

Clarifying marine invasions with molecular markers: an illustration based on mtDNA from mistaken calyptraeid gastropod identifications

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Abstract Species invasions are occurring at an increasing rate in coastal environments. Accurately identifying introductions is a critical issue to take full advantage of the new invasion databases. Further, life history differences between morphologically comparable species may require that different management strategies be instigated to effectively control different species. Facing this problem, we used molecular approaches and documented a case of mistaken identification in a group of marine invertebrates, the calyptraeid gastropods. Members of this group have repeatedly and successfully invaded new habitats after anthropogenic introduction, especially in estuaries and bays on the west coast of the United States of America. For example, *Crepidula fornicata*, native to the east coast of the USA, has been reported from at least five USA west coast estuaries. We

sequenced a fragment of the COI gene of a sample of putative *C. fornicata* from Humboldt Bay, California. By constructing a phylogeny of these and other calyptraeid gastropod sequences, we discovered that the individuals were *C. convexa*, the convex slippershell. In contrast to *C. fornicata*, *C. convexa* has large, demersal eggs and larvae are well developed at hatching. Its potential for dispersal is therefore lower as compared to *C. fornicata* and therefore any strategy to manage the invasion should take this into account. In the present study, we demonstrated the utility of molecular tools that can be used by non-taxonomic experts, to quickly and accurately identify alien species – an important first step in any study of invasion biology.

Keywords Invasion · Mollusc · mtDNA · *Crepidula*

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Introduction

The invasion of novel habitats by non-native species is a pervasive component of human induced global change (Vitousek et al. 1997; Mack et al. 2000; Cassey et al. 2005). This is true for the marine environment as much as the terrestrial. Indeed, translocations via both ballast water and commercial shellfish operations continue to be major propagule sources of potentially

invasive non-native species (Carlton and Geller 1993; Carlton 2000; Wolff and Reise 2002). There is widespread concern about the consequences of marine invasions and considerable effort has been made to predict potential marine invaders or marine habitats (Carlton 1996) and to reduce population density or eradicate invasive species. These are often unsuccessful due to insufficient knowledge about many parameters of the invasion process such as the life-history traits of the invader, invasion pathways – or the invader (Myers et al. 2000). Recently, it has been recognised that there is an increasing need to compile and to analyse databases that document non-native species introductions and invasions (Ricciardi et al. 2000; Cadotte et al. 2006). It is, of course, vital that these databases accurately report the species that are being introduced.

A group of highly and commonly invasive species are the calyptraeid gastropods (Collin 2003a). Since the late 1800s, members of this group have been (and continue to be) introduced in association with major commercial shellfish operations, and have proliferated in many seas of Europe, Asia and North America (Carlton 1999; Wolff and Reise 2002). *Crepidula* species are not cryptic species *sensu stricto* and thus their identification is possible based on shell and other anatomical characteristics (see key characters and pictures in Hoagland 1977). However, species of this group are phenotypically plastic and shells have a low number of informative characters (Collin 2003a), so misidentification of introduced *Crepidula* species have been made, and corrected, in the past. For example, *Crepidula fornicata* (Linnaeus 1758), a north-eastern USA native, was widely thought for many years to be present in many Asian ports, until correctly identified as *C. onyx* (Woodruff et al. 1986). Nevertheless, *C. fornicata* has been correctly identified as an invader of many European and north-west USA bays (Wolff and Reise 2002), where it has considerable ecological and economic impacts (Blanchard 1997; Thielges et al. 2003). Other calyptraeid gastropods have also been translocated and successfully established elsewhere – *C. onyx*, as previously mentioned, but also *C. convexa* and *C. plana* (Carlton 1999). If life history strategy is an important factor in the establishment and invasion

process, then this group shows that species with quite different life history strategies (Hoagland 1979; Collin 2003b) can become successful invaders: for example, *C. fornicata* is a protandrous hermaphrodite, has a high fecundity and an extended planktonic duration (Dupont et al. 2006; Viard et al. 2006 and references therein); in contrast, *C. convexa* has a lower fecundity and direct development (Collin 2003b). As part of a wider investigation of the invasion process of *C. fornicata* along the western coasts of USA and Europe, the aim of the present study was to use molecular tools to identify an introduction of *Crepidula* spp. Molecular tools have been widely used to elucidate the invasion vectors (e.g. Voisin et al. 2005), to identify small-size organisms (e.g. Patil et al. 2005) or reveal cryptic species (Stepien and Tumeo 2006). We here illustrate that some simple molecular tools can also be powerful agents to correctly identify historical samples (dried mollusc samples) and validate (or invalidate) previous morphological identification, thus being an efficient tool in the study and management of coastal marine invasions.

