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# Phylogeography of the Christmas Island blue crab, *Discoplax celeste* (Decapoda: Gecarcinidae) on Christmas Island, Indian Ocean

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*The land crab,* Discoplax celeste *(Gecarcinidae) is endemic to Christmas Island in the Indian Ocean. Due to a freshwater-dependant life history, in which the megalopae migrate from the ocean up freshwater streams to their adult terrestrial/freshwater habitat,* D. celeste *inhabits only a few isolated locations on the island. This restricted distribution is one of a number of factors which has previously highlighted the vulnerability of this species to outside threats. A number of anthropogenic factors including the introduction of multiple invasive species and habitat destruction have led to drastic ecosystem change on Christmas Island. The aim of this study was to investigate whether the restricted geographical distributions of* D. celeste *populations contribute to significant genetic structuring across Christmas Island, with an objective to inform future conservation strategies for this species on Christmas Island. Fragments of the mitochondrial cytochrome oxidase I gene and the control region were sequenced from 95 individuals collected from all five locations on Christmas Island known to be inhabited by* D. celeste. *Analyses using analysis of molecular variance revealed no evidence of population sub-structuring, indicating that despite any geographical isolation, there is a single population of* D. celeste *on Christmas Island. This lack of population differentiation is probably explained by the oceanic dispersal of larvae, rather than terrestrial migration of* D. celeste. *Therefore, based on these results, for conservation purposes,* D. celeste *on Christmas Island can be considered a single management unit.*

**Keywords:** *Discoplax celeste*, Christmas Island, population genetics, freshwater, COI, control region, endemic

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## INTRODUCTION

The gecarcinid land crab *Discoplax celeste* Ng & Davie, 2012 is endemic to the Australian Territory of Christmas Island, Indian Ocean (10°28′S 105°38′E) (Figure 1A). Previously misidentified on Christmas Island as *Discoplax hirtipes* (Dana, 1851), recent taxonomic study has recognized this crab as a distinct new species (Ng & Davie, 2012). *Discoplax celeste* has a porcelain blue carapace and chelae and it was previously thought that this species on Christmas Island was an endemic colour form of *D. hirtipes* (Hicks *et al.*, 1990). Distributed throughout the Indo-West Pacific, *D. hirtipes* has a brown to purplish-brown carapace and chelae (Hicks *et al.*, 1990; Ng & Guinot, 2001). However, there are additional colour forms that have also been identified as *D. hirtipes*, consequently further taxonomic study is required (see Ng & Davie (2012) for a detailed discussion).

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Recent work on *D. celeste* (as *D. hirtipes* or *Cardisoma hirtipes* (Ng & Guinot (2001) separated *Discoplax* A. Milne-Edwards, 1867, from *Cardisoma* Latreille, 1828)) has shown that the need for long term nitrogen, ion and water balance drives this crab into association with freshwater, therefore limiting the local distribution of this species (Greenaway, 1989; Adamczewska & Morris, 1996; Turner, 2010). Thus, unlike the other two most obvious and abundant land crab species on Christmas Island, the Christmas Island red crab, *Gecarcoidea natalis* (Pocock, 1888) and the robber crab *Birgus latro* (Linnaeus, 1767), *D. celeste* is restricted in its distribution during the dry season (April–October) to areas of freshwater seepages and springs, usually well inland (>1 km from and 150 m above the ocean) (Dela-Cruz & Morris, 1997a, b; Morris, 2005). Due to the underlying geology (Barrett, 2001), these areas of freshwater and thus established populations of *D. celeste* are found at only a few places on the island: the area around the Dales streams in the west and the springs between Waterfall Bay and the Ravine, which includes Ross Hill Gardens, and Dolly Beach on the south-east coast (Hicks *et al.*, 1990; Turner *et al.*, 2011) (Figure 1B). There have also been unconfirmed

