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Cryptic Diversity: a Long-lasting Issue for Diatomologists

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In 2007 we and our co-authors published a paper in *Protist* on species delimitation in the complexes of the marine planktonic pennate diatoms *Pseudo-nitzschia delicatissima* and *P. pseudodelicatissima* (Amato et al. 2007). As of the moment of this writing, it has been one of the most cited papers in *Protist*. This is why we provide a short reflection on what the paper was about, and how the research we and others conducted has been relevant, and continues to be, in various different fields of protistology and beyond.

"The term 'species' refers to a concrete phenomenon of nature" (Mayr 1996). Such a phenomenon is the "maintenance of a harmonic gene pool" within a population (Mayr 1996), or metapopulation or lineage (De Queiroz 2007 and literature therein). This relates to the establishment of barriers to gene flow between organisms belonging to different metapopulations, but several evolutionary processes, such as mosaic evolution, can make different characters evolve at a different pace. If morphology evolves more slowly than other features which characterise a 'species' then cryptic diversity can arise (Mayr 1996).

The identification of species, based on an array of different characters and criteria, constitutes a common language for both practical and academic purposes. Already primitive societies developed the ability to distinguish and name animals and plants because they could observe the morphology and behaviours of the organisms in the natural environment. We know the names of the plants in our gardens, of the insects and animals in the forest and we are also aware that some of them are still a nightmare for taxonomists, such as oaks, where the identification at the species level is highly problematic because different discriminating features were used (Denk et al. 2017). Scientists have been struggling for centuries to provide solid grounds and criteria to define 'species' and this struggle is even more evident for unicellular eukaryotes (protists). The ability to cultivate these organisms and the development of electron microscopy techniques allowed to visualise the complex structure of their cells and cell walls and the taxonomy of protists developed rooted on their morphological characters. The advent of molecular techniques, such as PCR amplification and sequencing, provided genetic support to phylogenetic relationships among morphologically delineated taxa and, in many cases, unveiled the presence of genetically distinct organisms within a single morpho-species. Numerous are the papers dealing with diatoms because the morphology and ultrastructure of the siliceous frustule – at the base of morpho-species delineation – is relatively easy to study. Moreover, diatoms are phototrophic and most of them are

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easy to cultivate to produce biomass and provide material for molecular studies.

Cryptic Diversity and Species Concepts in Diatoms

Mann (1999) provided an extensive historical overview of species concepts in diatoms, showing that the presence of phenotypic (varieties, forms, races) or ecological (ecotypes) variation at the intraspecific level was acknowledged long ago by taxonomists. Only at the end of the past century species complexes, i.e., groups of closely related species in which boundaries between them are unclear, gained the attention of diatomologists who started to integrate morphological studies with the application of molecular markers.

Examples of early studies were the separation of *Skeletonema pseudocostatum* from *S. costatum* based on valve ultrastructure and distinct ITS sequences (Medlin et al. 1991), or the erection at the species status of *Pseudo-nitzschia multiseries* (formerly a variety of *P. pungens*) based on morphometric and molecular data and toxin production differences (Manhart et al. 1995). Sexual compatibility was tested by crossing strains of the pennate epipelic freshwater diatom *Sellaphora pupula* and showed the presence of distinct 'gamodemes', i.e. reproductively isolated units within this species (Mann 1989). This was the first study in which the 'biological species concept' introduced by Mayr in 1942 was tested in diatoms; different entities can be considered as 'real biological units' once sexual compatibility among them and the capability to produce fertile progeny has been proven. This approach, however, applies only to pennate diatoms, which have a heterothallic (dioecious) mating system and sex can be induced when strains of opposite mating type are placed in contact (Amato 2010). The first promising results obtained with *Sellaphora* prompted Mann to recommend: '*The immediate priority is to gain a profound knowledge of the nature, origin, and maintenance of the variation pattern in several species complexes (model systems) selected from freshwater and marine habitats to represent the major lineages of diatoms, different growth forms, and different mating systems.*' (Mann 1999).

Studies coupling ultrastructural and molecular investigations flourished in the following years leading to the discovery of several cryptic – morphologically indistinguishable but genetically different taxa – and pseudo-cryptic – genetically different taxa that can be differentiated by subtle morphological differences sensu Mann and Evans (2007) – species. Various distinct *Skeletonema* species were described, hidden beyond the apparently ubiquitous *S. costatum* (Sarno et al. 2005, 2007; Zingone et al. 2005). Cryptic taxa were also uncovered within the widespread diatom genus *Leptocylindrus* (Nanjappa et al. 2013) and in the *Chaetoceros socialis* species complex (Chamnansinp et al. 2013; Gaonkar et al. 2017).

