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CORRECTED PROOF

The invasive slug *Deroceras invadens* Reise, Hutchinson, Schunack and Schlitt, 2011 occurs on Norfolk Island

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Abstract

Cytochrome *c* oxidase subunit I DNA sequences provide genetic confirmation of the presence of the invasive slug *Deroceras invadens* on Norfolk Island, an isolated Pacific island, where it has not previously been identified. The six sequences that were determined include two distinct haplotypes indicating that more than one individual has contributed genetic material to the invasive population. No close relatives of one of these haplotypes have yet been found in Europe, suggesting that the species' genetic diversity in its native range has not yet been fully sampled.

Key words: phylogeography, *Deroceras laeve*, DNA barcoding, multiple haplotypes

Introduction

Until recently, misidentifications prevented a clear understanding of the Australian distribution of the invasive slug *Deroceras laeve* (O. F. Müller, 1774) and another morphologically similar species, also invasive, from the genus. Further complications arose from the recognition that the second species, now described as *D. invadens* Reise, Hutchinson, Schunack and Schlitt, 2011, is genetically distinct to *D. panormitanum* (Lessona and Pollonera, 1882) the species to which the invasive populations had been referred (Reise et al. 2011). Anatomical studies (Hutchinson et al. 2014) have clarified the distributions of *D. laeve* and *D. invadens* in mainland Australia, indicating that the latter is widespread in the southeast of the continent and is also found in Western Australia. These studies also suggest that records of the presence of *D. panormitanum* on the isolated Pacific islands of Lord Howe Island and Norfolk Island were incorrect identifications of *D. laeve* (Hutchinson et al. 2014). However, Hutchinson et al. (2014) did identify *D. invadens* on Raoul Island in the Kermadecs, from which the nearest large land mass, New Zealand, is more than 1100 kilometres distant, and on the Chatham Islands 650 kilometres east of New Zealand.

Discriminating between *D. laeve* and *D. invadens* using external morphology is difficult. It has however been suggested that the profile of the tail is a character that potentially separates *D. laeve* from species in the *D. panormitanum* complex. De Winter (1988) distinguishes *D. panormitanum* as having a "rounded tail, that usually extends beyond the foot fringe". Reise et al. 2006) note that "the tail of *D. panormitanum* rises up from the sole vertically, or even curves backwards, whereas in *D. laeve* it slopes forward". Reise et al. (2006) also note that the difference is not clear in all specimens. Only one *Deroceras* slug from Norfolk Island has previously been definitely identified. Hutchinson et al. (2014) used internal anatomy to determine that a specimen from the island belonged to *D. laeve.*

The tail profile of specimens of *Deroceras* slugs from a recent quarantine survey of Norfolk Island did not definitely conform to that expected for *D. laeve* albeit that they were contorted by fixation in ethanol (Figure 1). This suggested the possibility that *D. invadens* might now also be present on the island. To check this, genetic information was collected from the mitochondrial cytochrome *c* oxidase subunit I ("COI") sequence of the slugs (Figure 2). These are the first sequences to be reported from Australian specimens of the genus.

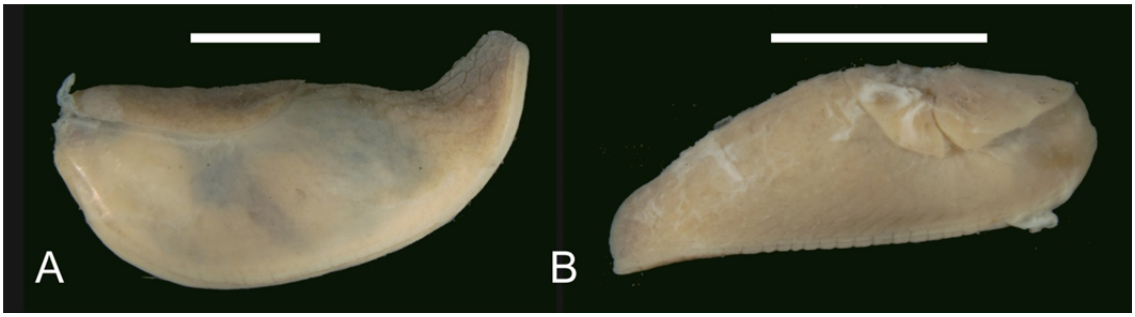

Figure 1. Photomicrographs of two sequenced *Deroceras invadens* after storage in ethanol. A: C.532716 viewed from the left side before the removal of mantle tissue for DNA extraction; B: C.532719 viewed from the right side. The scale bar represents 3.5 mm in each part. Both photographs were taken by Sue Lindsay.

