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OPINION PIECE

The need to employ reliable and reproducible species identifications in coralline algal research

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ABSTRACT: Coralline algae perform important ecological roles in nearshore marine ecosystems globally by promoting the settlement of invertebrate larvae and enhancing biodiversity by creating habitat. However, these roles are severely threatened by global environmental changes. Most coralline algae are extremely difficult to identify, and DNA sequencing has revealed rampant inaccuracy of morpho-anatomical approaches to distinguish species, and even genera. If appropriate identification methods are not reported, or even used, we will be left with an uninterpretable body of literature where the species-specific biology of coralline algae cannot be validated. This will make it difficult to determine the impact a changing ocean may have on these ecologically important species. We reveal the magnitude of the issue in coralline algal research — both the identification methods used and the reporting of identification protocols. An analysis of 341 articles over the past decade revealed that only 7.6% used molecular methods, with over 70% not reporting any details of how species were identified. While many coralline algal taxonomists understand that the majority of species cannot be identified morphologically, this message has not disseminated to the ecological and physiological community. We provide a series of guidelines for conducting DNA-based identifications and strongly recommend the use of these methods over less informative morpho-anatomical techniques. Most importantly, the methods of identification should be adequately reported. Without following these guidelines, research on coralline algae runs the risk of collecting uninterpretable data, and conducting irreproducible science, slowing our ability to determine how these important species will respond to future ocean conditions.

KEY WORDS: Species identification · DNA barcoding · Calcifying algae · Molecular tools · Rhodolith · Voucher specimens · Maerl

1. INTRODUCTION

Coralline algae (subclass Corallinophycideae) are calcifying red algae that play ecologically crucial roles from the tropics to the poles, stimulating settlement of many invertebrate larvae, cementing reefs, and providing nursery and protection for a host of other algal, invertebrate and fish species (Heyward & Negri 1999, Nelson 2009, Chenelot et al. 2011, McCoy & Kamenos 2015, O'Leary et al. 2017). Unfortunately, ocean acidification threatens the ability of coralline algae to provide these ecosystem services, as it can

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reduce the calcification rates of mature coralline algae (Martin & Gattuso 2009, Comeau et al. 2014, Cornwall et al. 2017) and severely decreases recruitment and post-settlement growth of juvenile corallines (Ordoñez et al. 2014, 2017, Guenther et al. 2018, Cornwall et al. 2020). Moreover, despite broad morphological similarities, species differ markedly not only in their physiology (Dethier & Steneck 2001, Fisher & Martone 2014), chemistry (Janot & Martone 2016) and ecology (O'Leary et al. 2017, Hind et al. 2019), but also in their response to environmental stress (Noisette et al. 2013, McCoy & Ragazzola 2014, Cornwall et al. 2017). With increasing scientific interest in understanding the impacts of climate change on these potentially sensitive species comes an urgent need to more accurately determine the mechanisms underlying the observed variation of species-specific responses and how ecological roles differ between species (Noisette et al. 2013, McCoy & Pfister 2014, Cornwall et al. 2017, McCoy & Kamenos 2018).

Coralline algae have long been regarded as difficult to identify (Steneck 1986, Woelkerling et al. 1993, Hind et al. 2014a). Thus, it should not be surprising that the advent of DNA sequencing has revolutionised our understanding of coralline algal systematics at all taxonomic ranks, and has revealed the inadequacy of morpho-anatomical techniques (summarised in Twist et al. 2019). DNA-based phylogenies employed over the past decade have revealed previously unrecognised diversity, new understanding of species distributions and ranges (geographic and ecological), and a re-evaluation of criteria for generic and species delimitation. Increasingly, sequence data are being obtained from type material — first used on geniculate coralline algae by Gabrielson et al. (2011) and subsequently on non-geniculate coralline algae by Sissini et al. (2014) — to clarify generic and species concepts, and the correct application of names. For example, *Porolithon onkodes* (Heydr.) Foslie was thought to be a widespread tropical coralline species, but DNA sequencing has revealed that more than 20 species were passing under this name (Gabrielson et al. 2018). The evidence has been clearly presented and articulately expressed by multiple authors in taxonomic papers: current morpho-anatomical methods are unreliable tools for identifying nearly all coralline algal species (e.g. Hind et al. 2014b, Melbourne et al. 2017, Richards et al. 2018, Torrano-Silva et al. 2018, Rindi et al. 2019, Twist et al. 2019). However, despite these findings, morpho-anatomical methods are still regularly employed, either due to a lack of understanding of the magnitude of the issue, or due to the cost, expertise and time associated with molecular identification compared to identifications based on gross morphology.

