergence between populations [10,69,70].

(d) Species ranges and total species richness

Geographical ranges of species were estimated using locality data of 265 specimens, identified based on *cox*2-3 sequence data. *Cox*2-3 was the marker of choice because it was more variable and showed faster coalescence within species lineages compared with the other two loci, and thus more accurately reflected species boundaries in *Portieria* (see the electronic supplementary material, table S3). Species richness based on our sampling was estimated using the incidence-based first-order jackknife estimator implemented in EstimateS v. 8.2 [71]. The values derive from an extrapolation of diversity based on the frequency of observing species restricted to a single locality. We provide a rough estimate of the total *Portieria* diversity in the Philippines by extrapolating the richness estimates beyond the current sample size. Therefore, various asymptotic functions (power, exponential, Monod, negative exponential, asymptotic regression, rational and Lecointre function) were fitted through the data using nonlinear least-squares estimates. Model selection was based on the Akaike information criterion (AIC) [72,73]. Nonlinear regressions and model comparison were carried out in R.

3. Results

(a) Species delimitation and phylogeny

In the single-locus method for species delimitation, branch lengths in the ultrametric gene trees were analysed to test species boundaries (figure 2). For the *cox*2-3 tree, the likelihood of the GMYC model was significantly higher ($p < 0.001$) than that of the null model of uniform coalescent branching rates (see the electronic supplementary material, table S3). In the *rbc*L and EF2 trees, the difference between these two models was marginally significant ($0.01 < p < 0.05$). The results indicate the presence of multiple species in our sample. For the *cox*2-3 data, the model estimates 21 species clusters, with a narrow confidence interval ranging from 20 to 25. Fitting the model on the *rbc*L and EF2 trees resulted in slightly fewer species clusters (20 and 18, respectively), with wider confidence intervals (see the electronic supplementary material, table S3).

Genealogical concordance was assessed between the *cox*2-3, *rbc*L and EF2 trees. Eleven reciprocally monophyletic clades were concordantly recovered in the three gene genealogies (open circles in figure 2), in addition to three singletons (B33, B35 and SP3). Incongruence was restricted to the V1 and V32 clades. The five V1 species clusters in the *cox*2-3 tree (V1A-E) were either para- or polyphyletic in the EF2 and *rbc*L trees. The V32 clade in the *cox*2-3 and EF2 gene trees was paraphyletic in the *rbc*L tree.

The multilocus Bayesian species delimitation results are indicated in the species phylogeny (guide tree) in figure 3*a*. When assuming that the 21 GMYC clusters correspond to species, the Bayesian species delimitation supported the guide tree with high speciation probabilities (1 or 0.99) on all nodes. The results were stable over a broad range of

