Extreme mitochondrial variation in the Atlantic gall crab *Opecarcinus hypostegus* (Decapoda: Cryptochiridae) reveals adaptive genetic divergence over *Agaricia* coral hosts

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The effectiveness of migration in marine species exhibiting a pelagic larval stage is determined by various factors, such as ocean currents, pelagic larval stage duration and active habitat selection. Direct measurement of larval movements is difficult and, consequently, factors determining the gene flow patterns remain poorly understood for many species. Patterns of gene flow play a key role in maintaining genetic homogeneity in a species by dampening the effects of local adaptation. Coral-dwelling gall crabs (Cryptochiridae) are obligate symbionts of stony corals (Scleractinia). Preliminary data showed high genetic diversity on the COI gene for 19 *Opecarcinus hypostegus* specimens collected off Curaçao. In this study, an additional 176 specimens were sequenced and used to characterize the population structure along the leeward side of Curaçao. Extremely high COI genetic variation was observed, with 146 polymorphic sites and 187 unique haplotypes. To determine the cause of this high genetic diversity, various gene flow scenarios (geographical distance along the coast, genetic partitioning over depth, and genetic differentiation by coral host) were examined. Adaptive genetic divergence across Agariciidae host species is suggested to be the main cause for the observed high intra-specific variance, hypothesised as early signs of speciation in *O. hypostegus*.

A central challenge in evolutionary biology is to establish the influence of spatial and ecological processes on the evolutionary patterns of species, including local adaptation, colonization and speciation. Gene flow is the genetically effective exchange of migrants among populations, depending on the rate of exchange and the migrants' fitness. Patterns of gene flow have a strong effect on the evolution of a species by dampening the genetic response to local selection, as they tend to make gene frequencies uniform among populations, whereas genetic drift and adaptation tend to diversify populations. It is easy to comprehend how in a terrestrial environment the landscape (e.g. mountains, rivers or forests) can act as a barrier to gene flow, and give rise to genetic divergence between conspecific populations. Understanding how genetic differentiation arises in a marine landscape is, however, a more challenging task. Consequently, the patterns of gene flow remain understudied for many marine species. Genetic methods are powerful tools to examine genetic connectivity among individuals and to determine the spatial population structure of marine species.

The population genetic structure in marine species can be affected by several mechanisms. Gene flow patterns may be proportional to geographic distance, whereby genetic differentiation increases with distance. Although oceanic currents can have a homogenizing effect on the genetic structure of populations, other geographical
Factors such as habitat discontinuity, local current systems and physical barriers can act as limitations to gene flow. Then again, gene flow may be higher among ecologically similar environments.

Many marine invertebrates exhibit a pelagic larval stage. The effectiveness of migration is determined by the duration of the pelagic larval phase and the strength of oceanic currents, together affecting the realized larval dispersal distance, as well as factors such as the survival and reproduction rate of the successfully dispersed larvae in a novel habitat. Because pelagic larvae can potentially disperse both horizontally and vertically, ecological differences over depth gradients, such as light, temperature and turbidity, may also give rise to different selection pressures resulting in genetic diversification in a marine environment. Correlations between genetic differentiation and depth distances have been measured for various corals; for instance, Pocillopora damicornis (Shaw and Hopkins, 1977) were collected off Curacao (n = 7). Opecarcinus hypostegus (Ellis and Solander, 1786) includes very shallow as well as deeper reefs down to at least 60 m. Transect data at 6 m, 12 m, and 18 m revealed a depth preference in O. hypostegus for the deeper reefs. Prevalence rates at 6 m were highest in Agaricia agaricites (Linnaeus, 1758) and at 12 m and 18 m highest in Agaricia lamarcki Milne Edwards and Haime, 1851. High genetic diversity was observed at the cytochrome-c oxidase I (COI) gene for the 19 collected specimens obtained from different localities along the Curacaoan coast, from various depths and five Agaricia coral hosts. Seventy-six polymorphic sites, resulting in a nucleotide diversity (π) of 0.02617 and a haplotype diversity (h) of 0.10 were retrieved (van der Meij, unpubl. data). These results were surprising, because most Indo-Pacific members of the Cryptochiridae show very low haplotype diversity at the COI gene across large distances and COI is most commonly used to infer phylogenetic relationships at species level, but see ref. 37.