Material and methods

Slugs were collected by hand by Dr Michael Gorton from four localities on Norfolk Island during the Quarantine Survey of 2013 (Australian Government Department of Agriculture 2015). The specimens were stored in ethanol (>70%). The Australian Museum Malacology collection registration numbers, field designations of the extracted specimens, locations, collection date and habitat were as follows:

1. AM C.532716 (field number MJG093b): −29.029603S, 167.932433E on 6/12/2013 from a cucumber plant in a greenhouse;
2. AM C.532719 (field number MJG159b): −29.054501S, 167.943761E on 9/22/2013 from a low lying stack of seasoned *Araucaria heterophylla* timber on pastoral margin;
3. AM C.532720.001 to C.532720.005 (field numbers in corresponding order MJG161-1 to MJG161-5): −29.022142S, 167.971795E, on 9/22/2013 amongst coastal weeds and debris and driftwood under boulders near road side at Cascade jetty;
4. AM C.532723.001 and C.532723.002 (field numbers in order MJG165c-1 and MJG165c-2): −29.022325S, 167.967159 on 9/22/2013 amongst rocks in very damp, weedy vegetation (principally *Colocasia esculenta*) near a disturbed muddy stream at Cockpit Waterfall reserve.

DNA extraction, from a small portion of the left hand side of the mantle, and PCR amplifications of COI were performed as detailed in Colgan and Da Costa (2009) using the Folmer et al. (1994) universal primers. The sequences were determined in both directions at Macrogen (Seoul, Korea).

Chromatograms were checked and sequences assembled using Sequencher™, version 4.9 (Gene Codes Corporation, Ann Arbor, Michigan). All *Deroceras laeve, D. reticulatum*, *D. panormitanum*, *D. golcheri* and *D. invadens* sequences in GenBank at 1 June, 2016 were downloaded for analyses to identify the Norfolk Island specimens. An alignment of the sequences was made using CLUSTAL X (Thompson et al. 1997). This was analysed using maximum parsimony and maximum likelihood ("ML") approaches. No differences in the placement of the Norfolk Island specimens were observed so only ML results are reported. These analyses were conducted by the RAxML Black Box (Stamatakis et al. 2008), partitioning the data by codon position for parameter estimation and assuming a gamma distribution for inter-position variation in substitution rates, with no sites considered invariable. One hundred bootstrap replicates were conducted.

Results

Full-length sequences were obtained for 6 of the 9 slugs from which extractions were attempted. Two haplotypes were observed among these, with one represented by one individual and the other by five. The GenBank accession numbers for these two haplotypes were KX977425 and KX977426. The ML topology (Figure 2) reveals that the Norfolk Island specimens were all resolved, with robust bootstrap support of 91%, within the *D. invadens* lineage confirming that this species is now present on the island. The *D. invadens* lineage was resolved (88% bootstrap support) as the sister group of the strongly supported (92%) clade comprising the species *D. panormitanum* and *D. golcheri*, each of which was strongly supported as monophyletic. *D. laeve* was not recovered as monophyletic in this analysis, possibly because of the large distance to the outgroup *D. reticulatum* on which the topology was rooted.

Blastn searches revealed that the closest available matches for the Norfolk Island sequences were with