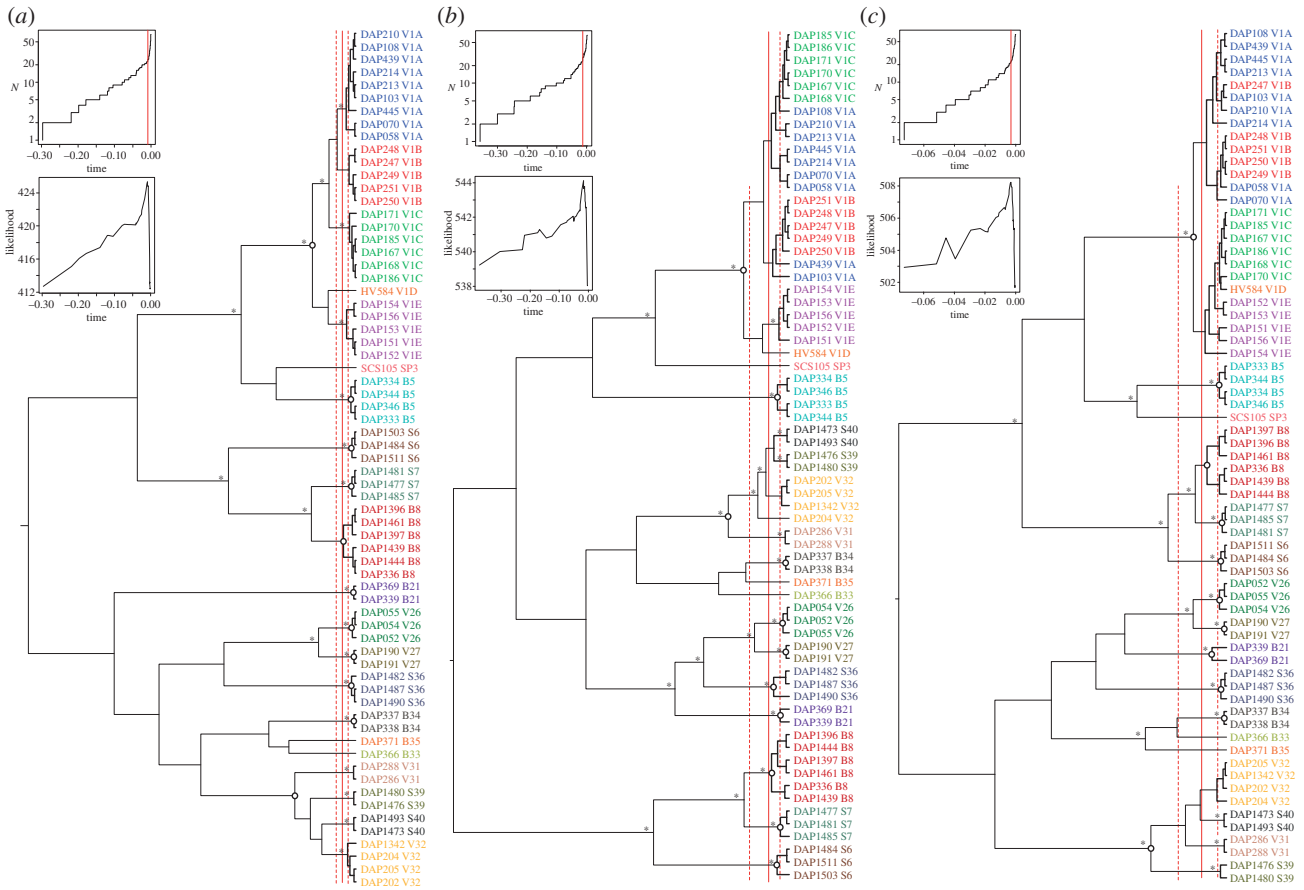

Figure 2. GMYC-based species delimitation based on the (*a*) *cox*2-3, (*c*) *rbc*L and (*b*) EF2 gene trees. Ultrametric trees were obtained by Bayesian relaxed molecular clock analyses. Open circles indicate reciprocal monophyletic terminal clades that were concordantly recovered in the three gene trees. Branches supported by posterior probabilities greater than 0.95 and ML bootstrap support greater than 80 are indicated with an asterisk. Lineages-through-time (left) and single-threshold GMYC likelihood profile plots (right) are shown for the three gene trees. The solid red lines indicate the maximum-likelihood transition point of the switch in branching rates from interspecific to intraspecific events, as estimated by the GMYC model; the confidence intervals are indicated by the dashed red lines. Results of the GMYC analyses are summarized in electronic supplementary material, table S3.

prior settings relating to effective population size and root ages. The Bayesian species delimitation did not support guide trees where the number of species had been increased (see the electronic supplementary material, figure S1), indicating that the species diversity was not underestimated by the GMYC analysis of the *cox*2-3 dataset.

Divergence time estimates (see the electronic supplementary material, figure S2) suggest that *Portieria* may have originated as early as the Late Eocene (around 35 Ma), and mainly diversified in the Oligo-Miocene, with possibly recent speciation events in the Plio-Pleistocene in clade V1.

