Species are hypotheses: avoid connectivity assessments based on pillars of sand

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Abstract.

Connectivity among populations determines the dynamics and evolution of marine populations, and its assessment is essential in ecology in general and in conservation biology in particular. The robust basis of any ecological study is the accurate delimitation of evolutionary units, such as populations, meta-populations and species. Yet a disconnect still persists between the work of taxonomists describing species as working hypotheses to be tested notably through molecular systematic studies, and the use of species delimitation by molecular ecologists interested in describing patterns of gene flow. This problem is particularly acute in the marine environment where the inventory of biodiversity is relatively delayed while molecular studies since two decades have shown a high prevalence of cryptic species. In this review we illustrate, based on a review of the marine population connectivity literature and case studies, how the failure to recognize evolutionary-relevant unit boundaries leads to heavily biased estimates of connectivity. We review the conceptual framework within which species delimitation can be formalized as falsifiable hypotheses, and show how connectivity studies can feed integrative taxonomic work, and *vice versa*. Finally, we suggest strategies for spatial, temporal and phylogenetic sampling to reduce the probability of inadequately delimiting evolutionary units when engaging in connectivity studies.

I. Context and problems.

Population connectivity, involving the demographic and/or evolutionary interdependence of populations through individual movements across their species range, has been subject to an increasing number of studies for the last two decades (Scopus search for "population connectivity", accessed on August 23th, 2014: 2,060 documents published since 1993, including 1,700 during the last 5 years). Connectivity studies have bearing on both applied and theoretical research. For instance, assessing the extent of connectivity among populations has become a key aspect in diverse areas of conservation biology. Applications of connectivity studies include: 1) identifying genetically isolated populations of protected or endangered species that should be monitored as separate conservation units (Palsbøll *et al.* 2007), 2) optimizing the size and location of protected areas to create well-connected reserve networks (Kritzer & Sale 2004; Jones *et al.* 2007), 3) identifying stocks for fisheries management (Fogarty & Botsford 2007; Waples *et al.* 2008; Reiss *et al.* 2009), and 4) evaluating the potential impacts of resource exploitation on population dynamics of local communities (Bors *et al.* 2012; Plouviez *et al.* 2013). Besides these important applications to management and conservation, connectivity studies can also help address long-standing questions. In ecology for example they allow understanding to what extent marine populations are closed or open systems (Cowen *et al.* 2000), in microevolution they can be used to identify factors that create and maintain genetic differentiation (e.g. Bilton *et al.* 2002; Shank 2010), or to understand how local adaptation can occur in high gene flow species (e.g. Nielsen *et al.* 2009).

In this review, we underline the crucial importance for connectivity studies of the state of taxonomic knowledge and correlated aspects on the available background knowledge on the biology of organisms. These factors are sometimes overlooked despite the fact that they should condition the sampling design and the inferences made from genetic data. One of the main considerations we develop is that properly estimating connectivity at the population level requires assessing the robustness of available taxonomic hypotheses. This is not trivial as for a large part of biodiversity, taxonomic knowledge is inadequate or even lacking. Indeed, a great portion of the world's biodiversity remains to be described, mainly in under-explored, difficult-to-access habitats such as tropical rainforests and the marine environment (e.g. Appeltans *et al.* 2012). In addition, in these habitats, species descriptions are often based on few specimens and species distribution ranges and ecological requirements are poorly known. The development of DNA-sequencing techniques has considerably accelerated the rate of discovery and the documentation of species distributions. This is notably true in the marine realm, especially for poorly studied eukaryotic phyla for which few or no other characters were available as reference for taxonomic delimitations (e.g. cryptic lineages of macro- and micro-algae that are subject to convergent evolution towards reduced morphologies; see for review Leliaert *et al.* 2014). However, turning these DNA-based discoveries (generally

only based on data such as those gathered from the Barcode of Life program), into robustly and formally named taxonomic entities remains a long and difficult process (Satler *et al.* 2013). Consequently, the literature includes a large number of undescribed and un-named cryptic or pseudo-cryptic species for which identification from one study to another is challenging in the absence of formal naming and description. As recently underlined by Fontaine *et al.* (2012), the time lapse between specimen collection and formal species description is extremely long (average "shelf life" of 21 years). A perhaps less appreciated issue is that even for abundant, commercially-important or ecologically well-studied taxa, nominal species were often described long ago and are not necessarily adequately delineated. This situation is particularly critical in the marine environment (e.g. Uthicke *et al.* 2010; Jaafar *et al.* 2012; Mantelatto *et al.* 2014; Thomas *et al.* 2014). For instance *Ciona intestinalis* (Linnaeus, 1767), is a model organism in evolutionary developmental biology and phylogeny and was among the first animals to have its genome fully sequenced (Dehal *et al.* 2002). However this taxon was only recently revealed as a complex of four cryptic species (Suzuki *et al.* 2005; Caputi *et al.* 2007; Nydam & Harrison 2007; Zhan *et al.* 2010), including two widespread species that diverged ca. 3-4 my ago (Roux *et al.* 2013) but that are not yet formally named.

Beyond the discovery of recently diverged species, examples of taxonomic confusions at higher ranks are also not rare. For instance in 2009, Johnson *et al.* revealed that three families of deep-sea fishes artificially separated juveniles, males and females. This confusion was resolved by examining the morphology of more specimens and by using molecular data. This taxonomic revision, in which the three families are synonymized (the Cetomimidae being the valid family name), greatly changes the understanding of connectivity in terms of life history traits and ecology but should also affect the strategy for sampling populations.

The main objective of this review is thus to stress the fact that, when conducting connectivity assessments, the taxonomic status of the studied organisms should be critically reassessed, and revised if necessary, in light of newly acquired biological data. This point is particularly crucial in marine systems, which are the focus of this review, both because the marine biota accumulates the greatest number of deep evolutionary lineages (i.e. phyla), but also because it remains vastly under-sampled (see Costello *et al.* 2010 for multicellular organisms and Not *et al.* 2012 for the phytoplankton). Additionally, we outline that the type of data collected for estimating connectivity using population genetic methods may in turn be used to refine the taxonomic knowledge at the species rank. Actually, cryptic species are typically revealed by population genetic studies or by phylogeographic analyses. In fact, the patterns observed in connectivity studies and species delimitations belong to the same divergence continuum because both result from the same ecological and micro-evolutionary processes. Furthermore, the lack of knowledge for marine organisms is so important (e.g. Knowlton 2000 or Webb *et al.* 2010) that the question of species delimitations does not only concern the limits between recently separated lineages (i.e. the 'emergent phylogenetic systems' as defined

in Carstens *et al.* 2013) but also in the definition of higher-rank classifications as illustrated with the above example of Cetomimidae. We here first illustrate the importance of accurate taxonomic evaluation in the analysis of connectivity by examining one specific question (i.e. the correlation between pelagic duration and genetic structure estimates). We then highlight the main issues in relation with taxonomic assignment and propose a framework (guidelines) for preventing errors due to faulty taxonomy when conducting connectivity studies.

An illustration of the problem: pelagic larval duration, genetic structure, and obscure species boundaries.

The issue of poor species delimitations extends beyond individual studies when we consider meta-analyses or reviews investigating ecological trends such as the relationship between life-history traits and connectivity levels. A first, fundamental, step before conducting such comparative studies should be to ensure that comparisons are made across equivalent evolutionary units. Note that these evolutionary units may or may not be named. For instance, comparative studies including measures of genetic structure such as F_{ST} may produce biased conclusions if taxonomically-unresolved taxa are included. For instance in *C. intestinalis* the value of F_{ST} computed with microsatellites is six times higher between two populations, each made of the different cryptic species attributed to this name, compared to the values computed between populations including only one of these cryptic species (Zhan *et al.* 2010). If cryptic species are not treated as distinct entities, an analysis of genetic diversity among different sampling locations could lead to confound extrinsic barriers to dispersal with intrinsic reproductive barriers. We illustrate this issue by critically examining some of the source data used in a series of meta-analyses comparing measures of genetic differentiation across multiple species (Weersing & Toonen 2009, Selkoe & Toonen 2011). These meta-analyses address the question of whether early life-history traits influence connectivity and if this influence may be evidenced by the analysis of neutral genetic markers. This question is debated in marine molecular ecology because for most marine multi-cellular organisms, early life-history traits greatly differ from adult life-history traits (especially in terms of dispersal ability), and thus putatively affect connectivity among populations (especially if the habitat is fragmented). Different reviews and comparative studies have reached conflicting conclusions (e.g. Kinlan & Gaines 2003; Siegel *et al.* 2003; Shanks 2009; Weersing & Toonen 2009; Riginos *et al.* 2011; Selkoe & Toonen 2011). Weersing & Toonen (2009) examined how well pelagic larval duration (PLD) correlates with genetic estimates of connectivity. Their meta-analysis was based on a pseudo-random selection of papers from hits with selected keywords on electronic searches. The analyzed dataset included 130 species from 87 papers. Contrasting with the general expectation, the authors found only a weak correlation between PLD and genetic structure as estimated by the overall F_{ST}. As emphasized by Selkoe & Toonen (2011), this result may be explained by various factors, including the metrics used for estimating effective dispersal (i.e. gene flow), the type of markers and the sampling design.

