Morphological and genetic analyses reveal a cryptic species complex in the echinoid *Echinocardium cordatum* and rule out a stabilizing selection explanation ☆

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ABSTRACT

Preliminary analyses revealed the presence of at least five mitochondrial clades within the widespread sea urchin *Echinocardium cordatum* (Spatangoida). In this study, we analyzed the genetic (two mitochondrial and two nuclear sequence loci) and morphological characteristics (20 indices) from worldwide samples of this taxon to establish the species limits, morphological diversity and differentiation. Co-occurring spatangoid species were also analyzed with mitochondrial DNA. The nuclear sequences confirm that mitochondrial lineages correspond to true genetic entities and reveal that two clades (named A and B1) hybridize in their sympatry area, although a more closely related pair of clades (B1 and B2), whose distributions widely overlap, does not display hybridization. The morphology of all *E. cordatum* clade pairs was significantly differentiated, but no morphological diagnostic character was evidenced. By contrast, other spatangoid species pairs that diverged more recently than the *E. cordatum* clades display clear diagnostic characters. Morphological diversity thus appears responsible for the absence of diagnostic characters, ruling out stabilizing selection, a classical explanation for cryptic species. Alternative classical explanations are (i) environmental plasticity or (ii) a high diversity of genes determining morphology, maintained by varying environmental conditions. We suggest a new hypothesis that the observed morphological diversity is selectively neutral and reflects high effective population sizes in the *E. cordatum* complex. It is supported by the higher abundance of this taxon compared with other taxa, a trend for the genetic and morphological diversity to be correlated in Europe, and the higher genetic and morphological diversities found in clades of *E cordatum* (except B1) than in other spatangoid samples in Europe. However, the Pacific clades do not confirm these trends.

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1. Introduction

Cryptic species cover a wide taxonomic spectrum (Knowlton, 1993, 2000; Klautau et al., 1999; Bickford et al., 2007; Pfenninger and Schwenk, 2007; Trontelj and Fišer, 2009), and are being discovered at an increasing rate. Nevertheless, few studies go beyond the raw identification, and even fewer propose hypotheses for the processes involved in cryptic speciation.

When the absence of diagnostic character is established, it may result from the fact that divergence is too recent or from a particular slowdown of morphological differentiation. Comparisons with taxa that are phylogenetically closely related to the cryptic species complex, for which divergence times are known, (Poore and Andreakis, 2011) can be used to disentangle the two above-mentioned hypotheses.

The most invoked adaptive process to explain crypticism (when recent speciation is ruled out) is stabilizing selection (Eldredge and Gould, 1972; Charlesworth et al., 1982; Colborn et al., 2001; Wiens and Graham, 2005; Caputi et al., 2007; Smith et al., 2011). Stabilizing selection is mostly expected for ecological specialists or taxa displaying restricted distribution ranges. Additionally, morphological conservatism may also result from developmental constraints (Wake et al., 1983; Maynard Smith et al., 1985). In such cases, low morphological variation is expected within each species.

Conversely, crypticism may also be frequent when morphological variation within species is high because the establishment of diagnostic differences among such species takes more time. High polymorphism is expected to be advantageous in environments

submitted to spatio-temporal fluctuations. Morphological polymorphism may be achieved by two different mechanisms: environmental plasticity (a single genotype may lead to a variety of morphologies) or genetic polymorphism. The regime of environmental fluctuations and the life history traits of the taxon play a major role in determining whether plasticity or high genetic diversity is the best strategy (Stearns, 1992). Genetic polymorphism has a cost (the selective load), but it may be sustainable in species with large fecundity and effective population sizes. Whatever the underlying determinism (plasticity or genetic polymorphism), high morphological variability is more likely for taxa with large geographical ranges inhabiting a wide variety of environments. This is the case of species with high dispersal potential.

Although the causes examined above directly involved natural selection, population genetics theory suggests that strictly neutral processes could also be involved. Higher polymorphism at neutral loci is expected for taxa with larger effective population sizes. If such a taxon splits into separate species, ancestral polymorphism will remain shared among the daughter species for a longer time than in taxa with lower effective population sizes. If the phenotypic characteristics studied to diagnose species are selectively neutral (Bijlsma et al., 1991; Ouborg et al., 1991), this leads to an absence of diagnostic characteristics for a longer time in the high effective population size taxon.

Marine benthic organisms with a pelagic phase in their life-cycle are good candidates to investigate factors explaining cryptic species. They are prone to dispersal and may undergo high environmental variations during early development stages, but they are also closely linked to a specific environment once settled on or in the bottom (as juveniles and adults). They are also expected to display large effective population sizes (Boissin et al., 2011; Foltz et al., 2003, 2004). These features may promote different paths toward cryptic speciation: (1) stabilizing selection, (2) diversifying selection or selection for plasticity and (3) large effective population sizes leading to high diversity and thus absence of diagnostic characteristics.