(b) Species distribution patterns

Of the 21 identified *Portieria* species in the Philippines, not a single one was found throughout the study area (see the electronic supplementary material, table S4 and figure S3; figure 3*b*). Twelve species were confined to one or two sites, while only four species were found at more than three sites (see the electronic supplementary material, figure S3). With three exceptions (V32, S6 and S39), specimens of a species were always found within 80 km from each other. The diversity found in Batanes was very distinct from the other sites, with none of the species occurring outside the area. The central Visayas shared a single species (V32) with Samar (approx. 350 km). Only two species (S6 and S39) are shared between Sorsogon and Samar (approx. 250 km).

In several sites and on most islands, different *Portieria* species occurred in sympatry. In many cases, these species were phylogenetically distantly related; for example, four species from four different clades co-occurred in Chavayan (Batanes; see figure 3*b* and electronic supplementary material, figure S3). Sister or closely related species were also found in sympatry. A striking example is the central Visayas, which harboured the closely related species V1A, V1B and V1C, along with the more distantly related species V32, V26 and V27.

Given the narrow geographical range of most species, it is highly unlikely that our sampling strategy resulted in a complete coverage of *Portieria* diversity. Extrapolating richness beyond the current sampling effort (i.e. 25 localities) to its asymptote resulted to an estimate of 45 (± 5) species (see the electronic supplementary material, figure S4).

4. Discussion

Molecular reassessment of the diversity of the red algal genus *Portieria* in the Philippines demonstrates that previous morphology-based species circumscriptions dramatically underestimated the diversity in the region. In contrast to the assumed presence of one to three species in the Philippines [74], this study showed the existence of at least 21 cryptic *Portieria* species. Below we discuss the implication of our