If our ability to understand the ecology, physiology and biogeography of coralline algae is to improve, we need to accurately ascribe responses in published research to actual species. Otherwise, we will be left with an uninterpretable body of literature from which clear conclusions cannot be drawn. While coralline systematics has been making significant strides, overall difficulties associated with coralline algal identification have not been adequately addressed in the non-specialist literature, and clear guidelines on employing and reporting the methods used to identify coralline algae are required.

Our aim was to provide guidelines on accurate reporting and identification of coralline algae (see Table S1 in Supplement 1 at www.int-res.com/articles/suppl/m654p225_supp.pdf). In order to first establish the scale of this issue, we reviewed published research on coralline algae over the past decade and evaluated how identifications were made and reported. We considered that this timeframe included the advent and proliferation of molecular identification approaches/tools. We sought to improve awareness of the scale of the problem, particularly amongst non-taxonomists who may not be aware of this problem, or who have underestimated its importance. Best practice guidelines are presented for both science practitioners as well as those reviewing publications involving coralline algae. The guidelines address the identification of specimens, the importance of voucher specimens, and recommendations on how to report the methods used. We also highlight solutions to improve access to molecular identification for non-taxonomists.

2. SCALE OF THE PROBLEM

We quantified the details of coralline algal identification methods reported in articles published since 2010 (excluding taxonomic, review and palaeontology articles). Articles were found by searching for the terms 'coralline algae', 'rhodolith' and 'maerl' in the Web of Science (www.webofknowledge.com/WOS) database. Articles were selected for analysis if authors attempted to identify coralline algae to genus or species for use in their study (e.g. for use in an experiment or to quantify diversity), resulting in a total of 341 articles between the start of 2010 and the end of 2019 (see Supplement 2 at www.int-res.com/articles/suppl/m654p225_supp.pdf). These selected articles did not include 54 additional articles

published since 2010 that intentionally grouped coralline algae together (making no attempt to differentiate species) in studies that appeared to address species-specific questions relating to coralline algae. We recorded whether authors reported using genetic tools (i.e. DNA sequence analysis), morphology (i.e. detailed description of morphological or anatomical characters and/or reference to a key/guide) or neither (i.e. no mention of any identification method) to identify coralline algae. Additional information was recorded on whether studies reported retaining voucher samples of specimens (in house or at a registered herbarium) and, when genetics were used, if DNA sequences were available in a public database.

Results show that, despite numerous taxonomic studies in recent years stating the need for DNA sequencing to make reliable identifications, only 7.6% of the studies used genetic identification (Fig. 1). However, the prevalence of the use of DNA sequencing increased from 5.5% in the years 2010–2014 to nearly double at 9.2% in the following 5 yr. Despite this, still over 70% of studies in the previous decade that attempted to identify coralline algae did not report how the species were identified, a trend that has not improved in recent years (Fig. 1). It is possible in these cases that morpho-anatomical identification was used, and/or taxonomic experts consulted. However, in the absence of information on how species were identified, species identifications cannot be confirmed. We point to a need for culture change in reporting identification protocols in published research, which is currently not common practice in the majority of coralline algal research papers.

Only 9.7% of studies that did not use genetic identification mentioned that they retained samples or deposited material in a registered herbarium. If representative specimen samples are retained and deposited in publicly accessible herbaria or collections, it is possible for them to be re-analysed in light of new technology and/or knowledge and emerging understanding of coralline algal diversity. In the absence of voucher material, the identity of specimens cannot be reassessed, and thus conclusions regarding species-specific understanding of the biology of taxa cannot be confirmed.