In addition, we suggest that one possible explanation for such a weak correlation may be that unrecognized cryptic species within organisms with high PLD have been included in the dataset. Indeed, if two cryptic species are combined under a same name, F_{ST} estimates might be high not because the sampled populations are geographically structured (i.e. limited gene flow) but simply because these populations do not belong to the same species. At least some of the selected papers include questionable taxonomic entities. For example, the reef fishes attributed to five different *Halichoeres* Rüppell, 1835 species had among the highest F_{ST} values and unexpectedly the highest PLD. Examination of the available data from the literature for these species shows that different taxonomic treatments have been applied for similar divergence patterns in this genus (Rocha *et al.* 2005; Rocha *et al.* 2008). For example the name *Halichoeres bivittatus* (Bloch, 1791) covers two divergent mitochondrial lineages whereas the names *Halichoeres brasiliensis* (Bloch, 1791) and *Halichoeres radiatus* (Linnaeus, 1758) are attributed to two mitochondrial lineages separated by a level of divergence similar to that of the lineages within *H. bivittatus.* The two mitochondrial lineages of *H. bivittatus* are allopatric, except in Bermuda where both are present, although not in the same environments. The high overall F_{ST} within *H. bivittatus* might thus result from endogenous reproductive isolation barriers rather than dispersal barriers within a metapopulation. The case of the fish *Elacatinus evelynae* (Böhlke & Robins, 1968) is another example. Several mitochondrial and nuclear genetic markers, and an adequate sampling of closely related species revealed three distinct and well-supported phylogenetic lineages (Taylor & Helberg 2005) but the very high F_{ST} value used in the meta-analysis by Weersing & Toonen (2009) was estimated over a sample that combines these three lineages (Taylor & Heldberg 2003). Whether the three lineages evidenced by the phylogenetic approach need to be described as distinct species is an open question but an important criterion would be to determine if the divergence is definitive (i.e. definitive cessation of reproductive exchanges whatever the causes). In the case of reproductive isolation, the correlation between F_{ST} estimates and PLD should be evaluated only within each lineage. These examples illustrate that depending on how the available results are interpreted in terms of taxonomic status, conclusions of comparative studies on patterns of connectivity can vary widely.

Theoretical framework for delimiting species: a primer.

This critical analysis underlines a crucial aspect of taxonomy: described taxa are not facts but testable hypotheses about the structure of biodiversity. This is true not only for higher taxonomic ranks, generally considered as arbitrary ranks, but also for the species rank, which is the only taxonomic category for which a "biological reality" is recognized by most scientists (see for example in Mishler 2009). The question is then how such hypotheses are formulated in reference to a theoretical background and how they are then revised in light of new empirical data. The literature about the species concept is very large and the debate has often been considered as unsolvable (often referred as "the species problem"). In this debate, de

Queiroz (1998) was among the first to identify that two questions of different nature are mingled. He indeed underlined that the question of the definition of the species category has to be distinguished from that of the question of the adequacy of the criteria used in practice in the definition of the taxa ranked as species. The definition of the species category is an ontological question that may be addressed based on the theoretical ground offered by the Evolutionary Theory (as discussed in Samadi & Barberousse 2006). How each species-taxon should be delineated is an epistemological question that requires evaluating how well empirical data and analysis methods allow the taxonomists to propose sets of organisms that fit the chosen definition of the species category. Following this line of thinking, species-taxa are viewed as scientific hypotheses (species-hypotheses) engaged in a process of falsification based on the acquisition of new evidence. Although this analysis was only recently accepted among biologists, it closely corresponds to the working process of taxonomic revisions, which is one of the major activities of taxonomists (see discussion in Barberousse & Samadi, 2010). This analysis is also the conceptual background of the presently active field of integrative taxonomy. In the integrative approach to taxonomy, various attributes (morphology, DNA, geographical range, habitat, behavior, etc…) and criteria (i.e. the so-called "species concepts" such as the biological, the phylogenetic or the phenetic criteria), analyzed using various methods, are taken into account to propose robust species-hypotheses, reproducible and testable (see Camargo & Sites 2013 for a review on the integration of many characters and methods to delimit species). As underlined by Yeates *et al.* (2011), among others, the way hypotheses are validated should not be primarily the intersection among the evidence from different datasets but rather the prioritization of alternative species-hypotheses based on their explanatory power as defined within the framework of Evolutionary Theory. In this process the quality or robustness of available species-hypotheses greatly depends on the state of knowledge about the concerned compartment of the tree of life. For instance the state of art of species delimitations in birds is far more stable than that of most marine organisms. In fact uncertainties about available species-hypotheses may have two different causes. First, the state of art of taxonomy may be so preliminary that different life-stages of a same organism might have been erroneously classified in different species or even families (see above in Johnson *et al.* 2009). Second, the evidence that two evolutionary lineages are engaged in definitive divergence may not be easy to evaluate when the time elapsed since the split between the emergent lineages is recent (the "grey zone" of de Queiroz, 1998). If the relevance of taxonomic knowledge needs to be evaluated before conducting any biological study on a given organism we also should underline that the results of connectivity studies, if carefully analyzed, can in turn provide evidence about the state of divergence among emergent lineages.

Where do we go from there?

Four main sources of difficulty may strongly influence measures of connectivity and taxonomic hypotheses: (1) the state of knowledge about the biology of the studied organisms, (2) the state of taxonomic treatments of the studied organisms, (3) the spatial and temporal scales of sampling, and (4) the characters used to infer connectivity patterns. In the following section we review these four points and give some guidelines through examples taken from diverse marine model systems. We also offer some guidelines for designing connectivity studies when little background knowledge is available.

II. Methodological considerations and guidelines for connectivity studies.

1. Background knowledge on the biology of organisms.

The availability of adequate background knowledge on the biology of organisms is crucial both for a robust taxonomic framework and for the study of connectivity. As discussed in the introduction, one of the life history traits commonly examined when studying connectivity data for marine organisms is pelagic larval duration (e.g. Cowen & Sponaugle 2009; Shanks 2009; Weersing & Toonen 2009; Selkoe & Toonen 2011). This biological character is also crucial for taxonomists when dealing with species delimitation (see an example in gastropods in box 5). However, other biological attributes such as reproductive strategies, sexual dimorphism or ecological specificities (i.e. habitat specialization, biological interactions, phenology, etc …) can significantly influence both interpretations of connectivity data and taxonomic decisions. For example, advances in understanding of the migratory behavior of some focal marine organisms, such as eels, salmons or whales (e.g. Bottom *et al.* 2005; Aarestrup *et al.* 2009; Baker *et al.* 2013) revealed the existence of seasonal migrations among breeding and feeding sites, or discrete breeding sites, that have bearing on connectivity estimates. For instance, sex-specific philopatry affects the pattern of genetic structure in sperm whales (Engelhaupt *et al.* 2009) and humpback whales (Baker *et al.* 2013). In both cases, patterns of genetic differentiation are interpreted as a consequence of sex-biased philopatry, because it was possible to determine the gender of each individual analyzed, which is not possible for many poorly known organisms. The importance of sexual dimorphism, season and location of sampling can greatly affect the observed patterns of genetic differentiation and has consequence in the evaluation of species diversity (illustrated by the example of *Lessonia* in box 2). While the migratory behavior of some well-studied marine species has been characterized, the existence of seasonal migrations or discrete breeding sites is poorly known in most marine groups. Thus, the unreported occurrence of seasonal migrations may bias interpretations obtained when samples are collected from a single season. In extreme cases, different life history stages or sexes can wrongly be attributed to different taxa (e.g. Johnson *et al.* 2009), confounding estimates of connectivity. When the different sexes or stages inhabit different environments (such as different depths, e.g. the case of

the Cetomimidae in Jonhson et al 2009), estimating dispersal from a single life-stage or sex can bias interpretation of connectivity patterns but also taxonomic interpretations. Other attributes, such as clonality or alternation of generations, can also complicate connectivity studies. In the case of clonal species such as seagrasses or some algae, neglecting such attributes would lead to a severe underestimation of gene flow or to overlook the presence of cryptic species (see in Box 4 and also Adjeroud *et al.* 2014 for another case in corals). Thus, the sampling design and interpretation of genetic data in connectivity studies and taxonomic revisions should take into account life history and ecological traits that could be relevant to estimating dispersal. If, as in many cases, such knowledge is limited or inexistent for the species of interest, one could examine information available for closely related taxa and/or incorporate this uncertainty in the sampling design (by sampling at different seasons, or different depths for example).