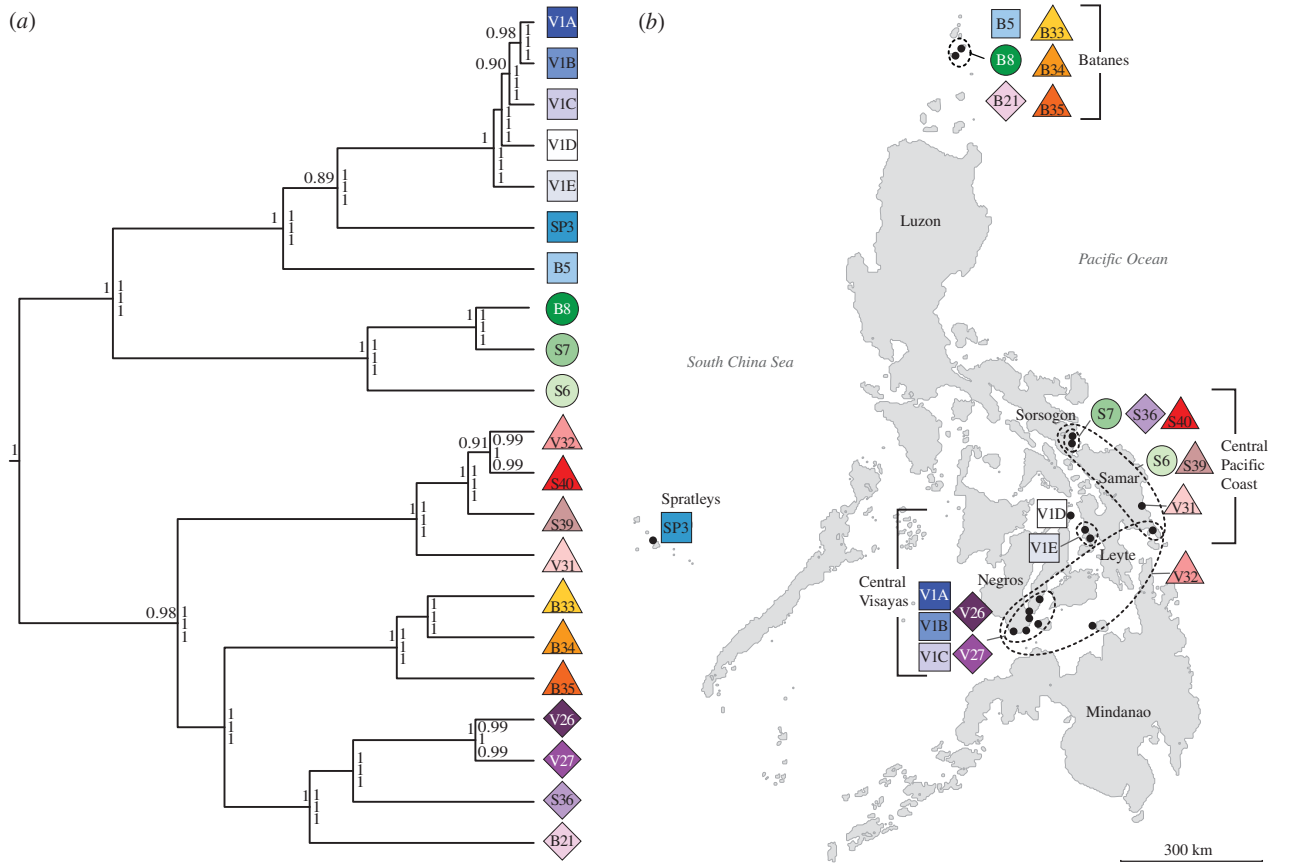

Figure 3. (*a*) Bayesian species tree inferred using *BEAST with numbers above branches representing posterior probability values (only values greater than 0.85 are shown). The speciation probabilities, analysed using BP&P, are provided for each node under three combinations of priors: top, assuming large population sizes $\Theta \sim G(1, 10)$ and deep divergences $\tau_0 \sim G(1,10)$; middle, assuming small population sizes $\Theta \sim G(2,2000)$ and shallow divergences $\tau_0 \sim G(2,2000)$; bottom, assuming large populations sizes $\Theta \sim G(1,10)$ and relatively shallow divergences $\tau_0 \sim G(2,2000)$. (*b*) Geographical distributions of *Portieria* species within the Philippines. Most species exhibit intra-archipelagic endemism; only three species are more widely distributed within the archipelago (S6, S39 and V32). Detailed species distributions are provided in the electronic supplementary material, figure S3.

results with respect to diversity estimates, intra-archipelagic endemism and speciation in the marine environment, and the consequences of small species ranges in relation to marine conservation.

(a) High levels of cryptic species diversity

Unveiling cryptic diversity in marine macroalgae is not uncommon [10,14,33–36], but the degree to which we do so here is unprecedented. The GMYC model approach based on the mitochondrial encoded *cox*2-3 spacer region resulted in 21 clusters of specimens which were reciprocally monophyletic and sufficiently distinct from other such lineages to regard them as separately evolving lineages ('species') [51]. Comparing GMYC results of the *cox*2-3 data with the chloroplast encoded *rbc*L and nuclear encoded EF2 gene trees, we observed a large degree of congruence, lending additional support to regard them as species [62]. Comparison of the mitochondrial, plastid and nuclear gene trees indicated that coalescence is faster for organellar DNA than for the nuclear encoded locus, an observation that is congruent with population genetic theory, which predicts that the effective population size of nuclear DNA is four times as high for diploid organisms compared with organellar DNA, which is haploid and uniparentally inherited [63].

The congruence that we observe between the single-locus (separate analyses of *cox*2-3, *rbc*L and EF2 data) and multilocus species delimitation analyses is an important finding. It gives credit to popular barcoding initiatives that mostly rely on some sort of genetic exclusivity criterion (e.g. reciprocal monophyly and genetic distance) and single-locus datasets (usually a mitochondrial marker). It is generally safe to ignore problems relating to incomplete lineage sorting and ancestral polymorphism in lineages that diverged long enough from one another. Given enough time, these species will be recognized using the criterion of reciprocal monophyly at each of the sampled loci [56,63,75]. Recently diverged taxa, however, are more likely to go unnoticed using exclusivity criteria (i.e. they have a higher false-negative rate). In such cases, it is important to select markers that show fast coalescence. In our datasets, the mitochondrial locus appeared to have a somewhat higher coalescent rate when compared with the chloroplast locus, but these results may be stochastic as there are no apparent differences in heritability of both organelle genomes.