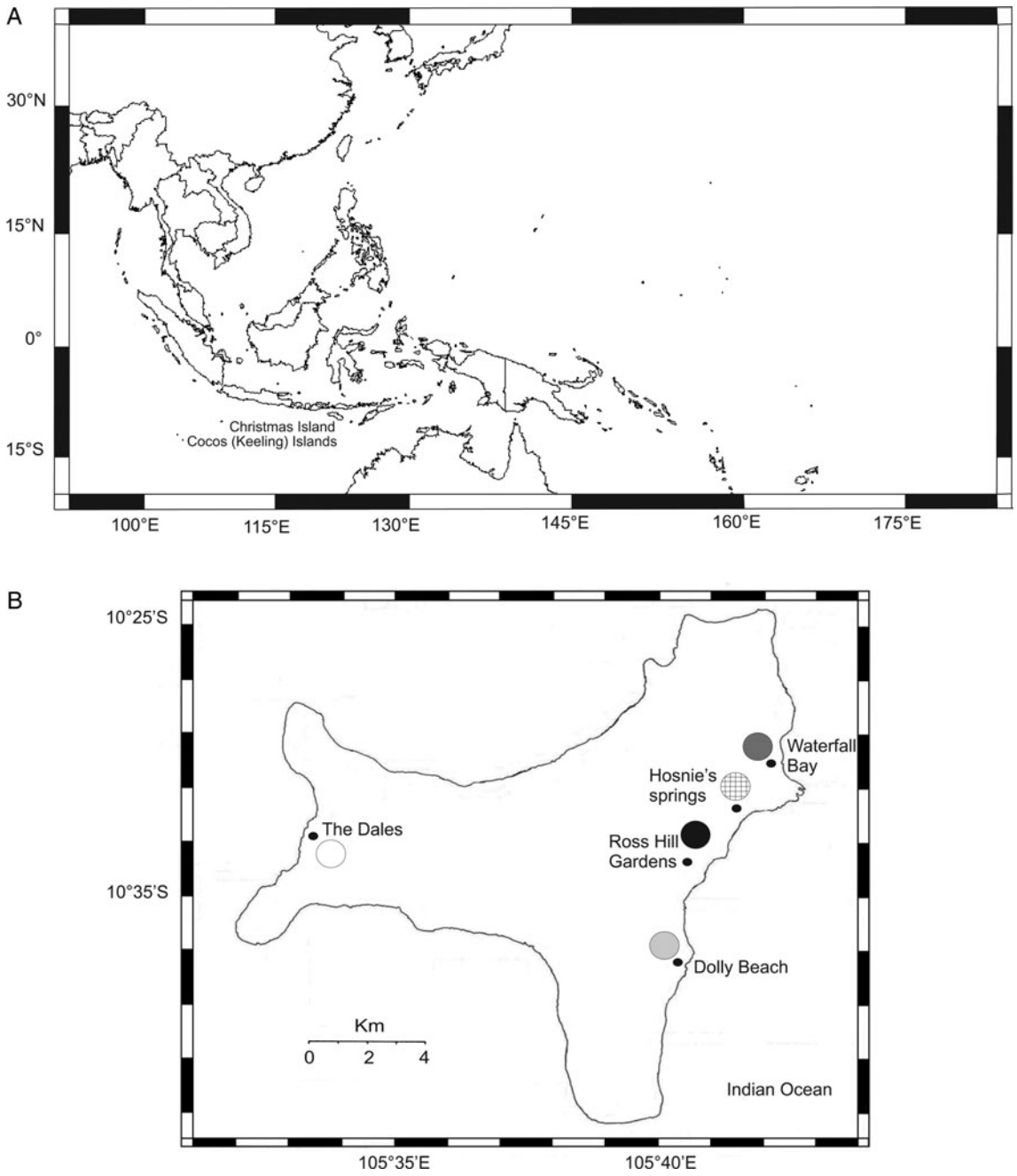


**Fig. 1.** (A) Map of the Indo-West Pacific showing the location of Christmas Island; (B) map of Christmas Island showing sampling sites (black dots) for *Discoplax celeste*. Each site is colour coded, and these colours correspond to colours used in the haplotype network (Figure 2).

reports of an established population at the springs near to the Blowholes on the south-west coast (S. Morris, personal observation).

When the wet season begins (November–March) *D. celeste* disperse over a wider area (Dela-Cruz & Morris, 1997a, b), but only once the wet season is well established (February–March) with the associated adequate supply of standing water do they undertake their breeding migration to the ocean. Despite being land crabs, *D. celeste* must return once a year to the ocean to reproduce as the species retains a marine larval stage (Greenaway, 1989; Morris, 2005). Following the migration of sexually mature crabs from their adult habitat to the lowermost shore terraces, mating occurs in burrows dug by the males (Hicks *et al.*, 1990). After mating the females remain in the burrows for several days whilst the eggs develop. Eventually, the females make their way down to the shoreline after dark where they cast their eggs into the ocean on the high tide just before the last quarter of the moon. The eggs hatch immediately on contact with the water (Hicks *et al.*, 1990).

After approximately 30 days at sea the megalopae return to land and migrate up the freshwater streams to their adult habitat. However, relatively little is known about the larval dispersal mechanisms of the land crabs on Christmas Island (Hicks, 1985; Davies & Beckley, 2010). In a similar manner to other land crab species, it has been previously assumed

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(Davies & Beckley, 2010), that the larvae of *D. celeste* are fully dispersed in the open ocean, at least in the immediate vicinity of Christmas Island, which would result in little genetic differentiation between the populations due to high levels of gene flow. However, there is also the suggestion that the larval stages of the Christmas Island gecarcinids, rather than being totally dispersed in the waters surrounding Christmas Island, may spend a significant amount of time sheltering in inshore waters on the fringing reef (Hicks, 1985; Hicks *et al.*, 1990; Gray, 1995; Davies & Beckley, 2010). This would potentially allow a megalopae migration back to an 'ancestral' habitat. Consequently, such a scenario could result in low levels of gene flow and high levels of genetic differentiation between populations. However, whilst several species of terrestrial crabs have been previously shown to exhibit various degrees of homing behaviour (see Vannini & Cannicci, 1995 for a review) to date, this has not been observed in gecarcinids.

The fact that *D. celeste* has such a limited and in places disjunct distribution on Christmas Island, coupled with the fact it has now been recognized as an endemic species (Ng & Davie, 2012), highlights the need for specialized conservation management of this land crab. To date no detailed examination of the genetic population structure of *D. celeste* on Christmas Island has been undertaken. Information regarding the degree of gene flow, genetic diversity and effective population size has been previously highlighted as the basis for the establishment of efficient conservation strategies (Frankham *et al.*, 2002; Oliveira-Neto *et al.*, 2008). The possible existence of discrete, genetically structured populations of *D. celeste* on Christmas Island was therefore investigated. If populations of *D. celeste*, despite having a marine larval stage, remain geographically isolated, divergent populations restricted to distinct freshwater seepages and streams will be present; the terrestrial dispersal abilities of these crabs are known to be limited due to the fact they are restricted in their distribution to areas of freshwater (Morris, 2005; Morris, unpublished radio-tracking data). Alternatively, if marine dispersal is an effective mechanism of among site dispersal there will be no genetic subdivision among individual streams. The cytochrome oxidase I (COI) gene and the control region were chosen for use in this study as they are characterized by high mutation rates and have been widely applied in population genetics studies, at both large and small spatial scales (Avise, 2000). Furthermore, the control region has previously been used to examine the phylogeography of the blue land crab, *Cardisoma guanhumi* Latreille, 1825 (cf. Oliveira-Neto *et al.*, 2008). Thus, the overall aim of this study was to investigate whether the restricted geographical distributions of *D. celeste* populations contribute to significant genetic structuring across Christmas Island, with an objective to inform future conservation strategies.

## MATERIALS AND METHODS

### Sampling locations and material

Due to the conservation status of *D. celeste*, sampling for this study was non-lethal. Adult *D. celeste* were collected from five locations on Christmas Island in August 2009: Ross Hill Gardens (RHG) (N = 26); the Dales (DAL) (N = 25); Dolly Beach (DOL) (N = 26); Waterfall Bay (WAT) (N = 11); and Hosnie's springs (HOS) (N = 7) (Figure 1B). The Dales and Dolly Beach are inside Christmas Island National Park, whilst the other locations are outside the Park's boundary. The crabs were hand caught and encouraged to autotomize one of the fourth pereiopods, from which a portion of muscle tissue was dissected and preserved in 98% ethanol. The crabs were then marked on their dorsal carapace with a non-toxic acrylic aerosol spray (AVT Paints, Springhill, Queensland, Australia) to avoid subsequent re-sampling and returned to the rainforest. In addition a leg muscle sample was also collected from *G. natalis* (from Christmas Island) and *Cardisoma carnifex* (Herbst, 1796) (from Cocos (Keeling) Islands) for use as outgroups. Samples were shipped to the UK and then stored at −20°C prior to DNA extraction.

### DNA extraction

Total genomic DNA was isolated using a modified CTAB protocol based on Sokolov (2000). Approximately 30 mg of muscle tissue was macerated using a sterile scalpel and added to an Eppendorf tube containing 500 µl of CTAB lysis buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4M NaCl) with 20 mg/ml of Proteinase K. The mixture was vortexed and incubated overnight at 55°C. Each sample was then extracted twice with an equal volume of chloroform:isoamyl-alcohol (24:1) and the final aqueous phase combined with 1/10 volume of 3M Na-acetate (pH 5.2) and 1.5 volumes of 100% ice-cold ethanol. The mixture was then placed at −20°C for 2 hours to precipitate the nucleic acids. Following precipitation nucleic acids were pelleted in a microcentrifuge, washed twice in 70% ethanol, and dried at 55°C for 10 minutes. The DNA pellet was re-suspended in 60 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Extractions were visually confirmed using 0.8% agarose gels stained with ethidium bromide. DNA extracts were subsequently diluted 1 in 5 in $ddH_2O$.