The purpose of this study is to examine the possible barriers that affect the genetic structure of O. hypostegus in more detail. COI sequence data was used to characterize O. hypostegus population structure and infer patterns of O. hypostegus gene flow along the leeward side of Curaçao. Factors that are expected to limit gene flow and increase genetic differentiation at this small geographical scale include: (I) geographical distance along the leeward side of Curaçao, (II) geographic differentiation over depth, or (III) genetic differentiation between individuals inhabiting different Agaricia species.

### Results

#### Patterns of polymorphism.

A 675 base pairs long fragment of the COI region was sequenced for a total of 195 individuals (Table S1). Across all collection sites, 146 nucleotide sites were polymorphic, yielding 187 unique haplotypes (h = 0.9994). Of these, 123 were third codon position changes, along with 23 first codon position changes and zero second codon position changes. Overall nucleotide diversity (π) = 0.02558 (Table 1). Translation of the sequences to amino acid data revealed only five polymorphisms in five individuals (RMNH. Crus.D.57581, 57456, 57557, 57559 and 57476; Table S1), all at different positions of the sequence. An Automatic Barcode Gap Discovery (ABGD) analysis shows that only one Molecular Operational Taxonomic Unit is present in O. hypostegus.

#### Population structure.

**Geographic differentiation.** A Mantel test revealed an isolation-by-distance pattern off Curaçao for O. hypostegus, with a relationship between the genetic differentiation (Fst) (Table S2) and the geographic distance (km) between the collection sites (Table S3) (r = 0.1408, P = 0.0587) (Fig. 1A). Partitioning the Isolation By Distance (IBD) analysis into groups of individuals collected from the same agariciid coral species, Agaricia lamarcki (n = 117), Agaricia agaricites (n = 66), Agaricia humilis Verrill, 1901 (n = 7), Agaricia

### Table 1. Number of polymorphisms (P), number of unique haplotypes (H), indexes of neutrality Fu and Li’s F, and Tajima’s D, haplotype diversity (h) and nucleotide diversity (π) for O. hypostegus individuals sampled from each of the agariciid host coral species based on COI sequence data. The values for A. humilis and A. grahamae are based on low numbers. *Not significant (P > 0.05).
grahamae Wells, 1973 (n = 4) or Agaricia fragilis Dana, 1846 (n = 1), revealed a significant relationship between genetic differentiation ($\Phi_{st}$) and geographic distance (km) for individuals sampled from A. agaricites ($r = 0.5439, P = 0.0022$) (Fig. 1B). No significant relationship between genetic differentiation ($\Phi_{st}$) and geographic distance (km) was found, however, for individuals sampled from A. lamarcki ($r = -0.0356, P = 0.3085$) (Fig. 1C). For the individuals sampled from the remaining host coral species, A. humilis, A. grahamae and A. fragilis, population sample sizes were too small or too few populations were sampled to perform a valid IBD analysis (Table S1).

Depth differentiation. A mantel test was used to test for a relationship between genetic differentiation ($\Phi_{st}$) and the difference in depth of collection (Table S1) between each sample, but no significant relationship was found ($r = 0.1063, P = 0.1426$). Hence, there is no statistical evidence for genetic isolation over depth (Fig. 2A). Partitioning the IBD analysis into individuals collected from the same host coral species had no effect on the outcome. No significant relationship was found between genetic differentiation ($\Phi_{st}$) and depth for individuals sampled from A. agaricites ($r = -0.1604, P = 0.8599$) (Fig. 2B), nor for individuals sampled from A. lamarcki ($r = -0.0356, P = 0.3085$) (Fig. 1C). For the individuals sampled from the remaining host coral species, A. humilis, A. grahamae and A. fragilis, population sample sizes were too small or too few populations were sampled to perform a valid IBD analysis (Table S1).