2. Taxonomic knowledge.

Connectivity studies generally consider the described species (i.e. available species hypotheses) as facts upon which the sampling scheme is established. In other words, the attributes associated with a species-hypothesis (either extracted from the original description or from other biological studies based on this species hypothesis) are considered as objective facts. Notably, the species range and habitat specificity are among the important attributes extracted from those hypotheses that are used in the design of a connectivity study. However, an important source of uncertainty in taxonomic hypotheses is the sampling of organisms and/or of characters upon which the species-hypotheses were initially drawn. Thus, data provided by a connectivity study are potentially a source of falsification of accepted taxonomic hypotheses. Consequently, for organisms for which no recent taxonomic revision is available, the sampling scheme should both follow the requirements of population biology but also that of phylogenetic systematics. For example, only a widespread sampling of a supposedly cosmopolitan species may reveal several diverging lineages restricted to isolated areas or distinct habitats as exemplified by the study of the supposedly ubiquitous and widespread amphipod *Eurythenes gryllus* (Lichtenstein in Mandt, 1822) (Havermans *et al.* 2013) or the copepods *Pleuromamma piseki* Farran, 1929 and *Pleuromamma gracilis* Claus, 1863 (Halbert *et al.* 2013). Collecting in under-sampled or unexplored areas may also reveal new divergent lineages, like in the cosmopolitan red algae *Asparagopsis armata* Harvey, 1855 and *Asparagopsis taxiformis* (Delile) Trevisan de Saint-Léon, 1845, for which human-mediated transports increase the uncertainty about taxon history and systematics (Dijoux *et al.* 2014). Sampling is also an issue for species described as geographically restricted or ecologically specialized: looking for (and sampling) the targeted species or a priori closely related species in other localities or habitats may help rejecting hypotheses of endemism and/or specialization. For example, a deep-sea chemosynthetic mussel attributed to a new *Idas* Jeffreys, 1876 species by Ritt *et al.* (2012), sampled in a deep cold seep site in the eastern Mediterranean sea, is included

in the same *cox1* cluster as the specimens attributed to *Idas simpsoni* (J. T. Marshall, 1900) and sampled at about 150 m depth in the north Atlantic on vertebrate bones (Thubaut *et al.* 2013). In this example the new species was considered as restricted to a given habitat that is highly fragmented and consequently other specimens sampled in a distant and distinct habitat were falsely assigned to a distinct species. Two sets of populations, identified as different species each associated with different habitats, may actually be populations of a single species connected through (even if rare) gene flow. Similar observations were also recently reported among chemiosynthetic shrimps that were considered as distinct in cold seeps and vents, while molecular analysis revealed the occurrence of a single species with high gene flow across the Atlantic (Box 3; Teixeira *et al.* 2013). These examples illustrate the fact that the definition of the targeted group (ingroup) may be biased due to a lack of samples from specimens a priori identified as belonging to a different (related) species. Such sampling biases correspond to higher rank classification errors and may for example exclude from genetic analyses a subset of populations despite its crucial role in shaping connectivity patterns. As underlined below, the risk of inadequacy of available species-hypotheses should not be ignored and sampling cannot be limited to the intra-specific level. Because of the poor state of taxonomic knowledge for most marine organisms, taxonomic hypotheses (i.e. accepted classifications) should thus be carefully evaluated prior to assessing connectivity.

3. Guidelines for spatial and temporal sampling of organisms.

Sampling should be guided by two objectives: adequately covering (1) both the putative geographic and ecological range of the species of interest (coverage at the population level), and (2) potentially closely-related species over a wide geographic and ecological range (taxonomic coverage). Coverage at the population level should also take into account potential temporal variability in the geographic location of individuals (e.g. different location and time of breeding and feeding) and in habitats (e.g. pelagic *versus* benthic).

As explained above, this two-level sampling (developed in the case study presented in Box 5) should ensure a proper estimation of the upper bound of intra-specific diversity and the lower bound of inter-specific diversity as to determine their potential overlap. The genetic distance between conspecifics and individuals from different species, known as the “barcoding gap” (Meyer & Paulay 2005), is often used as a threshold to delimit species (at least in a primary step – Puillandre *et al.* 2012a) but its detection and meaning highly depend on the sampling scheme. These sampling guidelines are of course difficult to follow in all situations, especially in the marine realm: rarity, endemism, habitat fragmentation, human-mediated transports (i.e. biological introduction), lack of knowledge on sister-species relationships for most taxa, technical difficulties in sampling in the deep sea, among others factors, make the estimation of intra- and inter-specific diversity difficult. Such strategy may point to new species-hypotheses and thus to the need of taxonomic revisions.

However, such revisions (i.e. providing named species-hypotheses) are possible only if the requirements of the codes of biological nomenclature are taken into account in the sampling scheme. Indeed, an important requirement for attributing species names to the redefined species hypotheses is the inclusion of type-material linked to available species-names (see for example Puillandre *et al.* 2011). This last step is often lacking in molecular studies and the literature is spoiled by arbitrarily-labeled species hypotheses (e.g. OTUs, MOTUs, n. sp etc …) that are difficult to compare from one study to another. As most name-bearing specimens (holotypes) cannot be integrated in molecular studies (due to their age, their poor conservation state, or simply because they were lost), sampling in type localities (as defined for example for animals in the article 76 of the International Code of Zoological Nomenclature) is a minimum requirement for naming species-hypotheses. This will both allow the corroboration (or the rejection) of available species-hypotheses and an adequate analysis of the pattern of connectivity within the targeted species. Implementation of the aforementioned sampling strategy and interpretation of the results should be guided as far as possible by this two-level method, especially when assessing the reliability of the inferred patterns of connectivity. In summary, these guidelines aim at identifying the adequate spatial and evolutionary scale for connectivity studies.

4. Guidelines for the sampling of characters.

Classical markers widely used for marine organisms include genes from the phylogenetic literature and notably those mobilized in the DNA barcoding initiatives (typically, the *cox1* gene for metazoans, Bucklin *et al.* 2011; *cox1* or RbcL genes for macro-algae, Leliaert *et al.* 2014), but also other mitochondrial and nuclear genes such as ribosomal genes or “universal” introns (e.g. Jarman *et al.* 2002; Chenuil *et al.* 2010; Gérard *et al.* 2013). By using a set of unlinked genetic markers, it is possible both to refine the phylogenetic relationships (phylogenetic criterion) and to test whether specimens from the same species are able to recombine and, conversely, whether specimens from different species cannot (biological criterion). This can be achieved by sequencing, for each specimen, at least two unlinked markers, such as one from the mitochondrial genome (or chloroplast genome in plants and algae) and one from the nuclear genome. It is now widely accepted that the use of a single marker, especially mitochondrial, is risky when delimiting species, and should be used only in a first step to quickly propose primary species-hypotheses. As underlined in the review of Carstens et al. (2013), multi-locus approaches are required especially when considering recently diverging lineages, notably because of the genome wide heterogeneity of introgression rates observed between such genomes (e.g. between the two tunicates *C. intestinalis* type A and type B, Roux *et al.* 2013; or between the two sibling species of red algae *Gracilaria gracilis* (Stackhouse) M.Steentoft, L.M.Irvine & W.F.Farnham, 1995 and *Gracilaria dura* (C.Agardh) J.Agardh, 1842, Destombe *et al.* 2010). Furthermore, in some taxa (e.g. cnidarians, sponges, plants, algae…), classical markers sometimes

lack the necessary resolution to distinguish closely related species and to infer connectivity (e.g. Calderón *et al.* 2006; Shearer & Coffroth 2008). Examples can be found in metazoan groups with remarkably low levels of mitochondrial evolution such as corals, when this sole genetic marker is considered (e.g. Shearer & Coffroth 2008; McFadden *et al.* 2011). However, for many poorly studied organisms, identifying a nuclear marker variable at the species level in addition to, for example, the *cox1* gene can be tricky (e.g. McFadden *et al.* 2010). In such taxa, genetic similarity among individuals may reflect above-species evolutionary relationships rather than demographic connectivity (e.g. Miller *et al.* 2010). Clearly, these technical difficulties should be overcome thanks to the new methods based on Next Generation Sequencing, such as the RAD-sequencing (Baird *et al.* 2008). In a single run, many independent loci covering a large part of the genome are sequenced. They are thus particularly appropriate to test for recombination among individuals and to unravel diagnostic molecular characters among taxa (e.g. Pante *et al*. accepted).

Even if DNA characters often provide reliable data for species delimitation, either using a single locus to propose primary species-hypotheses (e.g. "species discovery" by DNA barcoding) or multiple loci to test for recombination among individuals within a species, they may not always provide decisive answers. When genetic data are not fully conclusive, morphological, geographical and ecological data can help turn the scales toward alternative hypotheses. For example Kekkonen & Hebert (2014) use a geographic criterion (*i.e.* genetic units are or not in sympatry) to conclude about the status of the detected genetic units. Our goal is not here to list all the characters and methods that can be included in the integrative taxonomy loop (as illustrated in Figure 1), but just to emphasize the need to discuss alternate species hypotheses, referred as (M)OTUs ((Molecular) Operational Taxonomy Units), ESU (Evolutionary Significant Units), PSH (Primary Species Hypotheses) or candidate species, depending on the method and characters, using all the available data to converge to robust, reproducible and testable hypotheses.