### PCR amplification and DNA sequencing

A fragment (~650 base pairs (bp)) of the COI gene was amplified using the universal primers LCOI490, 5′–GGT CAA CAA ATC ATA AAG ATA TTG G–3′ and HCO2198, 5′–TAA ACT TCA GGG TGA CCA AAA AAT CA–3′ (Folmer *et al.*, 1994). Polymerase chain reactions (PCRs) were performed using 2.5 µl of template DNA, 5 pmol of each primer, 0.2 mM dNTPs, 1 × PCR buffer (Promega), 1.5 mM $MgCl_2$ (Promega) and 0.5 units of *Taq* polymerase in a final volume of 25 µl. Reactions were run on a GeneAmp PCR System 9700 with the following profile: 94°C for 2 minutes, then 30 cycles of 94°C for 30 seconds, 52°C for 30 seconds and 72°C for 1 minute, followed by a final extension step of 72°C for 2 minutes.

Polymerase chain reaction amplification and sequencing of a portion of the control region followed the protocol outlined by Oliveira-Neto *et al.* (2008) with some modifications. A ~1.6 kb fragment including the entire control region was initially amplified using the primers 12SUCAF3 (5′–CC AGTANRCCTACTATGTTACGACTTAT–3′) and ILEUC AR3 (5′–GCTATCCTTTTAAATCAGGCAC–3′) designed by Oliveira-Neto *et al.* (2007). PCR reactions were performed using 1 µl of template DNA, 1 µM of each primer, 0.25 mM dNTPs, 1 × PCR buffer, 3 mM $MgCl_2$ and 1 unit of *Taq* polymerase in a final volume of 25 µl. PCR conditions were as

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follows: 95°C for 2 minutes, then 35 cycles of 95°C for 20 seconds, 56°C for 30 seconds and 72°C for 90 seconds, followed by a final extension step of 72°C for 5 minutes.

Results of the PCRs were visually confirmed after electrophoresis on 1% agarose gels stained with ethidium bromide. Negative and positive controls were included with each batch of reactions. PCR products (20 µl) were purified using 0.5 U each of Exonuclease I (NEB) and Shrimp Alkaline Phosphatase (Promega), incubated at 37°C for 60 minutess and 80°C for 15 minutes. Purified samples were sent to Macrogen Inc. (Korea) for direct sequencing on an Applied Biosystems 3730xl capillary sequencer. Sequencing COI used the same primers as the initial PCR reactions. Sequencing the control region used a set of internal primers DLUSSAF1 (5′–GTATAACCGCGAATGCTGGCAC–3′) and ILEUCAR2 (5′–CCTTTTAAATCAGGCACTATA–3′) (Oliveira-Neto *et al.*, 2008) which resulted in a ~800 bp fragment of the control region. Both forward and reverse sequences were generated.

## Phylogenetic and statistical analysis

Analyses were carried out separately for the COI gene and the control region fragment. Sequences were verified as crustacean DNA using the GenBank™ BLASTn search (Altschul *et al.*, 1990). Sequences were then edited using the BIOEDIT 7.0.5.3 software package (Hall, 1999) and in the case of the control region sequences aligned using the MUSCLE algorithm (Edgar, 2004) within MEGA5 (Tamura *et al.*, 2011). Gap penalties were left at the default settings (gap open: −400, gap extend: 0). All alignments and base substitutions were confirmed visually. Homogeneity of base frequencies among sequences was examined using Chi-squared tests as implemented in PAUP* 4.b.10 (Swofford, 2002) and the degree of substitution saturation was examined, using the test of Xia *et al.* (2003), as implemented in the package DAMBE 5.2.15 (Xia & Xie, 2001). The Akaike information criterion in MODELTEST 3.7 (Posada & Crandall, 1998) was used to specify the proportion of invariable sites for this test. Standard population genetic analyses of the sequence data were performed using the program package ARLEQUIN 3.11 (Excoffier *et al.*, 2005). For each sample site and for the entire dataset the following parameters were used to estimate genetic variability: the number of haplotypes ($N_{hap}$), polymorphic sites ($N_{ps}$), average number of nucleotide differences and haplotype ($H_e$) and nucleotide diversity (Nei, 1987). Neutrality tests, Tajima's $D$ (Tajima, 1989a) and Fu's $F_s$ (Fu, 1997) were carried out using ARLEQUIN. $D$ and $F_s$ were calculated with an infinite sites model and significance was tested with 1000 simulated samples. These tests were used to determine departures from mutation-drift or mutation-selection equilibrium as well as the detection of changes in population size (Mousset *et al.*, 2004). Therefore significant negative values for Tajima's $D$ and Fu's $F_s$ indicate recent mutations that may indicate population expansion whilst positive values signify a lack of recent mutations that may have resulted from balancing selection processes (Ramírez-Soriano *et al.*, 2008).

Mismatch distributions (Rogers & Harpending, 1992; Slatkin & Hudson, 1999) were computed in ARLEQUIN for each sample site and for the global dataset. Using Rogers & Harpending's (1992) model enables three parameters to be estimated: $\theta_o = 2N_o u$, $\theta_1 = 2N_1 u$ and $\tau = 2ut$, where $N_o$ is the size of the initial population which is assumed to grow rapidly to a size of $N_1$ at a time $t$ generations before the present, whilst $u$ is the per-generation probability that a mutation strikes a particular nucleotide in the region being studied. The model parameters were estimated using the generalized non-linear least-square approach (Schneider & Excoffier, 1999). For each analysis 1000 permutations were used. Mismatch distributions allow assessment of the mixing of evolutionary divergent haplotype groups at the population level. Populations with a long and stable demographic balance have disordered mismatch distribution (Harpending, 1994). Distributions that appear more unimodal are indicative of populations that have passed through a recent demographic expansion, possibly following a bottleneck (Rogers & Harpending, 1992). The amount of correlation between the observed and expected mismatch distributions was tested with Harpending's (1994) raggedness statistic ($r$).