In order to have a clear view of the articles citing Amato et al. (2007) (Supplementary Material Table S1), its Google Scholar citation report was analysed (on May 31st 2018). All the 296 items citing Amato et al. 2007 (Master and PhD theses were not included) were classified into seven categories (Supplementary Material Table S1) then plotted against the year of publication of the citing item (Supplementary Material Fig. S1). Most of the citations were in papers about 'cryptic diversity and molecular taxonomy in diatoms' (28.7%). Yet, the second and third most abundant categories were 'ecology, diversity and distribution other organisms' (17.2%) and molecular taxonomy in organisms other than diatoms (16.6%), demonstrating the interest our study generated outside the 'diatom community'.

In the study by Amato et al. (2007), we applied a triphasic approach, comparing three species concepts – morphological, phylogenetic and biological – on a large set of strains identified by light microscopy as members of two species complexes: *P. pseudodelicatissima* and *P. delicatissima*. All strains were isolated at the same site, the LTER station in the Gulf of Naples (Mediterranean Sea). The distinctive characters in light microscopy (LM) were cell width and shape and overlapping pattern of cell tips in a chain. The ultrastructure of frustules (ornamentation of the valve faces and cingular bands) in TEM and morphometric analysis of the density of fibulae, striae and poroids was coupled with the genetic characterisation with four molecular markers (the nuclear ribosomal LSU, ITS1, ITS2 and the chloroplast *rbc*L). The secondary structure of ITS2 on which the number of Compensatory BaseChanges (CBCs) including Hemi-CBCs (HCBCs) was determined as well. When comparing two RNA structures, CBCs are base changes occurring on both sides of a helix, which do not produce any modification of the structure itself (Gutell 1994). For instance, if on one side an adenine changed to a guanine, then the base pairing with it on the other side of the helix has to be mutated accordingly in order to keep the structure unvaried. If the base change occurs only on one side of the helix but the structure is not altered because the base pairing is still allowed by non-canonical pairings (Gutell 1994) then a HCBC is recorded.

Our results revealed that these two species complexes consisted of eight ultrastructurally and genetically distinct entities or species which are now formally described (Amato and Montresor 2008; Lundholm et al. 2003, 2006; Quijano-Scheggia et al. 2009). More in general, the notion that such ‘cryptic’ species existed within recognised morpho-species was not new (see above), but the delineation of the biological species and the experimental correlation with the occurrence of CBCs in the complete secondary structure of the ITS2 as a proxy for species boundaries was still missing in diatoms (see Coleman 2007).

Sexual compatibility and ITS2 secondary structure were widely implemented inthe description of new *Pseudo-nitzschia* species (Ajani et al. 2018; Amato and Montresor 2008; Churro et al. 2009; Gai et al. 2018; Li et al. 2017; Lim et al. 2012, 2013; Lundholm et al. 2012; Orive et al. 2013; Percopo et al. 2016; Teng et al. 2014, 2015, 2016). In other systems this was an already established practice (e.g. Bischoff and White 2004; Müller et al. 2007) or has been relatively recently introduced (e.g. Di Capua et al. 2017; Engesmo et al. 2016; Li et al. 2013; Pröschold et al. 2017).

In most protists, it is no sinecure to entice strains to reproduce sexually, let alone, to demonstrate that taxa are reproductively isolated. We were fortunate to choose *Pseudo-nitzschia* as our model because breeding experiments with members of this genus are easy to design, conduct and control. As most pennates, *Pseudo-nitzschia* is heterothallic. Following a period of ongoing mitotic divisions, cells undergo gametogenesis, following well-orchestrated steps (e.g. Scalco et al. 2016) which lead to two functionally anisogamous gametes. The different stages in the sexual reproduction process are recognisable in LM, and the newly established F1 cells are markedly bigger (in terms mainly of cell length) than those belonging to their parental cohorts. Thus, results can be easily recorded.

Besides the ease to conduct mating experiments, *Pseudo-nitzschia* is an ecologically relevant genus, which includes species producing the neurotoxin domoic acid. To discriminate between toxic and non-toxic taxa has repercussions on human health and economy. There is often variability in the reports of toxin production within several *Pseudo-nitzschia* species (Bates et al. 2018): is toxin production a species-specific character or does the ability of producing toxins differ among distinct populations of the same species?

There are indications for the presence of species-specific metabolic differences among cryptic species that deserve further attention and systematic studies. An example is the high level of specificity, detected even among cryptic *Pseudo-nitzschia* species, for lipoxygenase enzymes mediating the metabolism of eicosapentaenoic acid (EPA, 20C:5) (Lamari et al. 2013). Another example is provided by marked differences in the metabolic fingerprinting of two cryptic species in the *Chaetoceros socialis* complex: the temperate *C. socialis* and the boreal *C. gelidus* (Huseby et al. 2012). Chemotaxonomy is largely used in other systems, such as thraustochytrids. In this stramenopile group of organisms, the ability to produce certain compounds like specific fatty acids or pigments, coupled with molecular phylogeny and morphology, is used as a supplementary discriminating feature among cryptic or closely related taxa (e.g. Dellero et al. 2018). But chemotaxonomy has to be considered with caution because strain-specific features have been observed as well (e.g. Di Dato et al. 2017).