Genetic subdivision between individuals collected from different host corals. Fu and Li’s F, and Tajima’s D were negative for the individuals sampled from the host coral species A. lamarcki, A. agaricites and A. grahamae, but not statistically significant (Table 1). An Analysis of Molecular Variance (AMOVA) indicated statistically significant genetic differentiation between individuals sampled from different host coral species (P < 0.00001) (Figs 3 and 4, Table 2). Pairwise Fst’s indicate significant genetic differentiation between the O. hypostegus individuals collected from different host coral species (Table 3). The strongest genetic differentiation was measured between A. humilis and A. agaricites (Fst 0.38309, P < 0.0001), followed by A. humilis and A. lamarcki (Fst 0.26133, P < 0.0001), A. agaricites and A. lamarcki (0.15726, P < 0.0001) and A. agaricites and A. grahamae (Fst 0.15070, P = 0.03604). Negligible differentiation was measured between A. grahamae and A. lamarcki (Fst 0.09234, P = 0.02703) (Table 3). No significant genetic differentiation was measured between individuals collected from A. grahamae (n = 4) and A. humilis (n = 7), possibly due to the small samples sizes. These results are in concordance with the median joining network (Fig. 3) that reveals a large mutation distance between individuals inhabiting A. humilis or A. agaricites and individuals collected from the other host corals. The largest mutation distance was found between O. hypostegus individuals inhabiting the coral hosts A. humilis and A. agaricites (Fig. 3).
groupings in the phylogenetic tree (Fig. 4) are in agreement with those of the haplotype network. The clade with *O. hypostegus* inhabiting *A. humilis* is retrieved with high support values and relatively long branch lengths. Within the large overall clade there are a few singletons, which are those individuals clustering closest to *A. humilis* in the haplotype network. Within the clade mostly associated with *A. agaricites*, little clustering is observed, which can be linked to the starlike structure in the network. Within the clade associated with *A. lamarcki*, more clustering is observed. The individuals inhabiting *A. grahamae* and *A. fragilis* are retrieved in various parts of the haplotype network and phylogenetic tree (Figs 3 and 4).

**Discussion**

**Patterns of polymorphism.** In the COI sequence data of the 195 *Opecarcinus hypostegus* specimens collected from Curaçao, 146 COI polymorphic sites were found and 187 unique haplotypes (Table 1). Strikingly, in Cryptochiridae collected from various locations in the Indo-Pacific hardly any polymorphic sites are present on the COI gene, even over distances as large as between the Indo-Malayan region and New Caledonia24, or the Red Sea and Japan36. Generally, COI sequence data shows high resolution at species level, and work well as a barcoding marker.

First and second COI codon positions are highly conserved, whereas third codon positions can evolve rapidly, making this locus a common choice for population genetics and phylogeography38,39. As expected, almost all variation in *O. hypostegus* inhabiting *A. humilis* is retrieved with high support values and relatively long branch lengths. Within the large overall clade there are a few singletons, which are those individuals clustering closest to *A. humilis* in the haplotype network. Within the clade mostly associated with *A. agaricites*, little clustering is observed, which can be linked to the starlike structure in the network. Within the clade associated with *A. lamarcki*, more clustering is observed. The individuals inhabiting *A. grahamae* and *A. fragilis* are retrieved in various parts of the haplotype network and phylogenetic tree (Figs 3 and 4).

The genetic diversity obtained in this study (mean $h = 0.9994$, mean $\pi = 0.02558$), from a very small geographic area, can be classified as an extreme level of intra-specific variance compared to the reported mean and median values for haplotype (0.63388 and 0.70130) and nucleotide diversity (0.00388 and 0.00356) for 23 animal species in a meta-analysis43, which showed a positive, non-linear relationship between the population-level estimates of $h$ and $\pi$. The values obtained in our study strongly deviate from their values, with $\pi$ being much higher. A combination of high nucleotide and haplotype diversities has been linked to large stable populations with a long evolutionary history and possible secondary contact between differentiated lineages44. In contrast, the negative
indexes of neutrality Fu and Li’s F, and Tajima’s D indicate a departure from neutral processes, which can be caused by demographic changes or selective events. Due to the non-significance of these values the hypothesis of neutrality can, however, not be rejected.