Genetic differentiation among sampling locations was assessed using Φ-indices in the analysis of molecular variance (AMOVA) framework (Excoffier *et al.*, 1992) as implemented in ARLEQUIN. Φ-indices incorporate haplotype frequency and divergence and were therefore used instead of F-statistics which incorporate haplotype frequency only (Excoffier *et al.*, 1992). Initially each sampling site was treated as an individual population to test for overall genetic subdivision between samples within populations ($\Phi_{ST}$) (tested by permuting haplotypes (10000) between populations). Next the AMOVA was calculated on two groups of sampling sites; individuals from Ross Hill Gardens, Dolly Beach, Hosnie's springs and Waterfall Bay were grouped as 'eastern samples', whereas those from the Dales were grouped as 'western samples' thus allowing the calculation of genetic subdivision between samples within a group of populations ($\Phi_{CT}$) and between samples within populations ($\Phi_{SC}$).

Evolutionary relationships among haplotypes (gene genealogies) were analysed through the construction of a haplotype network. The software TCS 1.21 (Clement *et al.*, 2000) was used to produce a haplotype network with maximum parsimony then applied to infer the most parsimonious branch connections between the haplotypes detected among all the sites sampled (Templeton *et al.*, 1992).

To further depict genetic differentiation between sampling sites a population tree was constructed (Posada & Crandall, 2001; Sotelo *et al.*, 2008). Genetic distances ($D_a$ distances (Nei, 1987)) between populations were calculated using the program DnaSP 5.10.01 (Librado & Rozas, 2009). The $D_a$ distance is the number of net nucleotide substitutions per site between populations. The $D_a$ distance was used in these analyses because it takes into account the intrapopulation variability. With the resulting distance matrix, a population tree was constructed with the neighbour-joining (NJ) algorithm (Saitou & Nei, 1987) using PAUP*.

# RESULTS

Separate analyses using COI and control region data yielded broadly similar results. Sequencing of 95 individuals yielded 658 bp of unambiguous COI sequences of which 15 bp (2.3%) were polymorphic. These polymorphisms resulted in 11 haplotypes being detected in the samples (GenBank accession numbers: HQ214613–HQ214623). COI was also sequenced from *G. natalis* (HQ214624) and *C. carnifex*

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(HQ214625). The data showed no indels or stop codons, as expected for a coding region and were not saturated either when whole sequences were compared or at third codon nucleotide sites (ISS < ISS.C, $P < 0.001$ in both cases (see Xia *et al.* (2003) for a detailed explanation)). All point mutations were transitions and percentage nucleotide composition was C: 21.3, T: 34, A: 27.2 and G: 17.4. Base frequencies were found to be homologous among sequences at all sites ($\chi^2 = 1.15$, $P = 1.00$), the 15 variable sites ($\chi^2 = 73.18$, $P = 1.00$) and the third codon sites ($\chi^2 = 7.79$, $P = 1.00$).

Overall, nucleotide diversity and haplotype diversity were both low and did not differ substantially among sampling sites nor were they significantly different from zero (Table 1). Of the 11 haplotypes detected, one haplotype (Dc2) was common to all sites and was the most numerous, constituting over 75% of the total individuals surveyed at each site. However, eight of the eleven haplotypes identified were exclusive (private) to one sampling site (Dc3, Dc4, Dc6, Dc7, Dc8, Dc9, Dc10 and Dc11) (Figure 2). All of the private haplotypes occurred at low numbers, with just one or two individuals per sampling site.

Sequencing of the control region, from 95 individuals yielded 796 bp of sequences of which 79 bp (9.9%) were polymorphic. These polymorphisms resulted in 62 haplotypes being detected in the samples (GenBank accession numbers: JF779688–JF779749). The control region was also sequenced from *G. natalis* (JF779843) and *C. carnifex* (JF779844). The data (whole sequences) were not saturated (ISS < ISS.C, $P < 0.001$). Seven of the 76 substitutions (9.2%) were transversions and percentage nucleotide composition was C: 15.6, T: 35.9, A: 41.3 and G: 7.2. Base frequencies were found to be homologous among sequences at all sites ($\chi^2 = 3.6$, $P = 1.00$) and the 79 variable sites ($\chi^2 = 29.19$, $P = 1.00$). Again, overall nucleotide diversity and haplotype diversity were both low, although values were slightly higher than for the COI data. Furthermore, there were no substantial differences among sampling sites for these parameters nor were they significantly different from zero (Table 1). Most (77.4%) of the 62 haplotypes detected were private haplotypes, with 95.8% of these containing just one individual.

The results of the AMOVAs (COI and control region) revealed no evidence of population sub-structuring either among all sample sites in the global dataset or when eastern and western sites were compared (Table 2). This was also demonstrated by the lack of structure present in the COI (Figure 2) and control region haplotype networks (control

**Table 1.** Sample size, molecular diversity, and population demographic statistics for *Discoplax celeste* for populations from each sample site for the cytochrome oxidase I (COI) gene and the control region. RHG, Ross Hill Gardens; DAL, the Dales; DOL, Dolly Beach; WAT, Waterfall Bay; HOS, Hosnie's springs.

**COI gene**

| | All samples | RHG | DAL | DOL | WAT | HOS |
|---|---|---|---|---|---|---|
| Sample size ($N$) | 95 | 26 | 25 | 26 | 11 | 7 |
| Number of haplotypes ($N_{hap}$) | 11 | 5 | 5 | 6 | 2 | 3 |
| Haplotype diversity ($H_e$) (SD) | 0.39 ± 0.063 | 0.41 ± 0.12 | 0.36 ± 0.12 | 0.47 ± 0.12 | 0.18 ± 0.14 | 0.52 ± 0.21 |
| Number of polymorphic sites ($N_{ps}$) | 15 | 10 | 8 | 11 | 5 | 8 |
| Average number of differences (SD) | 1.35 ± 0.84 | 1.60 ± 0.98 | 0.79 ± 0.59 | 1.6 ± 0.98 | 0.91 ± 0.68 | 2.48 ± 1.52 |
| Nucleotide diversity (SD) | 0.09 ± 0.06 | 0.11 ± 0.07 | 0.05 ± 0.04 | 0.11 ± 0.07 | 0.06 ± 0.05 | 0.17 ± 0.12 |
| Tajima's $D$ | −1.50 | −1.28 | −2.0 | −1.48 | −1.79 | −1.27 |
| $P_D$ | 0.04 | 0.09 | 0.00 | 0.07 | 0.01 | 0.10 |
| Fu's $F_s$ | −3.48 | 0.45 | −1.46 | −0.48 | 2.1 | 1.77 |
| $P_F$ | 0.06 | 0.63 | 0.11 | 0.37 | 0.82 | 0.84 |
| $\theta_o$ (95% CI) | 0.0 (0.0–0.22) | 0.0 (0.0–0.33) | 0.0 (0.0–0.52) | 0.0 (0.0–0.01) | 0.0 (0.0–0.01) | 0.0 (0.0–2.08) |
| $\theta_1$ (95% CI) | 0.52 (0.0–∞) | 0.54 (0.0–∞) | 0.53 (0.0–∞) | ∞ (∞–∞) | 0.12 (0.0–∞) | 1.89 (0.0–∞) |
| $\tau$ (95% CI) | 3.0 (0–4.84) | 3.25 (0–5.23) | 3.0 (0–11.5) | 0.0 (0.0–0.67) | 3.0 (0.43–3.0) | 5.91 (0.0–91.9) |
| Raggedness index ($r$) | 0.29 | 0.33 | 0.24 | 0.21 | 0.74 | 0.34 |
| $P_r$ | 0.48 | 0.48 | 0.57 | 0.99 | 0.68 | 0.22 |

