COMMENTARY / COMMENTAIRE

Comment on "Acquiring DNA sequence data from dried archival red algae (Florideophyceae) for the purpose of applying available names to contemporary genetic species: a critical assessment"[1]

Jeffery R. Hughey and Paul W. Gabrielson

Abstract: Saunders and McDevit recently reported their efforts to extract and amplify DNA by PCR in successively older red algal (Rhodophyta) herbarium specimens. They found that recent collections (4-11 years old) readily amplified but that archival material (decades to a century old) yielded contamination problems, diminished success correlated with age, or failed to amplify. As a solution, they proposed that epitypes be designated based on contemporary sequenced specimens. In response, we extracted and amplified in independent laboratories three loci (COI, ITS2, and *rbc*L) from the same 1836 *Sparlingia pertusa* specimen that Saunders and McDevit were unable to amplify. The use of Q-solution enhanced amplification success and likely is partly responsible for our achievements with archival specimens. These findings, along with data from the last 13 years in which we have sequenced over 100 historical and type specimens, indicate that with proper controls, amplifying DNA from red algal herbarium specimens of any age is practical and reproducible. The designation of contemporary epitypes should be a last resort, not an alternative to sequencing type material, and must be done with an understanding of the historical record of the species.

Key words: Rhodophyta, herbarium specimens, DNA type material, epitypification.

Résumé : Saunders et McDevit ont récemment fait état de leurs efforts pour extraire et amplifier l'ADN par RPC chez des spécimens d'algues rouges (Rhodophyta) de plus en plus âgés. Ils ont constaté que les collections récentes (âgées de 4 à 11 ans) permettent l'amplification, mais que le matériel d'archives (décades ou siècles) montre des problèmes de contamination, une diminution du succès avec l'âge ou l'absence d'amplification. Comme solution, ils ont proposé que l'on désigne les épitypes sur le base de spécimens contemporains séquencés. Suite à ce travail, les auteurs ont extrait et amplifié dans des laboratoires indépendants, trois lieux (COI, EIT2 et *rbc*L) à partir du même spécimen, *Sparlingia pertusa* 1836 que Saunders et McDevit n'ont pu amplifier. L'utilisation de la solution Q augmente le succès de l'amplification et explique partiellement le succès des auteurs avec des spécimens d'archives. Ces constatations, avec les données des 13 dernières années pour lesquelles les auteurs ont séquencé plus 100 spécimens types historiques, indiquent, qu'en utilisant des contrôles appropriés pour amplifier l'ADN de spécimens d'herbier d'algues rouges de tous âges, montrent leur intérêt pratique et reproductible. La désignation d'épitypes contemporains devrait être retenue en dernier essor, non pas comme une alternative au séquençage du matériel type, et doit être réalisée avec une compréhension des données historiques sur l'espèce.

Mots-clés : Rhodophyta, spécimens d'herbier, ADN de matériel type, épitypification.

[Traduit par la Rédaction]

Saunders and McDevit (2012) recently published their work detailing attempts to isolate and PCR amplify DNA from red algal herbarium specimens. Based on their efforts using different DNA extraction protocols (Hughey et al. 2001; Saunders 1993, 2008; Saunders and McDevit 2012), they demonstrated that younger herbarium specimens (4–11 years old) displayed greater than 90% amplification success for the eight PCR markers that they targeted (ranging in size from 151 to 563 bp, excluding primers). For the two archival collections of *Sparlingia pertusa*, first-round PCR was successful only

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with the 1955 specimen and only for two markers, ITS2 and *rbc*L, with no contamination; all first-round PCR amplifications failed for the 1836 specimen. Saunders and McDevit (2012) thus elected to use nested PCR techniques. These methods involved using PCR products from the first round of amplifications as the source for the second round. This extremely sensitive amplification technique resulted in some contamination but also in the successful amplification of target DNA from the 1955 specimen. In addition to the above, Saunders and McDevit (2012) analyzed 15 additional red algal specimens, most of which were collected in the 1800s. They targeted the ITS2, *rbc*L, and COI markers for this analysis and were successful in 5 of 45 amplifications. Their overriding conclusions were that (*i*) DNA analyses on archival specimens is not a practical way forward, given the very low rate of successful amplifications that they attained, and (*ii*) a more expedient solution is the designation of a contemporary epitype from which sequence data has been acquired. Based on our collective experience of working independently with red algal archival DNA over the last 10 years, we disagree with both conclusions.