Cryptic Diversity and Ecology

Cryptic diversity has considerable implications for ecological studies and vice versa ecological studies can provide additional relevant information for the circumscription of cryptic species. Different cryptic species can bloom in different periods of the year as shown by studies carried out on *Pseudo-nitzschia* species in the Gulf of Naples with a clone-library approach (e.g. McDonald et al. 2007; Ruggiero et al. 2015). Pseudo-cryptic diversity was recorded also in the widespread benthic diatom *Navicula phyllepta* and the three species present in the Westerschelde estuary (the Netherlands) showed to have different ecological niches as demonstrated by their different tolerance for low salinity and distinct preferences for sediment types and ammonium concentration (Vanelslander et al. 2009).

Differences among cryptic species can be remarkable also along a spatial scale. The ‘everything is everywhere’ hypothesis states that marine phytoplankton are genetically homogeneous throughout their distributional range. Nevertheless, the identification of cryptic diversity has questioned this hypothesis, demonstrating that there are biogeographic patterns also in the ocean (Casteleyn et al. 2010). As a consequence, what were supposed to be cosmopolitan species can actually be distinct entities, as shown by the different biogeographic ranges of distinct *Skeletonema* species (Kooistra et al. 2008). Increasing information derived from metabarcoding approaches could provide additional data that will refine our appreciation of the biogeography of unicellular microalgae and hopefully will add information on the level of genetic diversity of the distinct populations. But in order to obtain a good resolution power, molecular taxonomy – possibly based on a few universal molecular markers – should be improved and extended to a wider range of taxa.

Cryptic Speciation and Evolution

Crosses between strains of distinct mating types provided support for species delineation but in some cases they also provided evidence for the presence of hybrids between different taxa. This is the case of rare production of hybrids between *Pseudo-nitzschia arenysensis* and *P. pseudodelicatissima* (Amato and Orsini 2015) or the natural hybrids between two morphologically and genetically distinct varieties of *P. pungens* (var *pungens* and *var. cingulata*) recorded in the contact zone (Casteleyn et al. 2009). Very low rates of hybridization were recorded also amongst the 'slender', 'robust' and 'labile' semi-cryptic morpho/genotypes of the benthic diatom *Eunotia bilunaris* where hybrids had intermediate morphology but were sterile (Vanormelingen et al. 2008).

The examples above indicate that detailed studies can reveal the presence of hybridization and shed light on the presence/emergence of post-zygotic barriers between closely related cryptic species. In nature, a few plant, vertebrate and insect species have been molecularly identified as homoploid hybrids (Nieto Feliner et al. 2017 and literature therein). Homoploid Hybrid Speciation (HHS) is characterised by the establishment of a new species from hybridization of two distinct parental species with no ploidy alterations. To consider a hybrid as a new species three criteria must be fulfilled: i) the species has to be reproductively isolated from the parent species, ii) barriers to gene flow have to arise from the hybridization event (not from other factors like temporal or spatial segregation), and iii) the hybrid nature of the new species has to be genomically validated (Schumer et al. 2014). One example is the yeast *Saccharomyces paradoxus*. The hybrid between *SpB* and *SpC* displays intermediate phenotypes and occupies the contact zone of its parental lineages (Leducq et al. 2016). Hybrid speciation by polyploidization (allopolyploidy) or whole genome duplications (autopolyploidy) are very common in plants (e.g. Soltis et al. 2015). In diatoms, scanty are the reports of polyploidy, but a real validation of speciation by polyploidization is still missing. Evolutionary speaking the characterisation of allopolyploid, autopolyploid, and homoploid hybrids would be a milestone to understand mechanisms which have led to complex genomes, which characterise this group of organisms and to their ecological success.

Concluding Remarks

One of the main questions about cryptic species is whether they play different roles in the ecosystem and what would be the biological trigger that leads to cryptic or pseudocryptic species (e.g. Lahr et al. 2014; Struck et al. 2018). As recently stated in a very elegant review paper by Struck et al. (2018), a number of cryptic species probably need reanalysis in order to ascertain their status because scrutinising hundreds of works dealing with cryptic species description and identifying a quantitative framework to discriminate crypticity, it turned out that some of the supposed to be cryptic species possibly are actually not. The framework proposed to 'foster a shift from pattern- to process-driven research concerning cryptic species' and include phenotypic disparity in relation to divergence time (Struck et al. 2018).

New cases of cryptic species will continue to be discovered as morphological entities are molecularly analysed, but to date why 'selection fails to discriminate among genotypes that all could have the same phenotype' (Bonner 2013) still remains an intriguing evolutionary and biological question which is far to be addressed.

Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.protis.2018.09.005.

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