**Control region**

| | All samples | RHG | DAL | DOL | WAT | HOS |
|---|---|---|---|---|---|---|
| Sample size ($N$) | 95 | 26 | 25 | 26 | 11 | 7 |
| Number of haplotypes ($N_{hap}$) | 62 | 21 | 23 | 21 | 11 | 6 |
| Haplotype diversity ($H_e$) (SD) | 0.98 ± 0.01 | 0.98 ± 0.02 | 0.99 ± 0.01 | 0.98 ± 0.02 | 1.00 ± 0.04 | 0.95 ± 0.1 |
| Number of polymorphic sites ($N_{ps}$) | 79 | 47 | 48 | 57 | 34 | 31 |
| Average number of differences (SD) | 7.51 ± 3.54 | 6.95 ± 3.38 | 6.72 ± 3.28 | 8.32 ± 3.98 | 8.02 ± 4.04 | 9.91 ± 5.17 |
| Nucleotide diversity (SD) | 0.01 ± 0.05 | 0.09 ± 0.05 | 0.08 ± 0.05 | 0.11 ± 0.06 | 0.10 ± 0.05 | 0.13 ± 0.08 |
| Tajima's $D$ | −1.69 | −1.66 | −1.82 | −1.68 | −1.51 | −1.19 |
| $P_D$ | 0.01 | 0.03 | 0.02 | 0.02 | 0.07 | 0.11 |
| Fu's $F_s$ | −24.85 | −10.47 | −16.54 | -8.77 | −4.98 | 0.13 |
| $P_F$ | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.42 |
| $\theta_o$ (95% CI) | 4.03 (0.0–16.09) | 2.8 (0.0–11.16) | 0.0 (0.0–1.44) | 5.38 (0.0–18.78) | 0.0 (0.0–2.54) | 0.0 (0.0–1.62) |
| $\theta_1$ (95% CI) | 184.69 (12.96–∞) | 23.53 (8.27–∞) | ∞ (4.95–∞) | 187.81 (9.71–∞) | ∞ (6.92-∞) | ∞ (1.3–∞) |
| $\tau$ (95% CI) | 2.56 (0.64–14.34) | 4.35 (0.88–89.35) | 2.91 (1.24–4.4) | 2.18 (0.09–16.09) | 3.79 (0.49–6.01) | 1.75 (0.0–4.21) |
| Raggedness index ($r$) | 0.006 | 0.01 | 0.01 | 0.01 | 0.02 | 0.12 |
| $P_r$ | 0.82 | 0.83 | 0.98 | 0.62 | 0.96 | 0.61 |

SD, standard deviation; CI, confidence interval.

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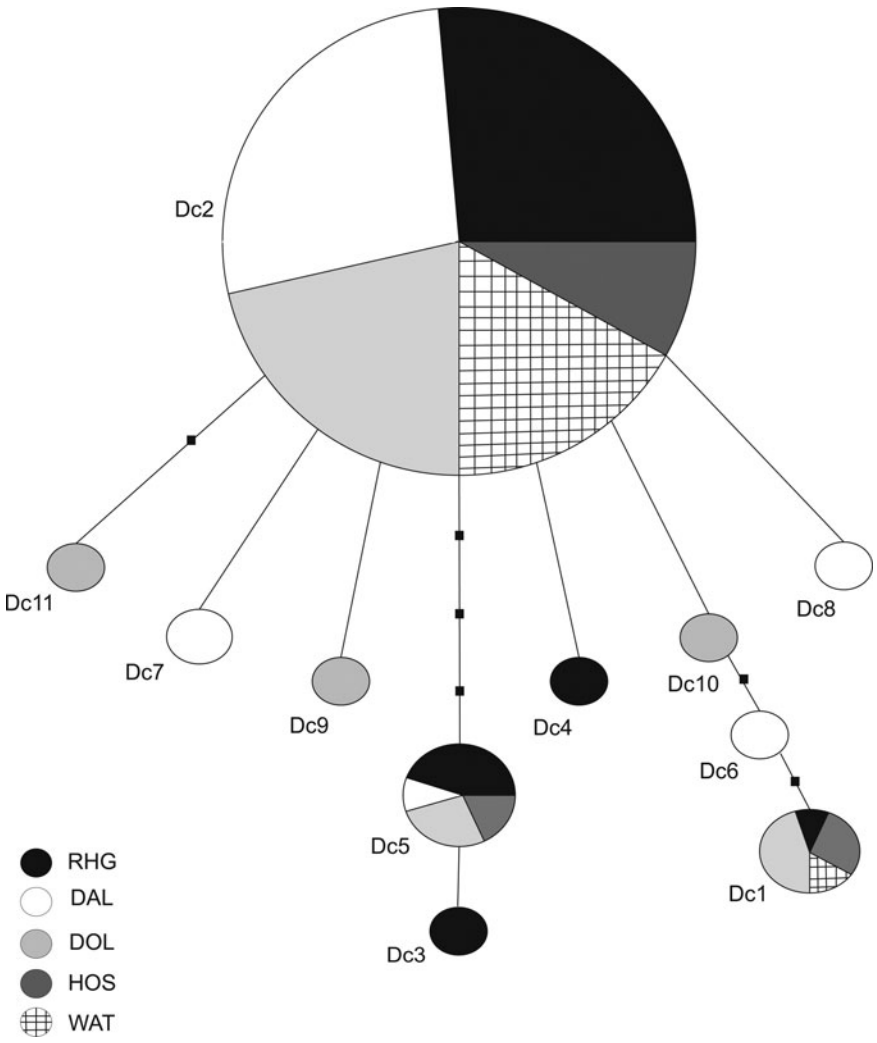


**Fig. 2.** Haplotype network for 11 cytochrome oxidase I haplotypes (from 95 individuals) of *Discoplax celeste* (i.e. Dc1 (*Discoplax celeste* haplotype 1)) from Christmas Island (Ross Hill Gardens (RHG), the Dales (DAL), Dolly Beach (DOL), Waterfall Bay (WAT) and Hosnie's springs (HOS)), constructed using the 'median joining' method and parsimony. Circles represent haplotypes, with the area of each circle representative of the frequency with which it occurred in the whole sample set, and pie charts indicate the distribution of the haplotype among the various populations. Connecting lines show mutational pathways among haplotypes. Small black squares represent missing, intermediate haplotypes.

region network not shown). These results were further confirmed by the lack of significant differences within the pairwise analyses of $\Phi_{ST}$ for both the COI and control region datasets (Table 3).