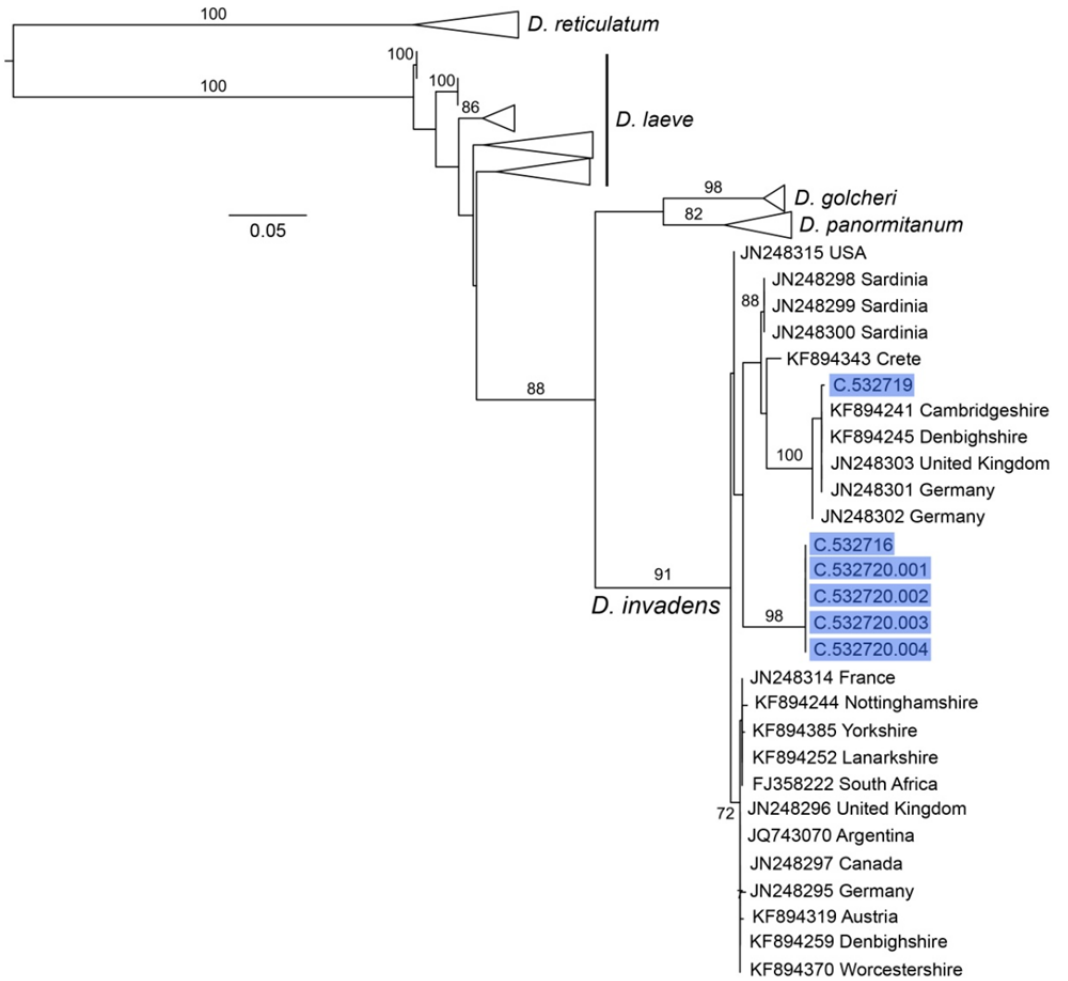

Figure 2. Maximum likelihood phylogeny showing the relationships of *Deroceras* sequences from Norfolk Island. Specimens from the island are highlighted in blue. Bootstrap support values greater than 70 are shown near the relevant branches. The scale bar is 0.05 substitutions per site. Sequences from *D. invadens* are identified by the relevant GenBank accession number. The localities for *D. invadens* specify the nation of provenance if no more detailed information is available or if there is only one representative from that country. The triangles represent multiple sequences from the nominated species.

D. invadens. The sequence found only in the slug C.532719 (Genbank accession KX977426) had similarity of 99% to several sequences from Europe but the more frequent haplotype (Genbank accession KX977425) had at most 96% identity with other sequences from the species. The closest matches from the sister group to *D. invadens* in the phylogenetic analyses was, for *D. panormitanum*, 92% for the slug C.532719 and 91% for the other sequences and for *D. golcheri* 90% for the slug C.532719 and 89% for the others.

The two Norfolk Island haplotypes of *D. invadens* differed at 26 of 655 positions (3.97%) indicating that the invasive population derived from multiple individuals. A short fragment was scored in one of the slugs from site MJG165c. This was sufficient to identify the species to which the individual belonged but too short to include in the phylogenetic analysis. The sequence differed from the more frequent Norfolk Island haplotype only at the three positions which could not be reliably determined in the 100 bases of available sequence.

Discussion

Two invasive species of *Deroceras* have been recorded from Norfolk Island although it is not known if they both still occur there. The specimen determined as *D. laeve* by Hutchinson et al. (2014) was collected more than 15 years ago whereas the

specimens confirmed as *D. invadens* by DNA sequencing were collected in 2013. The occurrence of *D. invadens* on Norfolk is the most northerly confirmed record from Australia by nearly 1.4 degrees of latitude, with the most northerly population on the mainland being at 30.4 degrees South (Hutchinson et al. 2014).

As *D. invadens* is capable of self-fertilising (Hutchinson et al. 2014), it is possible that an invasive population could be established by a single slug, as may have been the case for Marion Island in the Prince Edward Island group south-east of Africa. Only one COI haplotype was observed in the 25 individuals from the island that were sequenced by Lee et al. (2009). *D. invadens* is, however, represented by at least two haplotypes on Norfolk Island indicating that multiple individuals have successfully contributed genetically to the invasion.

Both of the *D. invadens* haplotypes found on Norfolk Island are relatively divergent within this species based on analysis of the sequences found here and GenBank data (Reise et al. 2011; Gutierrez Gregoric et al. 2013; Rowson et al. 2014). Sequences similar to one of the Norfolk Island haplotypes are known from the United Kingdom and Germany but no very close relatives of the other (the more common haplotype) have yet been found in Europe or elsewhere. This suggests that the species' genetic diversity in its native range has not yet been fully sampled. That range is proposed to be in Italy although it may not have included all of the country (Reise et al. 2011). Searching for the more divergent Norfolk Island haplotype of *D. invadens* might help characterise the native range of the species. The proposed range should include at least some of the areas in which the haplotype occurs in Europe.

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