5. Prioritization and workflow in multi-marker approaches.

Integrative taxonomy has been proposed as a solution to overcome apparent contradictions in species-hypotheses resulting from the use of different criteria or characters (Dayrat 2005, Will *et al.* 2005). This approach is now widely accepted, although not always implemented in a fully integrated way. This results from the fact that the different methodologies cannot be applied in a similar way to all taxonomic groups, since each of them has its own limitations. For example, ecological data, commonly used in species delimitation of terrestrial and shallow water organisms, are more difficult to gather when dealing with deep-sea species. However, some general rules have been set up, and now, the debate does not primarily concern the definition of the species concept or the suitability of an integrative approach, but rather the relative weight and priority of the different characters and criteria used for species delimitation (DeSalle *et al.* 2005; Padial *et al.* 2010; Yeates *et al.* 2011; Camargo & Sites 2013).

The principal source of difficulty for integrative taxonomy comes from cases of recent divergence ('emergent phylogenetic systems' as defined in Carstens *et al.* 2013). For example, under the hypothesis of a single species, high F_{ST} estimates are interpreted as population structure (low connectivity). If this species-hypothesis covers in fact two recently separated lineages, depending on the level of shared polymorphism among them, the F_{ST} estimates would be more or less close to one. Thus when taxonomy is uncertain, the two alternative hypotheses explaining such high F_{ST} estimates should be examined. Wang *et al.* (2008) illustrate this case by discussing how to discriminate between these two alternative hypotheses in their investigation of two morphotypes of finless porpoise (genus *Neophocaena*, Cetacea) that occur in sympatry in the strait of Taiwan. In their study, they apply population genetics methods to distinguish between a case of recent speciation and a case of low but recurrent gene flow. When simulating two populations with no gene flow during the last 18,000 years, simulated and observed F_{ST} and migration rate estimates were similar. Thus, the simulated scenario of a recent speciation event matched empirical data, leading the authors to conclude that the two morphotypes constitute two recent species that are currently in secondary contact. In this study the two species are not reciprocally monophyletic (phylogenetic criterion) but the population genetic approach supports the hypothesis of a complete breakage of gene flow (biological criterion) between the two morphotypes. Reciprocal monophyly is a criterion frequently used for species delimitation using molecular methods (e.g. Funk & Omland 2003) because it is much easier to evaluate than the biological criterion (inter-fecundity within species and inter-sterility among species). When the separation among studied species is ancient, the criterion of reciprocal monophyly is an operational method for detecting species. Conversely, among recently diverged species (i.e. 'emergent phylogenetic systems'), we recommend to give advantage to population genetic methods allowing the detection of lasting breakage of gene flow over the criterion of reciprocal monophyly. New methods of analysis, mostly based on coalescent theory, allow combining both criteria and should be as much as possible used in this context (e.g. Yang and Rannala 2010, Leaché et al 2014, Hsieh et al 2014). These methodologies coupled with new genotyping techniques allow the use, in a single analysis, of both the phylogenetic criterion (*i.e*. two sets of organisms have distinct evolutionary trajectories) and the biological criterion (*i.e.* the two sets of organisms are reproductively isolated) but were used only in a few pioneering studies (e.g. Leaché *et al.* 2014). Their applicability on poorly known organisms of the marine realm has yet to be explored but the robustness of such analyses will greatly depend on the quality of the sampling scheme relative to the state of taxonomic (and biological) knowledge.

III Conclusion and implications.

In many marine species, particularly those with high fecundity and large population sizes, population genetic studies have often revealed weak genetic structure, preventing accurate analyses of connectivity patterns. However, strong genetic structure patterns have also been revealed in marine systems. In this review, we argued that, in some cases, such observed patterns may actually be the outcome of incorrect sampling and/or incorrect taxonomic assignment.

We showed that unreliable definition of evolutionary units can lead to either overestimation or underestimation of connectivity. This situation is analogous to type I and type II statistical errors in a hypothesis-testing framework where full connectivity is the null hypothesis. In this review we illustrated two general cases, in which both types of errors may be encountered. These two cases correspond to the number of evolutionary units being (1) larger or (2) smaller than the number of species acknowledged in the study. In the first case, *i.e.* over-merging of evolutionary units, high genetic structure is equivalent to false positives and may emerge when populations from different, unrecognized or cryptic species are lumped together (e.g. Box 2 on the kelp genus *Lessonia* Bory de Saint-Vincent, 1825). This would lead to an increase in type I statistical error, in which the null hypothesis of a lack of barrier to gene flow among populations would be falsely rejected because species, and therefore populations, were not properly delimited. One simple example documented above is when falsely inflated F_{ST} -values due to mixing of specimens from different evolutionary entities are interpreted as picturing limited dispersal and increased genetic drift. The issue lies in the interpretation of the data rather than in the data themselves, and some examples were discussed above (e.g. Rocha *et al.* 2005; Taylor & Helberg 2005; Rocha *et al.* 2008). The second case, i.e. over-splitting of evolutionary units, results in the flawed *a priori* assumption of a complete lack of connectivity among taxa described as distinct species (see the example of the Atlantic shrimp named, depending on its habitat, *Alvinocaris muricola* Williams, 1988 or *Alvinocaris markensis* Williams, 1988 as detailed in Box 3), preventing their recognition as potentially interdependent entities and the will to engage in connectivity studies among their populations.

As illustrated by the examples discussed in this paper, the issue of unreliable delineations of evolutionary units has been exacerbated in the past two decades with the rise of molecular data, particularly in the framework of phylogeographic studies documenting an ever-increasing number of cryptic species in the marine realm (Appeltans *et al.* 2012). Species are hypotheses that need to be revised if necessary in light of new information. To anticipate the possibility of false taxonomic hypotheses, we highlighted several important aspects. First, the sampling design should encompass as much as possible 1) the entire known distribution range of the targeted organism and 2) closely related taxa, or even some distantly related taxa in taxonomic groups where the phylogeny is poorly known (e.g. deep-sea organisms). The latest point is rarely considered in connectivity studies although it is an efficient way to ensure the correct definition of

the focal (i.e. ingroup) species. Second, any connectivity study should be careful in its design regarding life-history traits: pelagic larval duration, reproductive system characteristics (sex-ratio, clonal reproduction, …etc) and social organization, which all have important consequences on connectivity patterns but also on species delimitations. Third, the types of markers used should be diversified; in particular the sole use of mitochondrial genes should be avoided. Altogether, the three points mentioned above are all relevant to the framework of integrative taxonomy. This approach has proven to be helpful in species delineation and meaningful when coupled with or used for connectivity studies.

Importantly, connectivity studies can reciprocally provide new data that can help defining new species in previously recognized nominal species. This is particularly well illustrated in the case of introduced or invasive marine species (see box 6). Human-driven dispersal adds to natural dispersal, thereby broadening species ranges, sometimes at a worldwide scale. Connectivity and phylogeography studies of marine invaders have shown that important and cosmopolitan invaders are actually species complexes (e.g. in colonial tunicates *Botryllus schlosseri* (Pallas, 1766), Bock *et al.* 2012; in solitary tunicates *C. intestinalis* Zhan *et al.* 2010; in the red alga *A. taxiformis* Dijoux *et al.* 2014). Cryptogenic, pseudo-indigenous species and cryptic species, as defined by Carlton (2009) are new issues deserving integrative taxonomy and barcoding studies, not only to verify the accuracy of invaders reports (e.g. McGlashan *et al.* 2008) or to quickly report newly introduced species (Bishop *et al.* 2013), but also to examine gene flow between cryptic species not fully reproductively isolated, in particular when invaders are morphologically similar to native species (e.g. Nydam & Harrison 2011). More generally, delineating species can be particularly challenging considering how rapidly species distribution can shift due to either introduction processes or global change. These issues increase the complexity of taxonomic assignment and biogeography studies.

Finally, poor species delimitation and the use of inadequate taxonomy assignment are not restricted to connectivity studies and have consequences on other fields of ecology and evolutionary studies, in particular experimental ecology, medicine, evolutionary developmental biology, speciation and phylogenetic studies, invasion biology research and biodiversity inventories (Bortolus 2008; Carlton 2009; Collins & Cruickshank 2013). Recent methodological and conceptual advances will contribute to avoid the pitfalls listed in this work, by taking advantage of the emergence of integrative taxonomy and the development of new molecular techniques such as NGS-based methods, to provide a more accurate exploration and understanding of the building-up of evolutionary units.

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Boxes.

Box 1. Species delineation blurred by hybridization and suboptimal sampling design: the case of *Fucus* Linnaeus, 1753.

The brown algae genus *Fucus* Linnaeus, 1753 is a well-known, textbook model in marine ecology, exemplifying the distribution of different morphological species in well-defined zones along a vertical intertidal gradient in the North Atlantic, from high- to low-water marks (Wahl *et al.* 2011). However, the recent use of molecular markers has deeply modified our understanding of both taxonomy and connectivity in this species complex.