Small scale geographical genetic differentiation. The leeward side of Curacao is about 65 km long from southeast to northwest. For the seaweed Sargassum polyceratium Montagne, 1837, fine-scale differentiation was retrieved around Curacao, with bays showing significant differentiation from each other21. A Mantel test revealed a relationship between the genetic similarity of certain individuals and geographical distance for O. hypostegus (Fig. 1A). Although the relationship was weak and not highly significant, this suggests a genetic structure within O. hypostegus individuals living in close spatial proximity being more genetically similar than expected under a random distribution of genotypes. Splitting the sample into groups of individuals collected from the same host coral species increased both the magnitude and significance of the isolation-by-distance pattern for individuals inhabiting Agaricia agaricites (Fig. 1B). For A. lamarcki, no statistical evidence for isolation-by-distance was retrieved (Fig. 1C). This difference may be explained by the higher abundance of A. agaricites corals off Curacao, compared to A. lamarcki34, providing suitable habitat closer to the natal site of the O. hypostegus larvae settling on A. agaricites. Furthermore, A. lamarcki has a wider depth distribution than A. agaricites19, which may influence the isolation-by-distance results.

Genetic partitioning over depth. No statistical evidence was found for genetic differentiation over depth in O. hypostegus (Fig. 2A), at least not within the studied depth range of this study (5–38 m). Partitioning the analysis into groups of individuals collected from the same host coral had no effect on the outcome, and individuals inhabiting A. agaricites or A. lamarcki did not show any significant genetic differentiation over depth (Fig. 2B,C).

Due to the technical limitations of scientific diving, our sampling was restricted to a maximum of 38 m depth. The depth distribution of O. hypostegus is, however, known to extend to the mesophotic zone (ca. 60 m), where an O. hypostegus individual was observed inhabiting an A. lamarcki coral33. We may argue that sampling over a depth range that is at least twice as large as in the present study, would reflect the total O. hypostegus distribution more completely. This could increase the likelihood of revealing genetic differentiation over depth, because sampled individuals living over a larger depth distribution face more variable environmental conditions.
Genetic differentiation over coral hosts. Under an ecology-driven gene flow scenario, gene flow may be strongest among similar environments\(^6,45–49\). This pattern may arise through mechanisms such as selection and local adaptation that will disrupt the patterns of isolation-by-distance\(^50\) or as a consequence of selection against maladapted immigrants from different environments\(^6\). In our study, significant genetic subdivisions between individuals inhabiting different host coral species were observed (Tables 2 and 3; Figs 3 and 4). There was statistical evidence for diversification across host coral species (i.e. environment) in \(O.\) hypostegus, which is expected to be an important alternative strategy to direct competition for the same host in Cryptochiridae\(^23\).

Cryptochirid males “visit” females inhabiting separate galls or pits, dubbed the “visiting” mating system\(^29,51\). It is unclear how far a male gall crab can travel to find a female partner. Many male and female gall crabs can inhabit the same coral colonies, especially if these are large in size. If a male \(O.\) hypostegus mates with females on the same coral colony, a genetic preference for that coral host might end up getting fixed in a population. The study on

Figure 4. Phylogram of \(O.\) hypostegus based on the Bayesian consensus tree showing Bayesian inference support values (left) and maximum likelihood bootstrap support values (right). The numbers refer to the collection numbers of the material (Table S1), the colours indicate the coral host species and correspond with those in Fig. 3.
Among host species | Sum of squares | Variance component | % | P-value |
--- | --- | --- | --- | --- |
A. agaricites | 0.26133 (p < 0.0001) | 0.01924 (p < 0.0001) | 1.0000 | 0.00000 |
A. lamarcki | 0.15726 (p < 0.0001) | 0.01232 (p < 0.0001) | 0.5341 | 0.00000 |
A. humilis | 0.38309 (p < 0.0001) | 0.02843 (p < 0.0001) | 0.7130 | 0.00000 |
A. grahamae | 0.10726 (p = 0.10811) | 0.00000 (p = 0.10811) | 0.0000 | 0.10811 |

Table 2. Analysis of molecular variance (AMOVA) among and within the groups of O. hypostegus individuals sampled from the agariciid host coral species, A. lamarcki, A. agaricites, A. humilis, A. grahamae or A. fragilis.

| Source of variation | df | Sum of squares | Variance component | % | P-value |
--- | --- | --- | --- | --- | --- |
among host species | 3 | 189.236 | 1.689 | 17.49 | < 0.00001 |
within host species | 191 | 1522.359 | 7.970 | 82.51 | < 0.00001 |

Table 3. Matrix of the pairwise Fst’s indicating the magnitude of genetic differentiation between the groups of O. hypostegus individuals sampled from the agariciid host coral species, A. lamarcki, A. agaricites, A. humilis, A. grahamae or A. fragilis.

gall crab occurrence rates on the leeward side of Curaçao revealed significant higher O. hypostegus prevalence in A. lamarcki compared to A. agaricites and A. humilis, suggesting a preference for inhabiting A. lamarcki.