At our request, Saunders generously sent us, in separate tubes, two large fragments of the 1836 *S. pertusa* specimen on which to perform our own extractions and PCR amplifications and for which all amplifications attempted by Saunders and McDevit (2012) failed outright. Material of this species has never been present in either of our laboratories, therefore processing in our facilities should not yield *Sparlingia* sequences as a result of contamination from an exogenous source. In the Hughey laboratory, two extractions were performed. The first included one cystocarp and vegetative tissue and the second included four excised cystocarps. Because reproductive tissue contains more DNA per thallus area, it may yield amplifiable DNA when vegetative tissue does not. Two negative controls also were processed in parallel with the samples following the extraction and amplification reagents of Lindstrom et al. (2011). Initially, three regions were targeted using the primers and cycling parameters published in Saunders and McDevit (2012) (total amplicon lengths: *rbc*L, 191 bp; COI, 264 bp; and ITS2, 302 bp). The *rbc*L region was successful in both extractions, but the COI and ITS2 regions failed. The controls were negative. Based on these findings, it was clear that the intact DNA in the 1836 specimen was ≤200 bp in length. To test this hypothesis, primers (Supplementary Table 1)[1] targeting two smaller fragments for each marker were designed (total amplicon lengths: 174 and 116 bp for the COI gene; 160 and 100 bp for ITS2). The first extract amplified successfully for the 116 bp COI gene but failed for all others. The second extraction amplified for all four of the other COI and ITS2 primer pairs. Sequences were compared with those previously published for *S. pertusa* and were found to be identical (Supplementary Table 2)[1], with the exception of the COI sequence, which differed by 1 bp from published data. In the Gabrielson laboratory, the sample and two negative controls were extracted using the Hughey et al. (2001) protocol, except that dithiothreitol was not included in the extraction process. PCR amplification was attempted with the F1152Pri–R1308Pri primer combination that Gabrielson (2008*a*, 2008*b*) used successfully with *Prionitis* spp., but this primer combination yielded no amplified product. A primer pair (Supplementary Table 1)[1] was designed for *S. pertusa* for the same segment of *rbc*L based on the one available sequence in GenBank (AB383123). This primer pair resulted in a fragment 177 bp in length (total amplicon length) that was identical in sequence to the one obtained by Hughey above. An attempt was made to amplify a longer target (~450 bp) using this new forward primer paired with R-rbcS (Freshwater and Rueness 1994), but this was unsuccessful. An additional PCR reaction was then performed with the primer pair that yielded the 177 bp fragment (four tubes with the 1836 *S. pertusa* and a negative control) both with and without Q-solution (Qiagen). All four tubes with Q-solution amplified well; only one tube without Q-solution amplified, but only weakly.

With respect to our first disagreement, these results conclusively demonstrate that DNA can be extracted and sequenced from a 19th century herbarium specimen in independent labs, implementing the negative extraction and PCR controls recommended by earlier workers (Cooper and Poinar 2000; Poinar 2003) and Saunders and McDevit (2012). Furthermore, previous studies amplifying DNA from more than 100 archival specimens also show that red algal herbarium material collected in the 18th, 19th, and early 20th centuries are amenable to genetic analysis (Hughey et al. 2001, 2002; Gabrielson 2008*a*, 2008*b*; Gabrielson et al. 2011; Lindstrom et al. 2011). Saunders and McDevit (2012) noted that only Hughey et al. (2001) stated that they used a DNA extraction control, but Gabrielson (2008*a*, 2008*b*) and Gabrielson et al. (2011) followed the Hughey et al. (2001) protocol as stated in their papers. We concur with Saunders and McDevit (2012) that the inclusion of several negative extraction and PCR amplification controls increases the likelihood of detecting contamination. In both of our labs, we routinely include negative PCR controls in all of our reactions. Although rare, we have found that with repeated use, PCR reagents may become contaminated.