Materials and methods

Sampling strategy and mtDNA sequence dataset

We obtained a sample of seven dried and preserved specimens collected in 1989 from Humboldt Bay, northern California: these were listed as *C. fornicata* in Carlton (1992). mtDNA analysis (see below) revealed unexpected sequences and we thus compared this sequence with mtDNA sequence data for other species of *Crepidula*. First, we analyzed five samples of *C. convexa* collected in August 2002 from the east coast of the USA (Virginia – Gloucester Point). Second, we used published sequences of different *Crepidula* species (Collin 2001): in our analyses we included all available unique sequences of *C. convexa* (Say 1822) (see Table 1) and *C. ustulatulina* (formerly *C. convexa*; Collin 2002) as well as all the unique sequences of *C. fornicata* from Collin (2001). As we were interested by species

Table 1 Genbank numbers and location of *C. convexa* sequences that were derived from Collin (2001). Additional *C. convexa* samples in Fig. 1 were collected for the present study

Genbank No.	Location
AF388741	Canada
AF388726	Rhode Island
AF388727	Rhode Island
AF388728	Rhode Island
AF388729	Rhode Island
AF388720	New Jersey
AF388721	New Jersey
AF388722	New Jersey
AF388723	New Jersey
AF388724	New Jersey
AF388725	New Jersey
AF388714	Georgia
AF388715	Georgia
AF388716	Georgia
AF388717	Georgia
AF388718	Georgia
AF388719	Georgia

identification, we also retrieved sequence data of all the *Crepidula* sp. available in GenBank at the time of the analysis and randomly selected individuals for each of the species: *C. cerithicola* (C.B. Adams 1852) (GenBank No. AF388698), *C. unguiformis* (Lamarck 1822) (AF178156), *C. striolata* (Menke 1851) (AF353123), *C. cf. perforans* (Valenciennes 1846) (AF178155), *C. plana* (Say 1822) (AF178127), *C. atrasolea* (R. Collin 2000) (AF178140) and *C. depressa* (Deshayes 1830) (AF178154). The total number of sequences used to generate the dataset was 73.

DNA sequencing

Total DNA was extracted from less than 15 mg of foot muscle using Dneasy Tissue Kit according to the manufacturer's protocol (Qiagen, Germany). The utility of the COI gene has been demonstrated by Hebert et al. (2003); we sequenced a ~710bp fragment of this gene using primers LCO1491 and HCO2198 of Folmer et al. (1994). PCR reactions were undertaken in 50 µl volumes which contained 5 µL of 10x reaction buffer (100 mM Tris-HCl pH 8.3 and 500 mM KCl) (Promega), 4 µL of 10 mM dNTPs (Promega), 5 µL of 25 mM MgCl₂, 1 unit of *Thermus aquaticus* DNA polymerase (Promega), 1–2.5 µL of

template DNA and 0.85 µL of each primer (10 mM), the balance being Milli-Q filtered and autoclaved water. PCR cycling conditions were altered slightly from Folmer et al. (1994) and were: 94°C for 2:00 min; 94°C for 35 s, 50°C for 35 s, 72°C for 70 s, 35 cycles; 72°C for 7:00 min. Double stranded PCR products were cleaned using MultiScreen-PCR MANU03010 plates (Millipore) and cycle-sequenced using ABI PRISM® BigDye Terminators v3.0 Cycle Sequencing Kit following the manufacturer's protocol (Applied Biosystems). Cycling conditions were: 96°C for 2 min, then 55 cycles of 96°C for 30 s, 50°C for 30 s, 60°C for 4 min. Unincorporated BigDye were removed using MultiScreen MAHVN4510 plates (Millipore). In order to minimize sequencing errors, both strands were sequenced for each individual using an ABI PRISM® 3100 Automated DNA Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA).

Phylogeny construction

Sequences were edited in BioEdit version 5.0.1 (Hall 1999) and aligned with Clustal V (Higgins et al. 1992). Once aligned, the appropriate model of DNA substitution was selected with the aid of Modeltest version 3.0 (Posada and Crandall 1998). Modeltest implemented a hierarchical likelihood ratio test for alternative models of DNA sequence evolution. Paup* version 4.0b8a (Swofford 2002) was used to infer phylogenetic relationships. The model of evolution selected by Modeltest was used to estimate a neighbour joining phylogenetic tree. Relative branch support was evaluated using non-parametric bootstrap analysis (Felsenstein 1985) based on 1000 bootstrap replicates.

Results

DNA from the seven dried samples, presumed to be *C. fornicata* from Humboldt Bay, was recovered with standard procedure as effectively and successfully as performed with individuals recently sampled and immediately kept in alcohol (i.e. *C. convexa* from Gloucester Bay (Virginia, USA) in this study; *C. fornicata* from west and east coasts of USA, unpublished data). GenBank

Accession Numbers for *C. convexa* from Humboldt Bay and Virginia are EF088806-EF088817.