Phylogenetic results showed in the median joining network (Fig. 3) and phylogenetic tree (Fig. 4) support the genetic differentiation across host species in O. hypostegus, with distinct clustering of individuals inhabiting the hosts A. lamarcki, A. agaricites and A. humilis. In the haplotype network, the observed groupings show different patterns. A star-shaped burst pattern (interlinked haplotypes with few mutation steps between them) can be observed for the individuals inhabiting A. agaricites (Fig. 3). These patterns appear due to high numbers of low frequency alleles with small average pair-wise distances between them, and may be evidence of a recent expansion from a small number of ancestors. In addition, the nucleotide diversity of specimens inhabiting A. agaricites is lower in comparison to other coral hosts (Table 1). In an expanding population, haplotype diversity and number of polymorphic sites can quickly increase, while nucleotide diversity usually lags behind. Indeed, high levels of h with moderate to low levels of π have frequently been attributed to recent divergence in marine species. Over time, when a population stops expanding and starts to stabilize, nucleotide diversity will increase. Newly created low frequency haplotypes either increase in the population or are lost, which increases the average number of segregating sites between haplotypes over time. In the A. lamarcki grouping, a higher number of segregating sites is observed between the haplotypes (Fig. 3), suggesting that this population is stabilizing.

A study on the historical evolutionary patterns of cryptochirids has indicated that the phylogeny of coral gall crabs is directed by the evolution of their scleractinian hosts. For yet unknown reasons, Indo-Pacific gall crab species show stricter host-specificity patterns than their Atlantic counterparts. The congeners of O. hypostegus in the Indo-Pacific are highly host specific and are often associated with one or several closely related coral species. Presumably, the current genetic diversification across host corals found in O. hypostegus may not only result in a stronger local-adaptation to ecological differences between coral hosts over time, but might even be strong enough to eventually foster speciation.

**Concluding remarks**

The main objective of the present study was to examine which spatial and ecological factors influence Opecarcinus hypostegus gene flow off Curaçao and can explain the observed high genetic diversity at the COI gene. Factors that were expected to influence the Opecarcinus hypostegus gene flow patterns were examined; we found a weak relationship for geographical genetic differentiation (mostly for the host coral A. agaricites) and no evidence for genetic differentiation over depth. The observed clustering in the haplotype network and phylogenetic tree, however, suggests that adaptive divergence over the coral hosts is present. We hypothesise that this divergence is an early sign of (sympatric) speciation. This divergence might result in several closely related species of Opecarcinus inhabiting Agaricia corals in the Caribbean, in a similar way to its congeners inhabiting various closely related Agariciidae corals in the Indo-Pacific. To further test this hypothesis, data from additional markers and localities is needed.

**Materials and Methods**

**Field sampling.** During field surveys in 2013 (16 Oct–9 Nov) and 2014 (12 Mar–28 Apr), specimens of Opecarcinus hypostegus were collected by chiselling off a small piece of their agariciid coral host from depths between 3 and 38 m at 29 localities on the leeward side of Curaçao (Dutch Caribbean, southern part of the Caribbean Sea). Four samples were collected at ca. 20 m depth from the island Klein Curaçao, located approximately 10 kilometres southeast of Curaçao (Fig. S1, Table 4, S1). The corals were visually identified to species level during the surveys using field guides, Coralpedia (http://coralpedia.bio.warwick.ac.uk) and the Coral ID tool (http://www.researchstationcarmabi.org). The leeward side of Curaçao stretches some 65 km from southeast to northwest with an almost continuous coral reef, providing uninterrupted suitable habitat for gall crab larvae settlement in which no clear geographical barriers appear to occur. In total, 210 O. hypostegus gall crab samples were collected from five Agaricia host coral species. Crabs were preserved in ethanol (80% in 2013, 96% in 2014).
All collected specimens are deposited in the Crustacea collection of the Naturalis Biodiversity Center in Leiden (formerly Rijksmuseum van Natuurlijke Historie, collection coded as RMNH.Crus.D).