The two closely related brown algae morphological species: *Fucus spiralis* Linnaeus, 1753 and *Fucus vesiculosus* Linnaeus, 1753 are found in sympatry along eastern Atlantic rocky shores, from northern Norway to Morocco. They differ mainly by their morphology, their vertical distribution on the shore and their reproductive system. *F. spiralis* is hermaphrodite and is found in the higher intertidal, whereas *F. vesiculosus* is dioecious and is located just lower on the shore. Molecular analyses of internal transcribed spacer of nuclear ribosomal DNA (ITS) were not efficient to separate these two taxa (Serrão *et al.* 1999) and their taxonomic status as two separate species was questioned. The use of five microsatellite loci on six populations of *F. spiralis* and ten of *F. vesiculosus*, separated by tens to hundreds of kilometers, showed that within each species individuals share diagnostic alleles whatever their geographic origin (Billard *et al.* 2005). Consequently, the phylogeographic approach of Billard *et al.* (2005) demonstrated that these two morphological species correspond to two different genetic entities. Using gene admixture detection methods, intermediate individuals between the two species were detected along shores where the distribution of the two species overlaps at the scale of few hundred meters (Engel *et al.* 2005). The authors suggested a recent divergence of the two taxa with retention of ancestral polymorphism or introgressive hybridization with varying levels of admixture.

Interestingly, using a more refined sampling (combining transects across the zonation of the shore and quadrats at the different tidal levels), a new genetic entity previously overlooked by Engel *et al.* (2005) was detected within the *F. spiralis* morphotype (Billard *et al.* 2010). These two entities were first labeled *F. spiralis* Low and *F. spiralis* High according to their vertical distribution on the shore. Based on an integrative approach combining molecular, physiological and morphological analyses, Zardi *et al.* (2011) proposed to elevate the new genetic entity, *F. spiralis* Low, to the species level using the name *F. guiryi* G.I.Zardi, K.R.Nicastro, E.S.Serrão & G.A.Pearson, 2011. The range distribution of *F. guiryi* was shown to occur from Brittany to Morocco (Coyer *et al.* 2011). Common garden experiments performed on the three sympatric genetic taxa*: F. spiralis, F. guiryi* and *F. vesiculosus* showed that physiological response to desiccation stress differed between species and was consistent with their respective vertical distribution on steep

environmental clines in exposure time (Zardi *et al.* 2011). The opportunity of hybridization between the three species was confirmed to be higher when they are physically close to each other on the shore while in southern Portugal, where *F. vesiculosus* is only found in estuaries and *F. guiryi* on the open coast, the two species never hybridize (Zardi *et al.* 2011). In addition to their ecology, it was proposed that selfing as well as limited gamete dispersal contribute to species integrity in the hermaphroditic species *F. spiralis* and *F. guiryi* (Engel *et al.* 2005; Billard *et al.* 2010; Zardi *et al.* 2011). Finally, a high level of asymmetric introgression was reported between these different species using a combination of nuclear and mitochondrial DNA markers (Coyer *et al.* 2011). A scenario of organelle capture via hybridization and spread of neutral nuclear alleles during range expansion was suggested to explain the common occurrence of introgression in this genus. This example clearly showed that organellar loci as barcoding tools should be used with care because of introgression.

Box 2. The integrative taxonomy loop: study case of two cryptic species in the kelp *Lessonia nigrescens* Bory de Saint-Vincent, 1826 complex.

The discovery of cryptic species does not always lead to a taxonomic reexamination and in many cases, cryptic species remain without taxonomic characterization (and therefore with no corresponding Latin name) long after their detection. Here we describe the case of the kelp *Lessonia nigrescens* Bory de Saint-Vincent, 1826 complex that was recently identified as two separate cryptic species thanks to a considerable sampling effort, combining phylogenetic, population genetics and ecological approaches. This kelp forms forests along the intertidal South Eastern Pacific coasts and was long considered as a unique species from Cape Horn in southern Chile (ca. 55° S) to central Peru (ca. 12° S; Santelices 1989). Two cryptic species were recently identified through a multigene phylogeographic study (using a combination of four markers located in the three genomic compartments - chloroplast, mitochondrion and nucleus - with 1,000 individuals covering more than 2,500 km of coastline) (Tellier *et al.* 2009). The two divergent genetic lineages show a parapatric latitudinal distribution: one species extends from southern Peru (17°S) to central Chile (30° S), and the other extends from central Chile (29° S) to Chiloe Island (42°S). Between 29° S and 30° S there is a narrow area where both lineages spatially overlap in discrete patches where individuals belong to either the northern or southern species. This contact zone between 29° S and 30° S has been described as a mosaic of sites occupied by one species or the other (Tellier *et al.* 2009), rather than a true gradual transition zone, with one species replacing the other. In addition, a detailed population genetic study of gene flow in the transition zone confirmed a total absence of hybrids and mixed populations (Tellier *et al.* 2011). Ecological differentiation between the two cryptic species was investigated, using different approaches (e.g. controlled response to different thermal and desiccation stresses in the lab, Oppliger *et al.* 2012; López-Cristoffanini *et al.* 2013). Both microscopic (haploid) and macroscopic (diploid) phases of the haploid-diploid kelp life cycle were studied. Results demonstrated adaptive divergence for both phases that could explain the geographic segregation of these two cryptic species. In addition, contrasted demographic processes could be observed at various stages of their life cycle: female gametophytes from the Northern species develop rapidly but give very few oogonia per gametophyte, whereas female gametophytes from the Southern species delay their maturation, grow vegetatively and then produce numerous oogonia per individual. These results strongly advocate that, even though the origin of the genetic differentiation of the two species is ancient, ecological and intrinsic differences are probably still important for the maintenance of reproductive isolation.

These two species were finally distinguished by subtle morphological differences leading to a formal description of two pseudo-cryptic species as *Lessonia berteroana* Montagne, 1842 and *Lessonia spicata* (Suhr) Santelices (Gonzalez *et al.* 2012).

Box 3: Taxonomic inflation results in the flawed *a priori* assumption of a complete lack of connectivity among morphologically distinct populations: an example with shrimps in the deep-sea environment.

As reported in this manuscript, most examples of taxonomic bias leading to distorted connectivity inferences are linked to the existence of hidden cryptic species or lineages. We here illustrate the opposite case in which the polymorphism of morphological characters led to taxonomic inflation and thus to a false inference of the connectivity pattern. This situation was found in the Alvinocaridae, a family of caridid shrimps specialized in deep chemiosynthetic ecosystems. In the Atlantic, two species were described in 1988 by Williams: *A. muricola* associated with cold seeps from Western Atlantic and *A. markensis* from hydrothermal vents of the Mid Atlantic Ridge. The two species were distinguished based on a slight difference in an abdominal segment being entire or obscurely serrate. This distinction was made on juvenile specimens and later confirmed on adults (Komai & Segonzac 2005), although on the basis of a distinct morphological criterion that was the more inflated and convex nature of anterior part of the branchial region in *A. muricola* compared to *A. markensis*. In 2005, Komai & Segonzac cautiously identified as *A. aff. muricola* shrimps sampled along the continental margins of Eastern Africa. These authors extensively discussed the pan Atlantic distribution of *A. muricola* given the puzzling absence of geographically intermediate population. These authors hypothesized either the occurrence of large-scale dispersal capacities or the existence of unknown suitable sites in the intermediate area. A barcoding approach based on the Cytochrome Oxydase I gene (*cox1*), initially aiming at retracing the phylogenetic history of the alvinocarid shrimp, casted doubt on the taxonomic status of those two species, showing entire sequence match among haplotypes of both species (Teixeira *et al.* 2013). Indeed, using both mitochondrial and nuclear sequences (*cox1* and 18S) together with a set of microsatellite loci, Teixeira *et al.* (2013) showed that all populations respectively attributed to these two names share a unique set of alleles at both types of genetic markers and display extremely weak genetic differentiation. This result eventually confirmed the hypothesis of intermediate populations raised by Komai & Segonzac (2005) while more importantly demonstrating that *A. markensis* and *A. muricola* should not be considered as two distinct species. Following this interpretation the two species have to be synonymized in a single species that has a wide geographic distribution but also a diversified range of suitable habitats in the deep sea. This example supports the recommendation of investigating the phylogenetic framework of taxa studied including closely-related taxa before engaging into comprehensive connectivity studies.

The same barcoding analysis also revealed that classification at both genus and species level should be revised within the Alvinocaridae with possible cases of synonymy of species described as distinct genera (e.g. *Rimicaris hybissae* Nye, Copley & Plouviez, 2012 reported in Eastern Atlantic seeps and *Chorocaris chacei* (Williams & Rona, 1986) and Mid-Atlantic vents).

Box 4. The implications of working on organisms with clonal populations and alternation of generations: the case of seagrass meadows and algae.