There was limited evidence for population expansion. When the entire sample set (of either COI or control region data) was compared, significant negative values were recorded for estimates of Tajima's *D*. Negative values were also recorded for Fu's $F_s$ although only in the case of the control region dataset was this result statistically significant (Table 1). Using the COI dataset, individuals from the Dales and Dolly Beach gave negative values for both neutrality tests, although again only the results for Tajima's *D* for the Dales sampling site was statistically significant. Using the control region dataset, individuals sampled at Ross Hill Gardens, the Dales, Dolly Beach and Waterfall Bay gave negative (statistically significant) values for both neutrality tests except that of Tajima's *D* for Waterfall Bay which was not statistically significant. However, none of the sampling sites showed any significantly positive *D* values (in either dataset) (Table 1) and therefore no evidence was recorded for a recent reduction in population size (bottleneck event). When the entire sample set was combined (in both the COI and control region analyses) as well as when each location was analysed separately the mismatch analyses (raggedness indices) were all non-significant (Table 1), which indicated a unimodal distribution consistent with population expansion. When the COI dataset was analysed, by far the smallest value for $\tau$ was reported from Dolly Beach (Table 1). However, when the control region dataset was analysed, the smallest values for $\tau$ were reported from Hosnie's springs (Table 1). Due to the small sample size for Hosnie's springs and large overlap among the confidence intervals this result should be treated with caution.

The population dendrograms of COI and the control region are shown in Figure 3A and 3B (population trees) respectively. These trees indicate there is a considerable genetic divide between the other gecarcinids sampled (*C. carnifex* and *G. natalis*) and *D. celeste* from Christmas Island. There is no evidence of any notable clustering of populations based on geographical location in the COI tree. Based on $D_a$ distance values alone, in the tree constructed using the

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**Table 2.** Analysis of molecular variance based on a $\Phi_{ST}$ matrix between haplotypes for the cytochrome oxidase I (COI) gene and the control region. The samples were separated between eastern (Ross Hill Gardens, Dolly Beach, Hosnie's springs and Waterfall Bay) and western samples (the Dales).

**COI gene**

| Group | Source of variation | df | SS | Variance components | Percentage of variation | $\Phi_{ST}$ | $\Phi_{SC}$ | $\Phi_{CT}$ |
|---|---|---|---|---|---|---|---|---|
| Global dataset | Among populations | 4 | 2.014 | 0.000 | 0.000 | 0.000 | | |
| | Within populations | 90 | 61.376 | 0.682 | 100.0 | | | |
| | Total | 94 | 63.389 | 0.682 | | | | |
| Eastern versus western | Among groups | 1 | 0.764 | 0.013 | 1.92 | 0.000 | | |
| | Among populations within groups | 3 | 1.250 | 0.000 | 0.000 | | 0.000 | |
| | Within populations | 90 | 61.376 | 0.682 | 98.08 | | | 0.019 |
| | Total | 94 | 63.389 | 0.695 | | | | |

**Control region**

| Group | Source of variation | df | SS | Variance components | Percentage of variation | $\Phi_{ST}$ | $\Phi_{SC}$ | $\Phi_{CT}$ |
|---|---|---|---|---|---|---|---|---|
| Global dataset | Among populations | 4 | 11.65 | 0.000 | 0.000 | 0.000 | | |
| | Within populations | 90 | 341.447 | 3.794 | 100.0 | | | |
| | Total | 94 | 353.095 | 3.745 | | | | |
| Eastern versus western | Among groups | 1 | 3.372 | 0.031 | 0.82 | 0.000 | | |
| | Among populations within groups | 3 | 8.276 | 0.000 | 0.000 | | 0.000 | |
| | Within populations | 90 | 341.447 | 3.794 | 99.18 | | | 0.008 |
| | Total | 94 | 353.095 | 3.760 | | | | |

NB: there were no significant differences between the values (in all comparisons $P > 0.05$).

control region data (Figure 3B) there appears to be a distinct separation between the population of *D. celeste* sampled from the Dales (at the western end of the island) and the other Christmas Island populations of *D. celeste* from the eastern side of Christmas Island.

## DISCUSSION

There is a high level of genetic variation in the populations of *D. celeste*, with this variation homogenously distributed across the various sampling locations. For instance, the results of the AMOVAs revealed no evidence of population sub-structuring either among all sample sites in the dataset, or when eastern and western sites were compared (Table 2). These AMOVA results were confirmed by the lack of any significant differences in the pairwise analyses of $\Phi_{ST}$ (Table 3) between populations and thus indicate that there is a single population of *D. celeste* on Christmas Island. Although *D. celeste* do disperse away from the immediate vicinity of their freshwater seepages and springs during the wet season, this appears to be driven ultimately by the need for these crabs to undertake their breeding migration to the ocean (Dela-Cruz & Morris, 1997a, b; Morris & Dela-Cruz, 1998; Morris, 2005). Therefore, whilst this would potentially enable some mixing between individuals from the sampling sites at the eastern end of the island, it is unlikely that there is mixing between these sites and the Dales at the western end of the island (~19 km away) (Figure 1B). Therefore, oceanic dispersal of larvae rather than long distance terrestrial movement of juvenile and/or adult crabs remain the more likely explanation for the results shown by these analyses and thus explain the lack of *D. celeste* population sub-structuring on Christmas Island. Analyses of the phylogeography of other terrestrial and semi-terrestrial crabs over larger geographical scales, for instance along the eastern coasts of South America (i.e. *Cardisoma guanhumi* Latreille, 1825 (Oliveira-Neto *et al.*, 2008) and *Ucides cordatus* (Linnaeus, 1763) (cf. Oliveira-Neto *et al.*, 2007)) and Africa (i.e. *Neosarmatium meinerti* de Man, 1887 (Ragionieri *et al.*, 2010) and *Perisesarma guttatum* (A. Milne-Edwards, 1869) (cf. Silva *et al.*, 2010)) have revealed a similar lack of population sub-structuring. In these other species, this lack of genetic structure has been explained by the fact that the marine larvae of these crabs are well mixed with the assistance of ocean currents. Conversely, the limited dispersal abilities of 'true' freshwater crabs are explained by the life history of these species: these crabs do not release free-swimming larvae,

**Table 3.** Pairwise $\Phi_{ST}$ among sites for the cytochrome oxidase I (COI) gene and the control region. RHG, Ross Hill Gardens; DAL, the Dales; DOL, Dolly Beach; WAT, Waterfall Bay; HOS, Hosnie's springs.