Sequence examinations were carried out over 73 sequences analysed over 614 base pairs. The general time reversible model (Rodríguez et al. 1990) was the model of sequence evolution selected for these data. The average nucleotide frequencies were: A = 0.2751, C = 0.1563, G = 0.1687 and T = 0.3999. Estimated rate parameters were R(a) [A-C] = 1.3186, R(b) [A-G] = 12.3413, R(c) [A-T] = 4.5075, R(d) [C-G] = 0.8274, R(e) [C-T] = 26.1538 and R(f) [G-T] = 1.0. The variable sites gamma distribution shape parameter (G) was estimated to be 2.0743 and the proportion of invariable sites (I) was 0.5699.

The phylogenetic tree was well resolved with high bootstrap support for the *C. convexa*, *C. ustulatulina* and *C. fornicata* clades (Fig. 1).

Surprisingly, all dried samples of putative *C. fornicata* collected from Humboldt Bay were within the clade that exclusively comprised individuals of *C. convexa*. Therefore, the samples obtained were highly unlikely to be *C. fornicata*. Also, the Humboldt Bay samples were also not *C. ustulatulina*, the most closely related species to *C. convexa*. Further, the samples were most likely to be derived populations, originally from north of Georgia, i.e. from the northeast of the United States (see Fig. 1).

Discussion

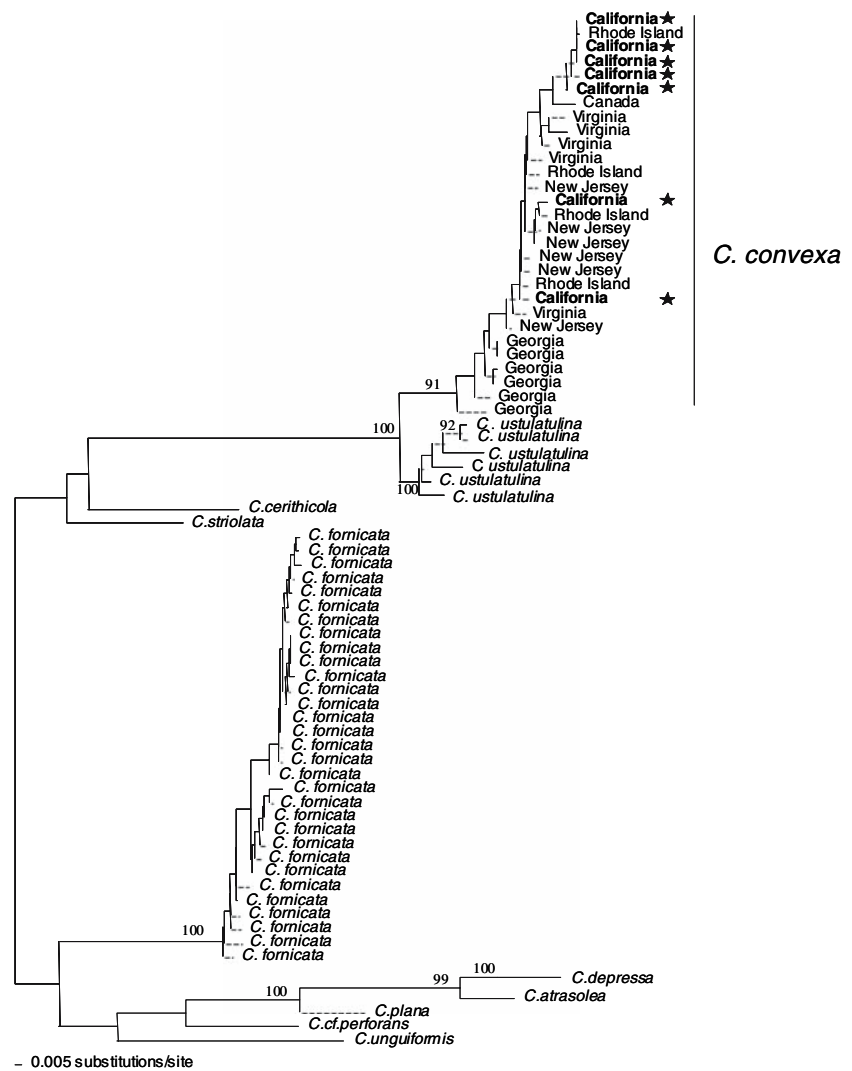
Sequencing of mtDNA and the construction of a robust phylogeny of several species of the genus *Crepidula* revealed that putative samples of *C. fornicata* collected in 1989 from Humboldt Bay, California, were most likely to be *C. convexa* (Fig. 1). Further, the enzyme cutter PstI can be used to discriminate simply (with a standard PCR-RFLP procedure) between *C. fornicata* and *C. convexa* COI sequences. The accurate identification of introduced species is important to the successful characterisation, management and understanding of the invasion process (Myers et al. 2000) and illustrated in other taxa at the subspecies level (e.g. *Carcinus maenas*; Geller et al. 1997) or at the species level (e.g. *Dreissena*