3. CONSEQUENCES OF NOT RELIABLY IDENTIFYING SPECIES

It is important to recognize that without the ability to identify specimens to species level, we may be unwittingly studying and comparing populations, species and higher-order phylogenetic clusters simultaneously, thereby lumping species-specific responses into intra-species variation. Clearly this has major implications for our study of species-specific traits and ecology (Mayr 1948), and possibly even our understanding of evolution and selection pressures in those species. These implications are amplified in groups with complex life histories, such as macro-

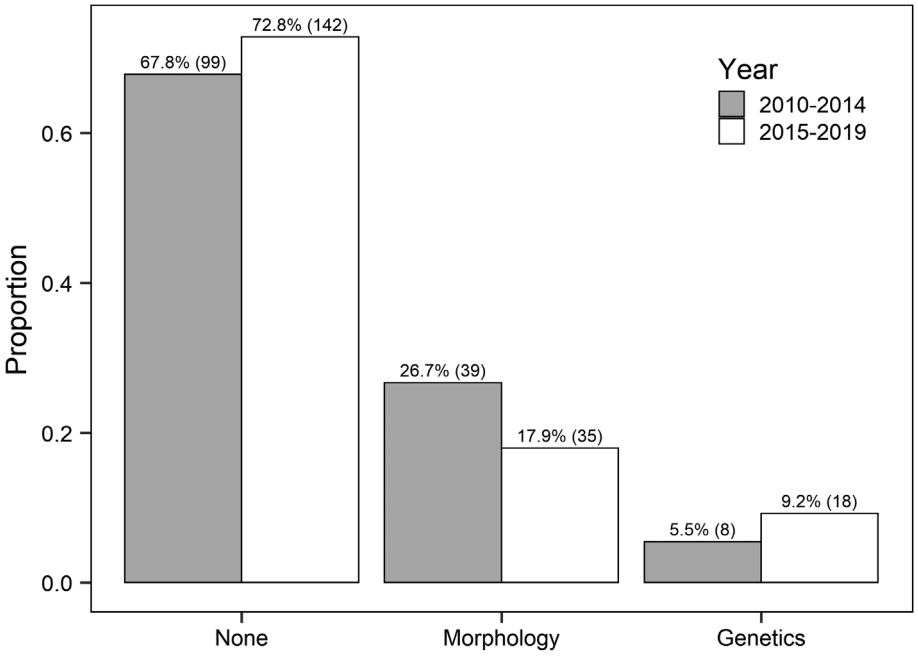

Fig. 1. The proportion of studies in each time period (2010–2014 and 2015–2019) that reported identifying coralline algae. None: no mention of any detail of how coralline species were identified; Morphology: detailed description of morphology or anatomical characters and/or reference to a key/guide; Genetics: use of genetic techniques for identification. Numbers at the top of bars are percentages followed by counts in brackets

algae, or symbioses, such as corals or lichens (e.g. brown algae *Lobophora* spp., Vieira et al. 2017; scleractinian corals *Lobophyllia* spp., Arrigoni et al. 2019; polyp stony coral *Acropora* spp., Ladner & Palumbi 2012). Such groups are inherently more difficult to study and may not fit squarely into current eco-evolutionary frameworks. For example, Hind et al. (2019) were able to show, through the identification of cryptic species, that although there is a higher abundance of coralline algae in sea urchin barrens, there is lower diversity compared to that present in kelp forests. These findings could have many consequences for understanding the functions of these different ecosystem states. Without understanding species-specific responses, or worse, combining an unknown number of species into some treatments and not others, species-specific ecological traits and accurate predictions of how coralline communities and the ecosystems they inhabit may respond to a changing ocean will continue to be hampered. We acknowledge that, in some cases, there may be guilds of responses, whereby morphologically similar species may respond similarly to the same environmental driver (e.g. McCoy & Ragazzola 2014, Barner et al. 2018). However, morphologically similar species would need to be tested before such an argument could be used. In many cases, grouping an unknown number of species within one treatment would increase sampling error-derived variance, and could confound species effects with treatments that cannot be systematically accounted for.