(b) Intra-archipelagic endemism and fine spatial scale of speciation

Species distributions were found to be highly structured within the Philippine archipelago. For example, none of the *Portieria* species were shared between the northern and central collecting sites, and there was limited species overlap between the central Visayan and the Central Pacific Coast sites (figure 3*b*). The distribution of species V32 presents

the largest geographical range of an individual *Portieria* species in the region, which stretches slightly over 300 km. At smaller spatial scales, similarity in species composition was higher.

Our study adds to the growing body of evidence that the geographical ranges of many marine species are more restricted then previously assumed. For example, the gastropod *Astralium rhodostomum*, with a perceived wide geographical range across the IWP, was found to consist of at least 30 cryptic species, all but one confined to a single archipelago [9]. Similar observations in molluscs, crustaceans and fishes indicate that archipelagic endemism is common in diverse groups of marine organisms [4,18,76,77].

The distribution pattern of *Portieria* in the Philippines demonstrates that species-level diversity may be geographically structured at even smaller scales (less than 100 km) within a single archipelago. The range sizes found in this study are unseen for marine benthic species, and are reminiscent of distribution patterns in terrestrial organisms where examples of intra-archipelagic endemism abound [24,25]. These results are even more interesting in view of a high physical connectivity between islands and basins [45,46], which would intuitively facilitate dispersal of marine organisms, resulting in much larger species distributions.

Our observation of fine-scale biogeographic structure suggests that dispersal limitation and speciation of marine macroalgae in archipelagos may act at much smaller geographical scales than is commonly assumed. Although several studies on marine invertebrates and fish have indicated population genetic structure among and within basins of the Philippine archipelago [78–80] and the Indo-Malayan region in general [81,82], the geographical scale at which these populations diverge is considerably larger compared with *Portieria*. Identical physical oceanographic processes probably yield markedly different results in organisms with different dispersal and/or life cycle strategies. As in many other seaweeds, the apparent absence of propagules in *Portieria* probably limits dispersal capacity, and this may reflect the spatial scale at which speciation takes place.

The high species diversity of *Portieria* in the Philippines is in line with the high biodiversity of other marine groups in the Coral Triangle [44]. Several hypotheses have been put forward to explain the high biodiversity in the region, including elevated local speciation rates (centre of origin hypothesis) and accumulation of species formed elsewhere (centre of accumulation hypothesis) [83,84]. Speciation at small spatial scales of the V1 clade may be attributed to the complex geography of the region and possibly took place during Pleistocene periods of glacially lowered sea level when seas (e.g. the South China, Sulu, Philippine, Celebes, Molucca and Banda seas) became landlocked, resulting in prolonged geographical isolation [2,44,83,85]. Accumulation of species may have resulted from integration of distinct biotas by tectonic movement over the past 50 million years, and more recent dispersal events [44,86–88]. The antiquity of the main *Portieria* clades (Oligo-Miocene) and the presence of more recent diversifications within these clades (Plio-Pleistocene) suggest that the evolution of *Portieria* in the Philippines may be the product of multiple processes, including accumulation and/or diversification over time frames of tens of millions of years, and more recent speciation events. Evidently, these hypotheses will need to be tested by additional taxon sampling and phylogenetic analysis of *Portieria* across its geographical range in the Indo-West Pacific.

(c) Marine conservation

Our findings have important consequences for marine conservation management in threatened reef ecosystems, such as those in the Philippine archipelago [89]. A traditional view held that marine species are more resilient to extinction because of their large geographical ranges, and therefore conserving a limited number of marine biodiversity hotspots would save most species from extinction [1,90]. The finding of fine-scale endemism implies that conservation efforts in archipelagos will need to focus on all islands rather than on a few presumed biodiversity hotspots [9].

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