**COI gene**

| | RHG | DAL | DOL | WAT | HOS |
|---|---|---|---|---|---|
| **RHG** | 0.000 | | | | |
| **DAL** | 0.015 | 0.000 | | | |
| **DOL** | 0.000 | 0.000 | 0.000 | | |
| **WAT** | 0.003 | 0.000 | 0.000 | 0.000 | |
| **HOS** | 0.000 | 0.035 | 0.000 | 0.000 | 0.000 |

**Control region**

| | RHG | DAL | DOL | WAT | HOS |
|---|---|---|---|---|---|
| **RHG** | 0.000 | | | | |
| **DAL** | 0.000 | 0.000 | | | |
| **DOL** | 0.000 | 0.000 | 0.000 | | |
| **WAT** | 0.000 | 0.000 | 0.000 | 0.000 | |
| **HOS** | 0.000 | 0.018 | 0.000 | 0.000 | 0.000 |

NB: there were no significant differences between the values (in all comparisons $P > 0.05$).

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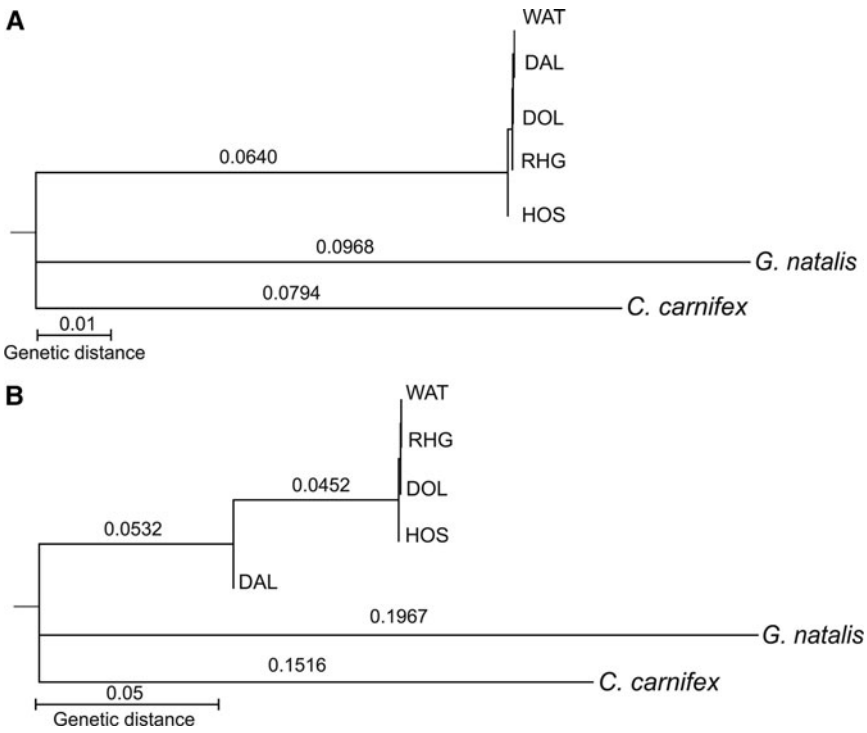


**Fig. 3.** Neighbour joining population tree based on (A) cytochrome oxidase I and (B) control region sequences from the different populations of *Discoplax celeste* on Christmas Island. *Cardisoma carnifex* and *Gecarcoidea natalis* were used as outgroups. Numbers on branches and lengths of branches are proportional to genetic ($D_a$) distances.

and instead have direct development, with brood care, carried out completely independent of the marine environment (Cumberlidge & Ng, 2009). This often results in endemism, often to the specific watercourse and/or island where they are found, with the result that populations display high amounts of genetic phylogeographic structuring (i.e. *Epilobocera sinuatifrons* (A. Milne-Edwards, 1866) (cf. Cook *et al.*, 2008), *Nautilothelphusa zimmeri* Balss, 1933 (cf. Schubart *et al.*, 2008) and *Candidiopotamon rathbunae* (de Man, 1914) (cf. Shih *et al.*, 2006)).

The results discussed above do not support any theory of *D. celeste* migrating back to an 'ancestral' habitat. However, there is potentially some difference between the larval dispersal of *D. celeste* compared to the rather more numerous gecarcinid present on Christmas Island, *G. natalis*. For instance, there is a population of *G. natalis* recorded from the North Keeling Island in the Cocos (Keeling) Islands (Hicks *et al.*, 1990; Environment Australia, 2004) approximately 900 km south-west of Christmas Island, which probably results due to larval drift from the Christmas Island population, or possibly as a consequence of anthropogenic activity between North Keeling and Christmas Islands (Tweedie, 1950). However, to date, *D. celeste* has not been recorded from any of the Cocos (Keeling) Islands, despite the presence of suitable habitat (Dela-Cruz, 1998), indicating that there could be differences in the mechanisms of larval dispersal between these two Christmas Island gecarcinids.

Indeed, the results of this study highlight the dearth of information about the larval biology as well as the larval dispersal mechanisms of land crabs both on Christmas Island (Hicks, 1985) and on other remote oceanic islands. Whilst the results of the current study do not support the idea that *D. celeste* megalopae migrate back to an 'ancestral' habitat, it is known that the megalopae follow freshwater streams from the shoreline to areas where they reside as juvenile and adult crabs (Hicks, 1985). By comparison, the megalopae of *G. natalis* come ashore at many places around the coast of Christmas Island and migrate through both wet and dry terrain to the rainforest plateau where they settle (Hicks, 1985). It therefore could be speculated that *D. celeste* megalopae are able to detect changes in the salinity of their environment, enabling them to locate sources of freshwater for their migration to their adult habitat. A number of recent investigations have suggested the importance of salinity detection by developing crab megalopae in estuarine environments, particularly in catadromous species (e.g. *Armases* spp. (Torres *et al.*, 2006; Anger *et al.*, 2008) and *Rhithropanopeus harrisii* (Forward, 2009)) as well as the Chinese mitten crab, *Eriocheir sinensis*, which as an invasive species has a varied lifecycle but always involves a migration through different salinities either as larvae, megalopae or adults (see Dittel & Epifanio (2009) for a recent review). Clearly further physiological investigations are needed to determine the mechanism(s) that facilitate this in *D. celeste* megalopae.