4. GUIDELINES AND RESOURCES

4.1. Molecular identification

Prior to molecular identification, appropriate collection, cleaning and storage techniques must be followed (discussed in further detail below in Section 4.3 and in Table S1). Many taxonomic articles provide detailed descriptions for undertaking DNA extractions and PCR amplifications (e.g. Hughey et al. 2001, Broom et al. 2008, Gabrielson et al. 2011, Rösler et al. 2016, Anglès d'Auriac et al. 2019, Twist et al. 2019). Here, we summarise their key suggestions (additional information provided in Table S1). Various commercially available extraction kits (e.g. Qiangen DNeasy kits, GenElute DNA kits, QuickExtract and NucleoSpin tissue kit) have demonstrated success in the extraction of DNA from coralline algal species. In non-taxonomic studies, the purpose of molecular identification is to determine if the biological units being studied belong to the same species, so they can be reliably compared to other distinctive species. Therefore, for DNA barcoding (or species identification), typically only one marker is needed to delineate species. Three markers — the plastid markers *psb*A and *rbc*L, and the mitochondrial marker COI-5P — are commonly used in coralline algal research (Table S1). The most commonly used marker is *psb*A (36.1% of all GenBank sequence entries for Corallinophycidae), as it is easily amplifiable and, in most cases, reliably separates recently diverged species (Broom et al. 2008). The marker *rbc*L (11.5% of all GenBank sequence entries for Corallinophycidae) has been demonstrated to be the most successful when amplifying DNA from type material from old herbarium specimens (Gabrielson et al. 2011, Hughey & Gabrielson 2012). The COI marker constitutes 30.7% of all GenBank sequence entries for Corallinophycidae. PCR products can be sent to various commercial vendors for Sanger sequencing. Once sequences have been obtained, these must be compared to publicly available libraries such as GenBank (https://www.ncbi.nlm.nih.gov/genbank/) by performing BLAST (https://blast.ncbi.nlm.nih.gov/) nucleotide searches (Table S1). Even if matches to sequences are not found in publicly available databases, archiving sequences in these databases makes them available to all researchers for future comparisons and identifications.

In some instances, it is not practical to perform molecular identifications on all specimens in a given study, nor does extracting and amplifying DNA work 100% of the time. In these circumstances, molecular analysis can be used to aid morphological placement of specimens. For example, O'Leary et al. (2017) used molecular analysis on a small subset (5–10 specimens) of each morphologically defined coralline group to confirm species identity and to determine that multiple species were not being grouped together. It is logical that most non-taxonomic studies should follow these subsampling procedures, especially in advanced physiological or geochemical studies where many samples are required to assess holistic organism-scale responses to stressors.

4.2. Morpho-anatomical identification

In very rare instances, such as where the molecular and morpho-anatomical identifications are reliably consistent, molecular identification may not be re-

quired (Table S1). However, for the vast majority of cases, using morpho-anatomical identification alone is not appropriate and often relies on the presence of reproductive features at particular stages of development. This is due to 2 main issues as previously alluded to: there are far more coralline species than previously thought based on morpho-anatomical identifications, and there are few morpho-anatomical characters that have been supported when tested with DNA sequencing (e.g. Hind et al. 2014a, Gabrielson et al. 2018, Richards et al. 2018, Pezzolesi et al. 2019).

There are currently only a few genera in restricted geographic areas that can be identified reliably based on morpho-anatomy, due to enough species having been sequenced to confirm diagnostic morpho-anatomical characters, e.g. non-geniculate *Phymatolithon* species in the Arctic and subarctic (Adey et al. 2018), geniculate *Calliarthron* species in the NE Pacific (Gabrielson et al. 2011), and the geniculate species *Jania sphaeroramosa* Twist, J. E. Sutherl. & W. A. Nelson in New Zealand (Twist et al. 2018). However, for the other >99% of species, morpho-anatomical species identification is not possible without first assessing whether morpho-anatomical and molecular identification match across their distribution.