Seagrass meadows were initially expected to exhibit large-scale panmixia due to the positive buoyancy of their seeds and rhizomatic fragments. In studying connectivity in those partially clonal organism, the first issue was the ability to recognize clonal lineages on the basis of their multi locus genotypes (MLG) in order to discard from the datasets the sampling units that are replicates of the same clonal lineage that would potentially artificially inflate structure estimates such as F_{ST} among meadows (Arnaud-Haond *et al.* 2005; Arnaud-Haond *et al.* 2007a). Even avoiding this potential bias, however, population genetic analysis across the range area of several seagrass populations suggested a systematic structure and limitation to gene flow across all geographical scales among almost all discrete meadows analyzed (Coyer 2004; Olsen, 2004, Arnaud-Haond *et al.* 2007b; Alberto *et al.* 2008). Secondarily however, fine grained analysis of the distribution of clonal lineages and genetic polymorphism in *Zostera marina* Linnaeus, 1753 meadows revealed a large influence of clonality, leading to a mosaic pattern of genetic patchiness largely driven by patchy events of recolonization followed by the occupation of space through clonal spread, despite the likely occurrence of much larger scale effective migration (Becheler *et al.* 2010; Becheler *et al.* 2014). These results are casting doubts on the accuracy of the genetic concept of populations in those partially clonal organisms, suggesting the ecological concept of distribution continuity (therefore the meadow scale) may be more accurate as a framework for connectivity analysis (Becheler *et al.* 2010) and genetic differentiation may not systematically reflect lack of connectivity. Additionally, the general lack of isolation by distance patterns reported across seagrass species was interpreted as a clue for scarce but significant events of large-scale dispersal influencing the dynamics, resilience and geographic distribution of genetic diversity in seagrasses (Kendrick *et al*. 2012). Whereas this may hold for short-lived species with a regular turn over and propagule recruitment, the decomposition of the genetic distance spectrum (Rozenfeld *et al.* 2007) among meadows of the long-lived seagrass *Posidonia oceanica* (Linnaeus) Delile, 1813 (Arnaud-Haond *et al.* 2012) also brings additional warning for the interpretation of genetic structure patterns in terms of contemporary gene flow. This approach of genetic data performed at the level of clonal lineages indeed showed that at the range scale, the pattern of genetic structure was reflecting ancient events of (re)colonization followed by long term clonal spread and accumulation of divergent mutations within meadows at the regional scale, rather than contemporary patterns of connectivity (Arnaud-Haond *et al.* 2014). Interestingly, in haploid-diploid species like many seaweeds, there is an alternation of haploid gametophytes and diploid sporophytes to complete the sexual life cycle. In such life cycle, dominance of clonal propagation can led to an uncoupling of haploid and diploid phases and thus to differences in allele frequency between gametophytes and tetrasporophytes (Sosa *et al.* 1998). This has been observed in species of red alga

649 reproducing mainly asexually (in *Gelidium arbusculum* Bory de Saint-Vincent ex Børgesen, 1927; Sosa *et al.*
650 1998; *Gracilaria chilensis* C.J.Bird, McLachlan & E.C.Oliveira, 1986; Guillemin *et al.* 2008) while the
651 occurrence of sex, even occasionally, in other species, explained the lack of significant differences between
652 haploid and diploid subpopulations (*Gelidium canariensis* (Grunow) Seoane Camba in Sosa & Garcia-Reina
653 1993; *Gracilaria gracilis* (Stackhouse) M.Steentoft, L.M.Irvine & W.F.Farnham 1995 in Engel *et al.* 2004;
654 *Cladophoropsis membranacea* (Hofman Bang ex C.Agardh) Børgesen, 1905 in van der Strate *et al.* 2002 and
655 *Chondrus crispus* Stackhouse, 1797 in Krueger *et al.* 2011). Genotyping both haploid and diploid individuals
656 in these species is necessary in order to detect the clonality and its effect on population structure.

Box 5. Large taxonomic and geographic sampling in the deep-sea gastropod *Bursa* Röding, 1798 helps delineating species before testing hypotheses on modes of speciation.

Deep-sea seamounts offer a multi-scale fragmented habitat for benthic organisms. For gastropods inhabiting these environments, connectivity patterns have been shown to widely depend on the type of larval development (Castelin *et al.* 2010; Castelin *et al.* 2012). Notably, the three deep-sea Bursidae species (*Bursa latitudo* Garrard, 1961, *Bursa fijiensis* (Watson, 1881) and *Bursa quirihorai* Beu, 1987) share a largely overlapping geographic range on the seamounts of the Norfolk and the Lord Howe ridges, and the continental slopes of New Caledonia, in the Southwest Pacific, and are not genetically structured over this area.

The prevalence of allopatric versus sympatric modes of speciation, were studied following Coyne & Orr (2004)'s biogeographic approach that involves studying current distribution patterns and phylogenetics of recently-separated sister species. In this approach, one of the first requirements is to show that the species pairs have a sister relationship and that reproductive isolation is complete (i.e. absence of hybridization between the sister-species). All Bursinae species have a long-lived planktonic-feeding larva and most of them have a wide distribution range. Moreover, most are geographically rare and the distribution of populations within the species range is usually sparse and highly fragmented. This leads to confusions in Bursidae taxonomy with some geographically-isolated populations attributed to distinct species names, or slightly distinct morphotypes collected at distant but not geographically isolated sites attributed to the same species name. The sampling strategy was thus designed to meet a three-fold objective: extending the taxonomic sampling without any *a priori* hypotheses about the systematics of the group, at both the genus and the species level (e.g. including all available individuals, populations, species and genera of the family Bursidae); extending the geographic sampling toward other oceans (Atlantic, Indian); and extending the ecological sampling by adding species from both shallow-water and deep-sea environments.

Starting from this large taxon sampling, "Primary Species Hypotheses" (PSH) were drawn from the shell morphology using the taxonomic literature and dry material of the Malacology collection at the French National Museum of Natural History (MNHN). ESUs were then defined using both the GMYC (Pons *et al.* 2006) and ABGD methods (Puillandre et *al.* 2012b) applied on *cox1* sequences and then strengthened with the analysis of a second mitochondrial gene (16S rRNA) and the nuclear 28S rRNA gene fragment. All sources of evidence generated from these four steps were integrated to propose "Retained Secondary Species Hypotheses" (RSSHs).

Over the sampled geographical range, the three morphologically diagnosable entities, *B. latitudo*, *B. quirihorai* and *B. fijiensis,* are reciprocally monophyletic based on all tested loci. The dataset also revealed cryptic lineages emphasizing that species diversity in the family Bursidae is underestimated and suggesting that a taxonomic revision of the family should be performed. Among New Caledonian species, *B. fijiensis*

691 and *B. quirihorai* are reciprocally monophyletic. These two species are the two most closely related species
692 in the inferred phylogeny. The current biogeographic ranges of these two species and the estimated time of
693 divergence make the scenario of sympatric speciation the most likely.

Box 6: Human-mediated transport may further increase complexity: from cryptogenic to cryptic species in marine invaders - delineating species in a changing world.

Besides the natural processes of expansion and contraction of the range colonized by a species over evolutionary times, sudden species-range expansions -often through human-mediated transport- have been observed at an increasing rate since the end of the 19th century. Since the original work by Elton published in 1958 (Elton 1958), biological invasions have received a growing set of attention from the scientific community (e.g. Blackburn *et al.* 2011 and Simberloff *et al.* 2013 for recent reviews and references herein). Tightly linked to human activities particularly through shipping and aquaculture, biological invasions severely alter biodiversity of marine ecosystems (Rilov & Crooks 2009). One of the important outcomes of marine biological invasion studies has been to document biotic homogenization at a global scale with the establishment of 'cosmopolitan' species and cryptogenic species (i.e. species that are neither demonstrably native nor demonstrably introduced as defined by Carlton (1996; Haydar 2012). Interestingly, many of these cosmopolitan invasive species actually appear to be composed of morphologically close taxa forming a species complex. This was recently exemplified in two model ascidians, the colonial tunicate *Botryllus schlosseri* (Bock *et al.* 2012) and the vase tunicate *Ciona intestinalis* (Nydam *et al.* 2011; Zhan *et al.* 2010). Both taxa are distributed worldwide. Phylogeography, phylogeny, population genetics and genomic studies showed for both taxa that they were actually characterized by marked genetic subdivision and large genealogical divergence (up to 4-8 million years for type A and type B in *C. intestinalis*, Roux *et al.* 2013). These two taxa are composed of (partially) reproductively isolated species, some of them came recently into secondary contact because of their introduction with human activities. This is notably the case for two members of the *Ciona intestinalis* species complex called type A and type B, which are living in sympatry after the presumably recent introduction of Type A in the Northern Atlantic; the two species are incompletely reproductively isolated in the English Channel where they are hybridizing (Nydam & Harrison 2011). The outcome of such secondary contact is unknown but may promote either adaptive introgression or speciation (Abbott *et al.* 2013). Bock *et al.* (2012) also pointed out that the different cryptic species of the *Botryllus schlosseri* complex are not characterized by similar invasive abilities questioning the reasons for such differential abilities. Integrative taxonomy and molecular approaches are thus critical research to carry out in biological invasions. They may help identify new cryptic introduced species with different invasion abilities, understand the evolutionary history of cryptogenic species (e.g. the tunicate *Molgula manhattensis manhattensis* (De Kay, 1843) (Haydar *et al.* 2011) and the fate of secondary contact between previously isolated taxa. For management purpose, they are also helpful to identify new invaders (Bishop *et al.* 2013) and verify the accuracy of published records of invasive species (e.g. McGlashan *et al.* 2008) - especially for morphologically cryptic cosmopolitan taxa.