There was some evidence uncovered suggesting that *D. celeste* on Christmas Island has undergone population expansion during its recent evolutionary history (Ramírez-Soriano *et al.*, 2008). For each complete dataset, significant negative values were recorded for estimates of Tajima's *D*. Negative values were also recorded for Fu's *F*, although only in the case of the control region dataset was this value statistically significant ($-24.85$ ($P < 0.001$) (Table 1)). Similar results were noted when the population genetics of the closely related gecarcinid *C. guanhumi* as well as the mangrove crab *U. cordatus* were investigated from populations along the Brazilian coast, with, for example Fu's *F* values

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recorded that were very similar (−23.97 ($P = 0.001$), *C. guanhumi* (Oliveira-Neto *et al.*, 2008) and −24.01 ($P < 0.001$) (Oliveira-Neto *et al.*, 2007)) to those recorded for *D. celeste* on Christmas Island. Clearly, despite the differences in the geographical ranges sampled (several 1000 km of continental coast versus a small 137 km$^2$ isolated oceanic island), these species have all undergone population expansion in the recent geological past. However, the values recorded for $\tau$ for *D. celeste* from Christmas Island were much smaller (3.00 (COI) 2.56 (control region) (Table 1)), than those reported for either *C. guanhumi* (21.5 (Oliveira-Neto *et al.*, 2008)) or *U. cordatus* (16.62 (Oliveira-Neto *et al.*, 2007)) indicating that the expansions of *C. guanhumi* and *U. cordatus* along the Brazilian coast are much older than that of *D. celeste* on Christmas Island, which possibly indicates a relatively recent colonization of Christmas Island by *D. celeste*.

Tropical freshwater ecosystems have been identified as particularly vulnerable and often contain a diverse endemic fauna, including many species of freshwater crabs (Cumberlidge *et al.*, 2009). *Discoplax celeste* and the freshwater habitat on Christmas Island, as well as the Christmas Island ecosystem as a whole is no exception, and is currently facing a number of specific threats, all of which are directly related to human activities on the island and include habitat loss as a result of land clearance for the mining industry, overexploitation of groundwater, as well as ecosystem change due to the introduction of numerous invasive species, including the 'yellow crazy ant' *Anoplolepis gracilipes* (Smith, 1857) (cf. O'Dowd *et al.*, 2003). It has previously been demonstrated that due to the restricted distribution of *D. celeste* on Christmas Island, the species is especially sensitive to any environmental changes (Hicks *et al.*, 1990). In the past, the diversion to storage tanks of one of the Christmas Island seaward bound streams that was previously home to a large *D. celeste* population resulted in this population becoming severely depleted (Gibson-Hill, 1947). In recent years the human population of Christmas Island has increased (Chambers, 2011) and at present there is no environmental framework in place to monitor the use of groundwater for human consumption. It is not known what impact the current rate of water usage is having on the Christmas Island ecosystem (Barrett, 2001) but based on previous observations it is imperative that the island's freshwater supply is managed effectively to ensure the long term future of this species. In addition, due to their large body size (Turner *et al.*, 2011) and relative ease of capture *D. celeste* has previously been overexploited (Hicks *et al.*, 1990) which resulted in several populations of the crab on Christmas Island being reduced to very low numbers. However, a particularly encouraging result from the present study was the fact that none of the sampling sites showed any significantly positive Tajima's *D* or Fu's $F_s$ values (in either dataset) (Table 1). Therefore, there was no evidence for any recent bottleneck events (reduction in population size) (Tajima, 1989a, b). At present not all known *D. celeste* habitats are currently inside the National Park boundary, but the species is now totally protected on Christmas Island under the Australian Government's Environment Protection and Biodiversity Conservation Act (1999). The low level of spatial structuring in the genetic diversity of *D. celeste* on Christmas Island (as shown by the results of the AMOVAs (Table 2)) is significant in terms of the conservation management of this species. Thus, based on the results of this study, for conservation purposes *D. celeste* can be considered as a single management unit (Frankham *et al.*, 2002). This means that in the future any area that became severely depleted of this species could be restocked, either by adults sourced from other areas on Christmas Island and/or theoretically by rearing juveniles in a laboratory setting (Oliveira-Neto *et al.*, 2008). It should be pointed out though that, to date, the successful rearing of gecarcinid larvae in the laboratory remains technically challenging (Shokita & Shikatani, 1990; Davies & Beckley, 2010) and therefore can only be thought of as a last resort.

Within the *D. hirtipes* species complex there are potentially further additional species that will be described in the future; for example *Discoplax* aff. *hirtipes* from the Andaman and Nicobar Islands has a dark violet carapace and cinnabar red chelae (Alcock, 1900; Hicks *et al.*, 1990) and specimens with the same coloration have also been recorded from Christmas Island (Ng & Davie, 2012). Following the revelation that *D. celeste* had been misidentified on Christmas Island as an endemic colour form of *D. hirtipes* (Ng & Davie, 2012) it will be useful to investigate the relationship between this species and other colour forms/species in the *D. hirtipes* species complex at the genetic level. Future studies should utilize a combination of molecular markers, potentially including nuclear markers, microsatellites and/or SNPs (single nucleotide polymorphisms) which are likely to be much more highly variable compared to the mitochondrial sequences used in the present study (Wan *et al.*, 2004). This would hopefully allow a clearer picture of the link between these colour forms/species, as well as the phylogeographic structure present in the wider *D. hirtipes* species complex to be uncovered.

A fundamental principle in island biogeography states that remote oceanic islands support a high number of endemic species (MacArthur & Wilson, 1967). Besides *D. celeste* there are a number of gecarcinids that exhibit island endemism, i.e. *Johngarthia malpilensis* (Faxon, 1893) which is only found on Malpelo, an island off the Pacific coast of Colombia (Lopez-Victoria & Werding, 2008), *Johngarthia cocoensis* Perger, Vargas & Wall, 2011 found on Cocos Island off the Pacific coast of Costa Rica (Perger *et al.*, 2011), and *G. natalis* which is also endemic to Christmas Island (Hicks, 1985; Hartnoll, 1988; Hicks *et al.*, 1990). Species which are endemic to remote oceanic islands are particularly vulnerable to population decline, primarily due to habitat deterioration, often as a direct result of human habitation (Triantis *et al.*, 2010). Thus, in light of the recent change in taxonomic status of *D. celeste* from an endemic colour form of a widely distributed species, to being described as an endemic species in its own right, it is important that these crabs are protected. Any conservation strategy formulated must take into account the fact that *D. celeste* has its own conservation needs, specifically the provision of a terrestrial/freshwater habitat. To date, two of the wetland areas that support *D. celeste* (the Dales and Hosnie's springs) have been listed as wetlands of international significance under the Ramsar Convention (Navid, 1984), thus highlighting the importance of these areas not only on Christmas Island, but in a global context. This reinforces the broader implications of managing and conserving *D. celeste*, as they, like the other Christmas Island crabs (i.e. *G. natalis*) play an important ecological role in maintaining these ecosystems of international significance (O'Dowd & Lake, 1989; O'Dowd *et al.*, 2003).

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