If morpho-anatomical characters must be used, the most recent taxonomic articles should be consulted and reference given to their use, as species complexes are rapidly changing in light of new molecular evidence. Harvey et al. (2005) provided a resource outlining coralline morphology and anatomy, along with laboratory procedures (e.g. decalcification, sectioning and staining) for conducting anatomical investigations on coralline algal specimens (although the species key and names provided in this guide should not be used for identification purposes). However, many of these anatomical identification techniques can be difficult and time consuming, and undertaking DNA sequencing is more accurate and typically more time efficient if the appropriate procedures outlined here are followed.

4.3. Specimen collection and voucher specimen storage

Coralline algae can be collected and preserved in several different ways for DNA extraction and long-term storage. The best preservation method for future DNA sequencing is desiccation in non-toxic silica gel. Less ideal is air drying, but 19th and early 20th century historical air-dried specimens have been sequenced with success (Gabrielson et al. 2011, 2018, Hind et al. 2014a, Richards et al. 2018). Prior to DNA extraction, samples should be carefully cleaned to remove epiphytes and endophytes that are visible (Table S1). Sampling strategies need to be designed that enable sufficient material for both destructive analytical techniques where required (e.g. in geochemical analyses; Cornwall et al. 2017, McCoy & Kamenos 2018), as well as the retention of material for future comparative studies and/or verification. Thus, we suggest that voucher specimens be deposited in herbaria or publicly accessible institutional collections and that these details be reported together with data archiving information (Table S1).

4.4. Training courses

Unfortunately, the frequency and geographical spread of training courses in phycological identification have declined over time, and many courses are available only to students enrolled in graduate or undergraduate programs of study. Accessibility and participation in these courses for researchers of all career stages conducting either field and/or laboratory-based research on coralline algae need to be revived. It is essential that instruction in DNA barcoding methods be included in these courses, without exception. Additional funding is needed for these courses as is wider advertisement of their availability.

4.5. Reviewers and editors

We recommend to reviewers and editors that reporting on identification and retention of vouchers be a requirement for publication. We advocate that the following points should be addressed by authors, possibly presented in a checklist required before submission: what methods were used to identify specimens, an explanation of any uncertainty in the species identification, where samples are deposited/kept, and whether molecular results are stored in a repository. In addition, citations of key reference material should be included, as well as acknowledgements of expert taxonomists when consulted. This information needs to be obtained to determine whether the method of identification that has been documented is appropriate for the hypotheses being tested and enables the conclusions of the study.

4.6. Grants

We advocate the need for specific funds to be allocated for accurate identification of coralline algae. Ideally, provisions within grant systems that specifically allow for additional funding to be requested for molecular analysis and/or training if researchers propose to work with coralline species. This way funding opportunities would not disadvantage coralline algal biologists, or even worse, cause existing coralline algal biologists to change their focus to different taxa. When reviewing grant proposals where molecular identifications are not, or cannot be, implemented, we propose that reviewers ask the question of how the samples will be identified. This should not be done in ways that remove coralline algal researchers from the pool of potentially successful candidates, but rather indicates to applicants that molecular work is required. The addition of DNA barcoding is likely to substantially affect proposal budgets, particularly for ecological research that is traditionally lower cost. However, the cost of molecular work is declining, and with appropriate study designs (e.g. selecting a subsample of specimens to sequence) the cost can be manageable.

5. CONCLUSIONS

While systematic research on coralline algae over the past decade has resulted in a surge of species discoveries and vastly altered understanding of coralline diversity and distribution, the data presented here provide evidence that these discoveries and the implications of taxonomic work are not being reflected in other coralline research. The lack of reporting of methods used to identify coralline algae and the slow uptake of appropriate DNA identification methods to identify specimens is concerning. A series of guidelines has been presented for accurately reporting species identification protocols, for conducting molecular based identifications, and for the retention of voucher specimens. Although coralline algal taxonomy is still in flux, voucher specimens and/or DNA sequences are fixed and, even if they do not match a currently described species, they still provide a permanent record of the experimental unit that can be validated at a later time point if needed. Additionally, the cost for conducting DNA sequencing is declining and researchers should be aware that at present, molecular identifications are the best method for identifying coralline algae. These guidelines will support coralline algal research by ensuring increasingly repeatable and reliable results.

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