Our results and those of Saunders and McDevit (2012) indicate that the DNA from the 1836 specimen of *S. pertusa* is best characterized as poor to average. We could only amplify <200 bp fragments of DNA (all attempts at amplifying longer fragments of all three markers failed), and only the extracted fragment with more cystocarps, hence more DNA/unit area, amplified for all three regions. Because biomolecules in herbarium specimens degrade over time (Staats et al. 2011), attempts to analyze DNA from herbarium specimens should be made on a case by case basis, not by attempting to amplify large PCR targets and hoping for outstanding preservation (300+ bp in length). Saunders and McDevit (2012) encountered the two main problems associated with analyzing archival DNA. The first is that biomolecular preservation is variable among both taxa and herbarium specimens, even for the same collection housed in different herbaria. In our experience of working with type specimens, intact DNA is, on average, around 200 bp for fleshy red algae, with poorly preserved DNA in the 100 bp range and well-preserved DNA (fe.g., in Corallinales and Sporolithales (Hughey et al. 2008; P.W. Gabrielson, personal observation)) in the 300–400 bp range. Despite numerous attempts, neither of our labs has ever successfully amplified DNA fragments longer than 400 bp from type material collected in the 18th and 19th centu-

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/b2012-102.

ries. The second problem, which Saunders and McDevit (2012) demonstrated quite conclusively, is that contamination may be a major issue and, as they suggested, needs assessment through the use of DNA extraction and PCR controls.

With respect to our second disagreement, the designation of contemporary epitypes, we wish to direct the authors to the most recent paper by Lindstrom et al. (2011) on species of *Mastocarpus* from Pacific North America. In this paper, type materials were obtained and successfully analyzed in a laboratory that had not previously been used to process modern *Mastocarpus* DNA. The authors were able to analyze 12 type specimens and correctly assign type materials and their names to a bewilderingly complex group of cryptic and highly polymorphic species. Epitypification (McNeill et al. 2006, Vienna Code, Chapter II, Article 9.7) requires that all of the original material be "demonstrably ambiguous and cannot be critically identified for purposes of the precise application of the name of a taxon." Saunders and McDevit (2012) cite the same species of *Mastocarpus*, which were deciphered using archival DNA from herbarium types (Lindstrom et al. 2011), as a prime example for the use of epitypes! Earlier work by Hughey et al. (2001, 2002, 2008) on Gigartinaceae included the analysis of 40 type specimens. The results indicated that some of the specimens were given the incorrect provenance when collected and were said to have come from Pacific Northwest America, when in fact they came from the southern hemisphere. Many were incorrectly placed in synonymy under older names, and some were originally classified in the wrong order. Even for a type specimen with an unknown provenance, where designating an epitype is completely impractical, sequencing type material, along with historical research, can reveal the identity of the specimen. Gabrielson (2008*a*) demonstrated this by sequencing type material of *Sphaerococcus sternbergii* C. Agardh (= *Prionitis sternbergii* (C. Agardh) P.W. Gabrielson), a specimen of unknown provenance that neither of the two arguably most pre-eminent 20th century phycologists, Harald Kylin and E. Yale Dawson, were able to correctly identify based on morphology. Yet, DNA sequence data unambiguously resulted in the designation of this type material as the earliest available name for a species previously known as *Prionitis lyallii* Harvey. These data strongly suggest that had epitypes been selected and DNA barcodes published representing "types," many of the sequences would have been done in error. Epitypification for the purpose of barcoding should be a last resort, not an acceptable alternative, and needs to be done with careful attention to the historical record. Epitypes should be, at the very least, topotype material (Gabrielson et al. 2011), not a specimen from another locality that appears morphologically similar to the type material, nor should epitypes be material for which preservation status is unknown, e.g., many collections from the 1940s through the early 1990s were preserved in formalin, making the extracted DNA very challenging, if not impossible, to amplify.