**DNA analyses.** For 195 specimens, the DNA was isolated from muscle tissue of the fifth pereiopod using the NucleoMag 96 Kit (Machery-Nagel) according to the manufacturer’s protocol for animal tissue. The coral hosts of these 195 individuals were: *A. agaricites* (*n* = 117), *A. humilis* (*n* = 66), *A. grahamae* (*n* = 4) and *A. fragilis* (*n* = 1). Polymerase chain reaction was carried out with the following conditions; PCR CoralLoad Buffer (containing 15 mM MgCl₂), 0.5 µL dNTPs (2.5 mM), 1.0 µL of each primer, LCO-1490 and HCO-21988, 0.3 µL Taq polymerase (15 units per µL), 18.7 µL of extra pure PCR water and 1.0 µL DNA template. Thermal cycling was performed by initial denaturation at 95°C for 3 min, followed by 39 cycles of 95°C for 10 s, 48°C for 1 min and 72°C for 1 min, and a final elongation step of 5 min at 72°C. PCR products were sequenced by BaseClear BV (Leiden, The Netherlands). Sequences were assembled and edited in Sequencher® v.5.3 (Gene Codes Corporation, Ann Arbor, MI USA) and aligned with ClustalW in BioEdit 7.2.557. Sequences are deposited in GenBank under accession numbers KU041838, and KY026220-KY026413 (Table S1).

**Molecular analyses.** The number of polymorphisms (P), nucleotide diversity (π), haplotype diversity (h), Fu and Li's F and Tajima's D values were calculated using DnaSP 5.10.01. Nucleotide diversity is defined as the average number of nucleotide differences per site between two randomly chosen DNA sequences. Haplotype diversity is defined as the probability that two randomly chosen haplotypes are different. The Fu and Li's F, and Tajima's D values are used to determine whether the population evolves neutrally.

Prior to the model-based phylogenetic analysis, the best-fit model of nucleotide substitution was identified for each gene partition by means of the corrected Akaike Information Criterion (AICc) calculated with MEGA 6.06, resulting in GTR+I+G as the most suitable model of nucleotide substitution. A maximum likelihood analysis (GTR+I+G; 1000 bootstraps) was carried out with Phymil 3.1 and using the Seaview platform. Bayesian inferences coupled with Markov chain Monte Carlo techniques (six chains) were run for 5,000,000 generations in MrBayes 3.2.6, with a sample tree saved every 1000 generations and the burnin set to 25%. Consensus trees were visualized in FigTree v.1.3.1.

A median joining haplotype network, to display the COI sequence variation, was build using PopART v.1.7 (http://popart.otago.ac.nz). For continuously distributed populations a Mantel's test between the genetic
Differentiation and geographical distance can detect an isolation-by-distance pattern. A Mantel’s test was performed in the program IBDWS v. 3.23 using Fst as a measure of genetic differentiation, which incorporates sequence distance information. Significance was determined by permuting the data 30,000 times. The population structure was described with an analysis of molecular variance method (AMOVA) implemented in ARLEQUIN. Significance was determined with 10,000 random permutations of the data. Arlequin was also used to calculate the pairwise Fst values between individuals collected from different host coral species.

The web version of ABGD was used to estimate the genetic distance corresponding to the difference between a speciation process versus intra-specific variation in O. hypostegus. Runs were performed using the default range of priors (pmin = 0.001, pmax = 0.10) using the JCG9 Jukes–Cantor measure of distance. The analysis involved 195 sequences with a total of 675 positions in the final dataset.

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K.M.v.T. and S.E.T.v.d.M. conceived the study, contributed material, and collected data in the field; K.M.v.T. analysed the majority of the data; K.M.v.T. and S.E.T.v.d.M. prepared final figures, and wrote the paper. Both authors approved the final version of the manuscript.

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