727 **Figure1: the integrative taxonomy loop**

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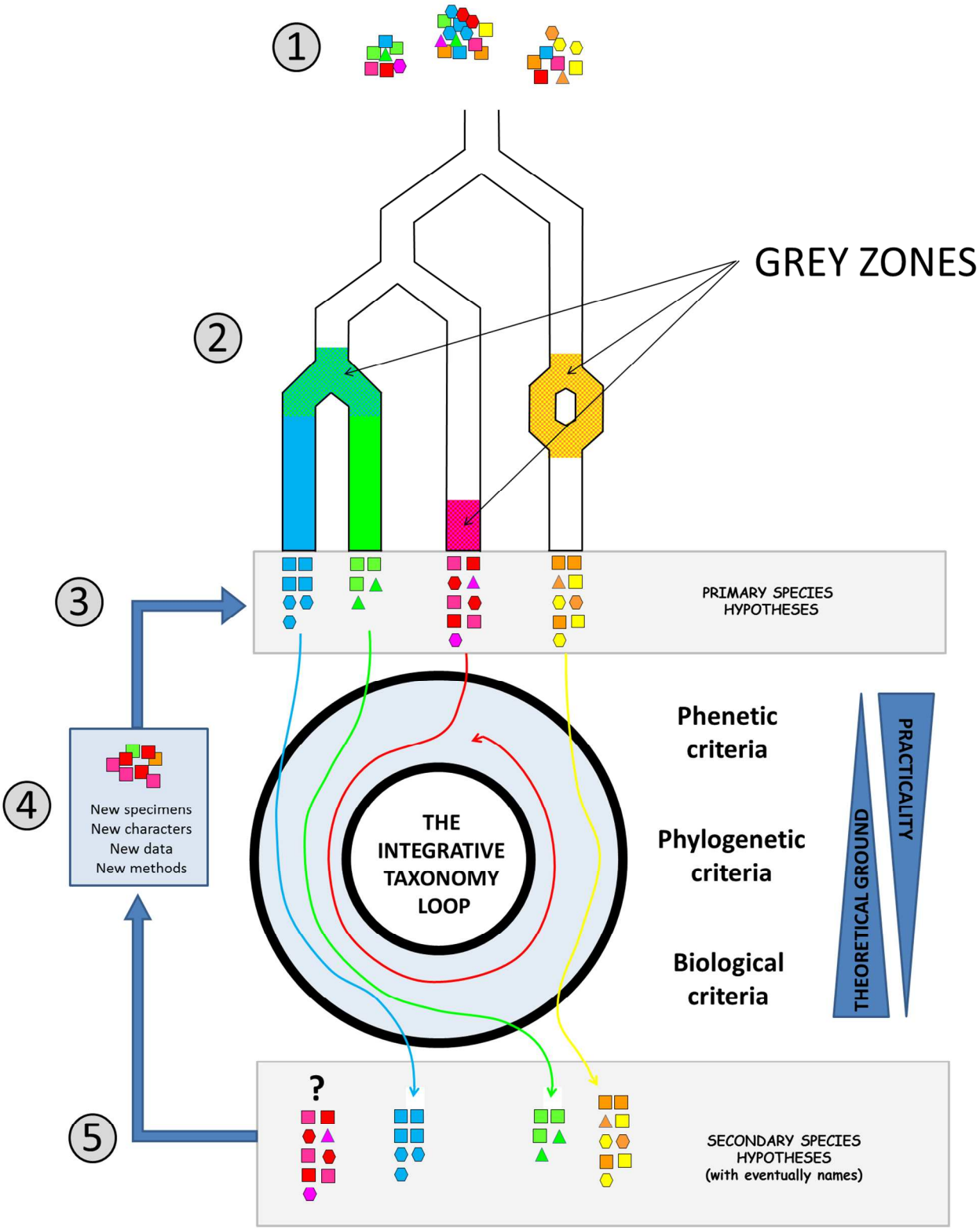

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731 **Box figure 1.**
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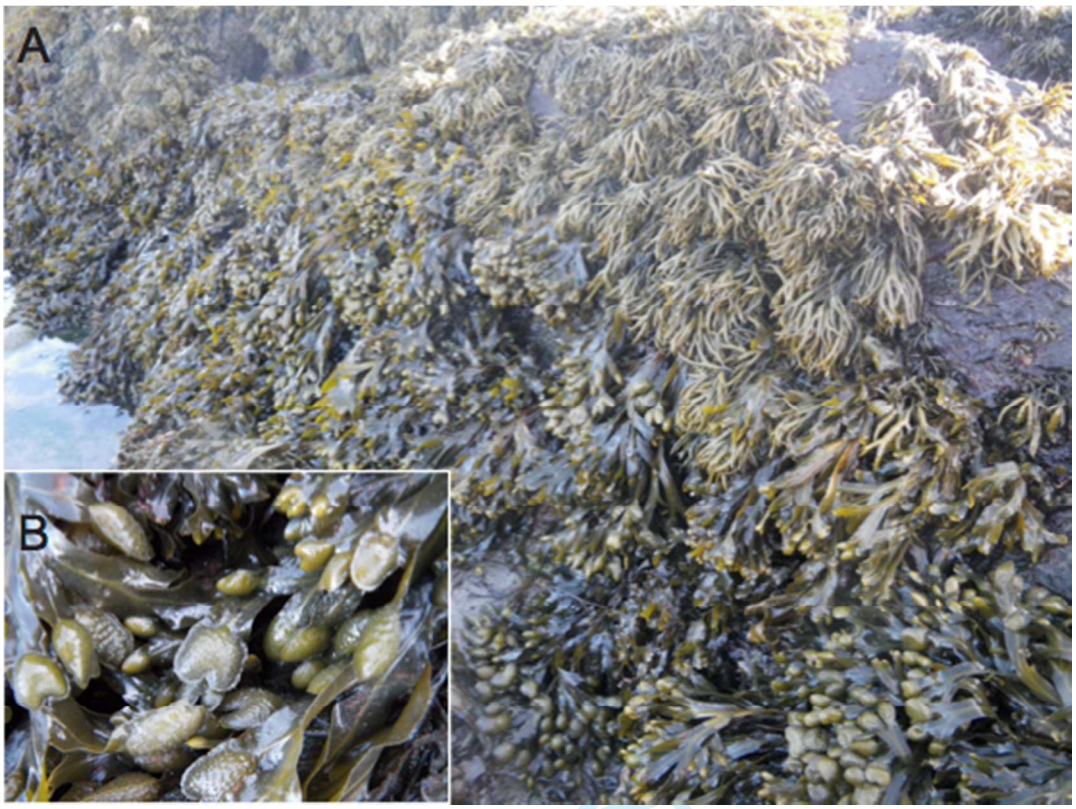

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735 **Box figure 2.**
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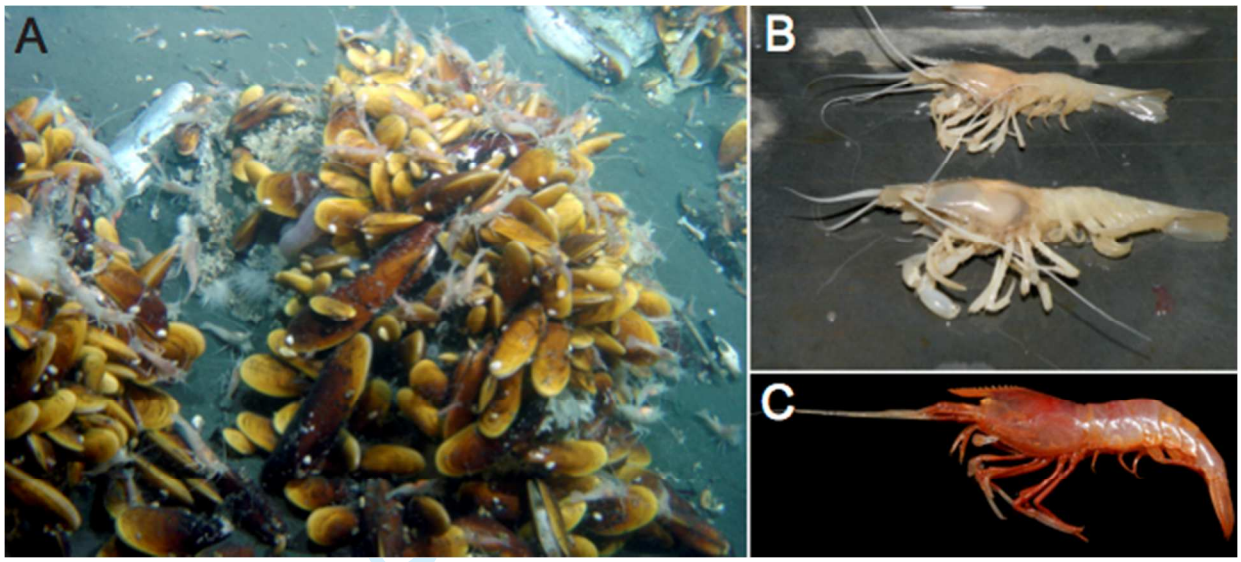
Box figure 3.