Sequencing type material is not a trivial matter. Herbaria are reluctant, at best, and many remain hostile to researchers wanting to sequence type material. Hughey's original extraction method (Hughey et al. 2001) was designed to use small amounts of material (~5 mm^2 or less, see figure 4 of Gabrielson 2008*b*), similar to that typically required for anatomical examination. However, in many cases, anatomical sections are insufficient to objectively assign a specimen to species. At present, the majority of cookbook methods that are employed such as the Hughey et al. (2001) and Saunders (1993, 2008) methods, as well as the standard commercial DNA extraction kits, use procedures that require even less material than is needed to obtain good anatomical cross sections. Thus, for morphologically plastic species with relatively few anatomical characters to reliably segregate them and specimens that frequently are not reproductive (this describes many red algal type specimens), sequencing type material is the only method that can unequivocally link a name to a type specimen (Hughey et al. 2001; Gabrielson 2008*a*).

We agree with many of the recommendations of Saunders and McDevit (2012) for working with archival DNA, because they were taken from scientists who work in the field of ancient DNA (a discipline that operates under very strict authentication criteria). In their conclusions, Saunders and McDevit (2012) cite the pivotal note by Cooper and Poinar (2000). This paper was later elaborated on by Poinar (2003) to include 10 authentication standards, some of which are relevant to herbarium specimens (physically isolated work area, PCR control amplifications, test the molecular behavior, reproducibility, independent replication, phylogenetic sense). We feel obliged, having successfully validated our results with *S. pertusa*, as well as with many other red algal specimens, to address the closing of this comment to those researchers interested in considering a study that incorporates DNA data from older algal herbarium specimens. We propose the following, which incorporates many of the guidelines originally proposed by Cooper and Poinar (2000).

1. Know your taxa and their historical records. It is essential that all of the relevant species that might be confused with the target species in a given geographic locality (and sometimes beyond) be sequenced based on contemporary material and then all relevant type specimens be sequenced and their historical records researched. It is in this context that sequenced type material must be understood.
2. Demonstrate competency in analyzing DNA from herbarium specimens. Before researchers request type material from herbaria, they should document and be able to demonstrate to curators that they are able to isolate and amplify DNA from non-type herbarium specimens of similar or older age than the material being requested. Only then should type material be requested and loaned. Herbarium curators need to ask researchers if they have fulfilled this criterion, and if they have, then material should be loaned.
3. Perform the genetic analysis in a laboratory that has not processed specimens from the taxa under study. If this is not a possibility, and modern DNA already exists for taxa for which the proposed work is to be undertaken, a feasibility study should be performed to determine the level of contamination. This can be accomplished by performing four–six negative control extractions, as recommended by Saunders and McDevit (2012), and attempting amplifications using the primers planned for the herbarium specimen study.
4. Use equipment that is dedicated to pre- and post-PCR analysis. The use of dedicated centrifuges and pipettes can prevent cross and carryover contamination.
5. Use a commercial DNA extraction kit. It is unlikely that DNA from seaweeds will be present in any of these products.

6. Use newly purchased, unopened consumables and reagents. The use of barrier tips and new reagents such as *Taq* polymerase, primers, and water can prevent contamination issues.
7. Perform multiple negative controls when analyzing herbarium specimens. This is a necessary precaution to monitor for contamination.
8. Use Q-solution when performing PCR reactions. As stated by Hughey et al. (2001) and demonstrated by Gabrielson above, Q-solution improves amplification success (Henke et al. 1997).
9. Target small (100 bp), medium (200 bp), and large (400 bp) fragments when performing amplifications.

In conclusion, the isolation and amplification of authenthic, endogenous red algal DNA from type specimens is a practical, necessary, and reproducible technique that allows taxonomists to unequivocally match modern specimens to archival material with a high degree of success. Although there are numerous pitfalls, most notably contamination and DNA degradation, type specimens contain sufficiently intact chloroplast, mitochondrion, and nuclear genetic material that can be analyzed. Every effort should be made to determine the precise application of a name through a thorough analysis (morphological or preferably genetic) of the type material prior to any proposal for epitypification.

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