Echinocardium is a genus of the Loveniidae family (order Spatangoida) composed of five contemporaneous species: *E. mediterraneum* (Forbes, 1844) in the Mediterranean Sea and on the southern coasts of Portugal and Northern coasts of Morocco; *E. flavescens* (Müller, 1776) in the Atlantic Ocean from the southern coasts of Iceland to the Azores; *E. pennatifidum* Norman, 1868 from Norway to Spain; *E. capense* Mortensen, 1907 in the Mediterranean Sea, Japan and Southern Africa (but see Mironov, 2006) and *E. cordatum* (Pennant, 1777) presenting an antitropical distribution covering European Seas, from Norway to Greece, the Pacific Ocean in the Northern (Sea of Japan) and Southern hemisphere (Australia and New Zealand), and the Southern Atlantic Ocean (Southern Africa). In addition to these five well-established species, six other forms have long been considered as distinct species, [*E. mortenseni* Thiéry, 1909, *E. laevigaster* Agassiz, 1869, *E. lymani* (Lambert & Thiéry, 1917), *E. fenauxi* Péquignat, 1963, *E. australe* Gray, 1851 and *E. zealandicum* Gray, 1851], but their specific status has since been invalidated (Mortensen, 1951; Higgins, 1974; David and Laurin, 1996; Féral et al., 1998).

Echinocardium cordatum occupies temperate sandy to muddy habitats, ranging from the intertidal to more than 250 m deep, but it is absent from American coasts (both Atlantic and Pacific) (Mortensen, 1951; Buchanan, 1963, 1966; Higgins, 1974; Féral et al., 1998; Kashenko, 2007). Its abundance and efficient burrowing activity make it a key ecological species (Osinga et al., 1995; Lohrer et al., 2004, 2005), which can represent up to 60% of the macrobenthic biomass (Nakamura, 2001). *E. cordatum* has a bentho-pelagic life cycle with external fertilization leading to a pelagic planktotrophic larva that settles and metamorphoses into a benthic juvenile. The echinopluteus larva can stay between 15

and 40 days into the water column (Kashenko, 2007; Nunes and Jangoux, 2007). During the first 12 days, the larvae remain distant from the bottom and can, in theory, drift over large distances (Shanks et al., 2003; Shanks, 2009) potentially contributing to genetic homogeneity. *E. cordatum* is probably the most studied Spatangoid, for its general biology (Moore, 1935, 1936; Goodwin and Srisukh, 1951; Buchanan, 1966; Duineveld and Jenness, 1984; De Ridder et al., 1985; Yakovlev, 1987; De Ridder and Jangoux, 1993; Osinga et al., 1995; Nunes and Jangoux, 2004; Vopel et al., 2007; Egea et al., 2011), ecology (Thatje et al., 1999; Dautov, 2000; Rosenberg et al., 2002; Ozolin'sh and Nekrasova, 2003; Lohrer et al., 2004, 2005; Gilbert et al., 2007; Zhou et al., 2007; Dashfield et al., 2008; Egea et al., 2011), development (David and Laurin, 1991; Saucede et al., 2006; Kashenko, 2007; Nunes and Jangoux, 2007), morphology (Higgins, 1974; De Ridder et al., 1987; Ghyoot et al., 1987; David et al., 1988, 1999; Hanot et al., 1990; David and Laurin, 1996) and genetics (Féral et al., 1995, 1998; Chenuil and Féral, 2003; Chenuil et al., 2008). Nevertheless our understanding of the evolutionary history of this taxon remains incomplete.

E. cordatum has long been considered a cosmopolitan species; its broad distribution is consistent with its reproduction mode, although the other species of the genus, which have similar life cycles, are only recorded in European waters (except *E. capense*). Preliminary analyses based on the 16S ribosomal gene of the mitochondrial DNA revealed that *E. cordatum* is split into several mitochondrial lineages. Three lineages were identified in Europe (clade A on the Atlantic coasts, clade B1 in the Mediterranean Sea and the Atlantic coasts of the Iberian Peninsula, and clade B2 in the Mediterranean Sea (Chenuil and Féral, 2003)).

Although molecular approaches, particularly mitochondrial DNA, have proved useful in species delimitation ((Avise, 2000; Hebert et al., 2003; Vogler and Monaghan, 2007; Meier, 2008) but see also (Galtier et al., 2009)), the occurrence of a deep divergence between mitochondrial lineages does not prove that they are distinct non-interbreeding species because demographic (e.g. a bottleneck) and/or selective events (e.g. several selective sweeps) can generate such topologies within a single panmictic unit (Wakeley, 2006).

The aims of the present study were to confirm whether mitochondrial clades uncovered in *E. cordatum* by Chenuil and Féral (2003) represent true cryptic species and whether they hybridize. We used both mitochondrial and nuclear markers, combined with a large morphological survey. We compared the genetic and morphological diversity, putting the results in perspective with more or less closely related spatangoid species presenting wide distributions and comparable ecologies. Our hypothesis that morphological diversity could be neutral in *E. cordatum* and that large effective population sizes may be associated with a type of cryptic species will be discussed.

2. Material and methods

2.1. Specimen collection & study strategy

The samples studied in this paper were mostly collected by scuba-diving, allowing morphological identification of the specimens according to Mortensen's (1951) identification key. The sampled sites cover most of the European coastline (Fig. 1) and a wide spectrum of environments, from fine silty-sandy (eg. Bodø) to coarse sediment (eg. Marseille).

For the purposes of our study, we considered mitochondrial, nuclear and morphological markers at different scales. First, at the widest geographical and taxonomic scale, mitochondrial 16S and COI sequences were obtained from several *E. cordatum*