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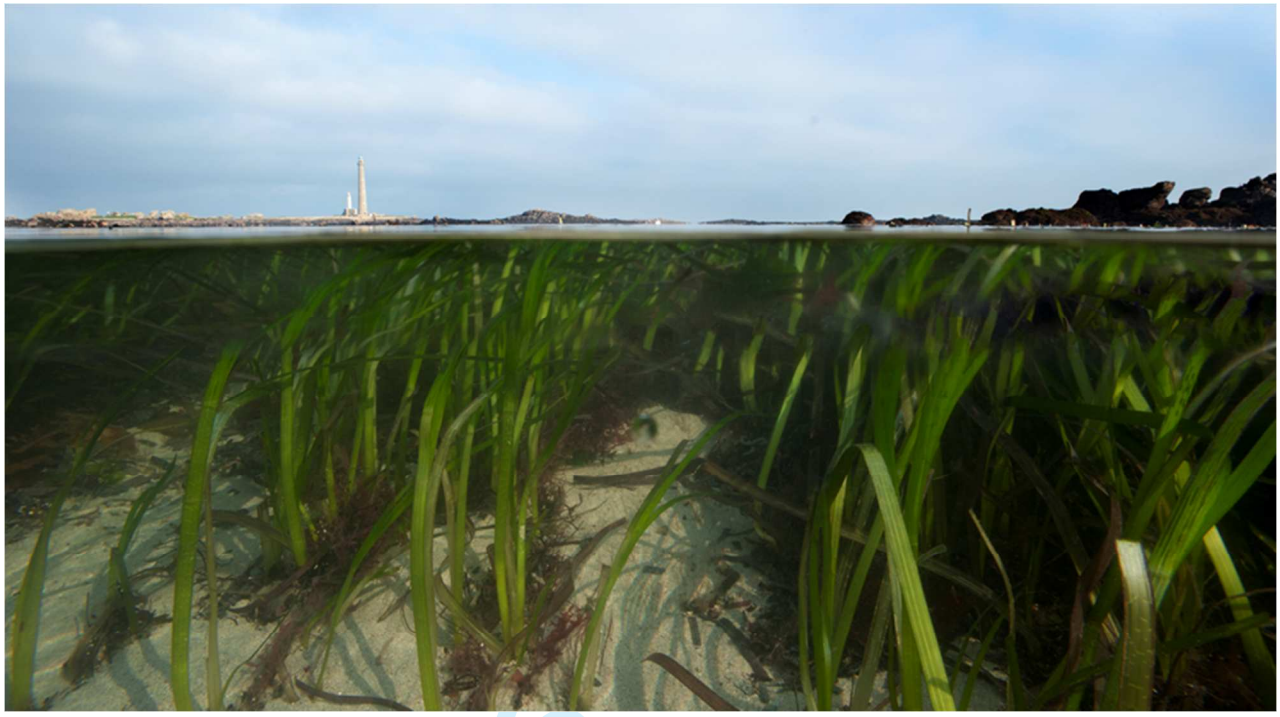

Box figure 5.

751 **Box figure 6.**
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Figure captions

Figure 1: The integrative taxonomy loop. Integrative taxonomy consists in analyzing different characters, with different methods, and applying different criteria of species delimitation to propose species hypotheses that are as robust as possible. The different families of criteria (as discussed in Samadi & Barberousse 2014) are listed right of the loop, with the more theoretically grounded on the bottom, and the more operational (i.e. easy to test practically) on the top. Within each family of criteria different kind of characters (i.e. morphology, ethology, ecology, biochemical, genetic, etc…) and methods (i.e. distances, maximum parsimony, maximum likelihood, population genetics inferences, crossing experiments, observations, etc…) may be applied. The different steps are as follows. 1. Populations (putatively within-species) and phylogenetic (putative sister-species) sampling. 2. Sampled species may be highly differentiated (blue and green), recently diverged species that are still in a "grey zone" (see Glossary) with most characters undifferentiated (pink and red), or a single species that went through a temporal split into several temporary lineages (yellow/orange). 3. Primary Species Hypotheses (PSH) are proposed. 4. PSH are engaged in the integrative taxonomy loop and are evaluated, possibly with the addition of new material, using different criteria for species delimitation (the more theoretically-grounded biological criteria being tested directly using cross experiments or indirectly with unlinked markers, and complemented with more operational (i.e. practical) criteria). 5. When possible, taxonomic decisions are taken (PSH are turned in Secondary Species Hypotheses - SSH) and are named. Some lineages (i.e. the pink/red lineage) may stay in the loop, needing more conclusive data before being turned into SSHs. Most of the literature and methods for species delimitation focus on species that are currently in the grey zone (cf. Carstens *et al.* 2013), even though most delimitation cases fall outside of this range.

Box figure 1. Species delineation in *Fucus*. A: Vertical distribution of fucoid species on rocky shore from high to low-water mark. Site: Viana do Castelo (GPS coordinates: 41° 41' 51''N, 8° 51' 07'' W, northern Portugal), photograph: Christophe Destombe. B: Detail of reproductive structure of *Fucus guiryi* showing a typical receptacle sterile rim (arrow). Photograph: Christophe Destombe.

Box figure 2. The pseudocryptic kelp species *Lessonia spicata.* A: This species (on the left of the picture) is found in central-south Chile (29°-41°S) in areas exposed to wave actions. Sporophyte individuals can be more than 2 meters long and are anchored to the rock by a holdfast. Site Los Molles (GPS coordinates: 32°14'S-71°31'W, central Chile), photograph: Pablo Balzo. B: The gametophytes of this species are microscopic and show a sexual dimorphism with males being smaller and more branched than females. Photograph: Valeria Oppliger.

Box figure 3. The deep-sea shrimp Alvinocaris muricola and A. markensis. A. *Alvinocaris muricola* in situ, on a mussel bed in in Western African cold seeps. (c) Ifremer, ROV Victor 6000, cruise Biozaïre. B-C. specimens of *A. muricola* (B) and *A. markensis* (c) sampled respectively on Western African cold seeps (cruise WACS, photograph Dominique Cowart) and Mid Atlantic Ridge hydrothermal vents (cruise BICOSE, photograph Laure Corbari) (c) Ifremer.

Box figure 4. Zostera marina meadows in Brittany. Individual shoots (ramets) are easily distinguished while rhizomatic connections linking ramets belonging to the same clonal lineage (genet) are hidden below the sandy sédiment (photograph: Ifremer/Olivier Dugornay).

Box figure 5. The deep-sea gastropod Bursa. A specimen of *Bursa sp.* sampled in New Caledonia using a dredge. The other organisms sampled in the same dredge are typical of hard bottoms (e.g. stylasterids, deep-water solitary stony coral, etc…). Photograph MNHN/Pierre Lozouet.

Box figure 6. The ascidian *Ciona.* *C. intestinalis* type A is established in the English Channel, where it shares habitats with its congener *C. intestinalis* type B. Photograph: Wilfried Thomas.

Glossary.

Biological nomenclature: set of rules that determine how names are given to taxa. In the binominal system, when a new species is described based on a given set of biological criteria applied over a set of specimens, one of them is designated as the holotype (the name-bearing specimen). When the taxonomy is revised using new material (additional specimens) and/or new biological criteria the position of the name-bearing specimens corresponding to the available names for the studied taxa is used to name the new taxonomic hypotheses. When it is not possible to study the holotype for the new character being proposed (e.g. molecular data), other categories of type material might be used (paratypes, syntypes, etc…)

Cryptic species: **(see also pseudo-cryptic species)**: two or more species lacking (obvious) morphological diagnostic features that are classified as a single nominal species; it is the opposite situation as compared to synonyms for which different scientific names are given to the same species.

DNA barcoding: identification of an unknown specimen by comparison of a standard DNA fragment (e.g. Barcode fragment of the *cox1* gene for metazoans) to a database of sequences linked to identified vouchers. By extension, use of a standard DNA fragment for biodiversity studies (e.g. to discover new species; see Collins & Cruickshank (2013) for a distinction between "specimen identification" and "species discovery and delimitation").

Divergence continuum: Refers to the observation that differentiation between two emerging lineages occurs through a series of changes that accumulate during the speciation process rather than abrupt genetic changes. For a recent genomic example, see Lexer *et al.* (2014).

Evolutionary significant unit (ESU): defined in 1994 by Moritz to explicitly take into account the genetic component of biodiversity in conservation, ESU is defining historically isolated groups of populations showing reciprocal monophyly and significant genetic divergence at nuclear loci.

Grey zone: temporal zone of the genealogical network during which two lineages are definitively diverging, but the criteria used for identifying divergence might not be applicable or in agreement (see in de Queiroz 1998). During this period of time, characters might differentiate at a different pace, leading to incompatible species hypotheses and justifying the need for integrative taxonomy.

Integrative taxonomy: species delimitation process in which species-hypotheses are proposed based on the integration of several lines of evidence (characters, criteria, methods) in a reproducible and falsifiable framework.

Phylogeography: the spatial distribution of phylogenetic lineages, particularly within and among closely related species (Avise 2009).

Population connectivity: Demographic- and evolutionary-dependence among isolated patches of individuals from a given species produced by the movement of individuals among patches.

843 **Pseudocryptic species**: morphologically overlooked species distinguished using other lines of evidence (e.g.
844 DNA) that proved to be finally distinguished by subtle morphological differences (e.g. Box 2 on the
845 kelp *Lessonia*).

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