bugensis; Müller and Griebeler 2002). Molecular tools to identify introduced species can be particularly useful in cases of species complexes or sibling species (e.g. *Marenzelleria* sp.; Müller and Griebeler 2002) when morphological characteristics are difficult to monitor in the field or require the help of taxonomic experts. In principle, the identification of *C. crepidula* and *C. convexa* can be made using either standard visual methods (e.g. shell characteristics or life-history traits; Hoagland 1977; Collin 2003a, b). However, it is noteworthy that specimens of *C. fornicata* have been mistaken in the past (Hoagland 1977; Woodruff et al. 1986). Our results also illustrate that the taxonomic intransigence of the Calyptraeidae (Collin 2003a) can turn into mis-identification of a non-native species in the published literature.

Several authors have underlined the usefulness and lack of molecular data for the study of biological invasions (Lee and Bell 1999; Holland 2000; Ricciardi and MacIsaac 2000). In non-native marine species, genetic studies have been used to trace the origin/vectors of introduction (e.g. *Undaria pinnatifida*; Voisin et al. 2005), to assess dispersal strategies (e.g. *Dreissena bugensis*; Wilson et al. 1999) or to investigate the occurrence or the patterns of hybridization with native species (e.g. *Spartina* sp.; Baumel et al. 2001). In *C. fornicata*, genetic markers have been successfully used to compare introduced and native populations (Hoagland 1984), to test hypotheses about founder events and effective dispersal in the range of introduction (Dupont et al. 2003; Viard et al. 2006) and examine reproductive strategies (Dupont et al. 2006). As shown in this study, genetic data can also be used as a rapid diagnostic tool for identifying previously un-recognized/mis-identified alien species. Further, we have demonstrated that samples that have been dried and stored for many years can be successfully sequenced, and so the techniques that we have presented here can be used in correctly classifying invasions from recent historical samples as recently demonstrated in terrestrial plants from herbarium samples, (e.g. *Phragmites australis*, Saltonstall 2002).

The origin of *C. convexa* collected from Humboldt Bay was most likely (ultimately) to be from the northeast of the US, based on the

Fig. 1 Neighbor joining phylogram showing bootstrap support. Samples from California (marked with a star) were putatively *C. fornicata* (Carlton 1992). Specific sequence identities and dataset are available from the authors



resolved phylogeny (Fig. 1). This is consistent with other bays in California that have likewise been invaded by north-east US coast species (e.g. Carlton 1992; Boyd et al. 2002). The mechanisms and vectors behind this introduction remain unknown. However, the transportations of *Crassostrea virginica*, *Crassostrea gigas* and *Ostrea edulis* for shellfish industry at the beginning of the 20th century are the likely vectors of introduction of *C. fornicata* along the US west coast (e.g. Puget Sound). A simultaneous introduction of *C. convexa* can thus be hypothesized in lights of our results. Given the low dispersal capabilities of this species, as compared to *C. fornicata*, human-mediated transportations between oyster culture sites along the US west

coast is likely to be responsible of the spread of *C. convexa* among basins of the US west coast. This scenario could be tested with the molecular methods described herein. The life history characteristics of a given species may be an important factor in determining an effective management strategy for ameliorating the effects of and controlling an exotic invasive species. In the specific case here, *C. fornicata*, a species with high fecundity and pelagic larval dispersal would be expected to undergo a rapid range expansion compared to a species that has low fecundity and limited larval dispersal – such as *C. convexa*. It may nevertheless be possible that other records of *C. fornicata* on the US west coast and elsewhere are in fact a different species. This

has indeed been shown to be the case in Asia (Woodruff et al. 1986).

There is some evidence that the transportation and establishment of invasive species may still be increasing (Simberloff 2004). Coastal marine and freshwater environments provide clear examples in which the number of established exotic species continues to accumulate. Indeed Cohen & Carlton (1998) found that non-native species have been arriving and establishing in San Francisco Bay Estuary at an accelerating rate. It is imperative that researchers are able to accurately identify non-native (and potential pest) species at the earliest stage of the introduction pathway (*sensu* Williamson 1996; Cassey et al. 2004). Characterising a problem is the first step in any potential ameliorating action; lists and databases are often compiled for this purpose (e.g. Carlton 1992; Ruiz et al. 1997; Reise et al. 1999; Gouletquer et al. 2002) and subsequently used in macroecological analyses (Cadotte et al. 2006). Our results clearly highlight that the published records of invasive species are not always accurate and that genetic techniques can be used to verify the accuracy of putative invasions - especially for morphologically cryptic or difficult to identify groups.

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