POINT OF VIEW

Conservation of microalgal type material: Approaches needed for 21st century science

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Abstract The current edition of the *International code of botanical nomenclature* (*Vienna Code*) accepts cultures of fungi and algae as types provided that they are preserved in a metabolically inactive state. In this article the authors outline a procedure for cryopreserving type cultures at ultra-low temperatures (<–135°C), thus ensuring that the material is held in a stable, metabolically inactive state. This procedure would allow direct access to live ex-type cultures, facilitating traditional research as well as molecular, metabolomic and proteomic studies.

Keywords cryobanking; cryopreservation; epitype; holotype; microalgae

INTRODUCTION

The type method was incorporated in the *International code of botanical nomenclature* by the Fifth International Botanical Congress meeting in Cambridge in 1930. Previously, the application of names was determined by circumscriptions. Anchoring a name to a type was intended to increase stability of nomenclature. Although the *Code* is basically designed to accommodate macroscopic plants, the need for special treatment of microscopic algae and fungi was recognized at the time that the type method was adopted. Article 18 of the *Cambridge Code* (Briquet, 1935) states in part "where permanent preservation of a specimen or preparation is impossible, the application of the name of a species or subdivision of a species is determined by means of the original description or figure." Essentially the same provision is found in all subsequent editions of the *Code* until the *Tokyo Code* (Greuter & al., 1994), which disallowed the use of a description as a type (Art. 8.3). The proscription of designating a living culture as a type first appeared as Art. 9, Note 3, in the *Montreal Code* (Lanjouw & al., 1961), a provision that notably included "Bacteria and Fungi" as exceptions. The exception for fungi was deleted in the *Seattle Code* (Stafleu & al., 1972) and the exception for bacteria was deleted in the *Leningrad Code* (Stafleu & al., 1978), by which time a separate bacteriological code had become well established (Lapage & al., 1976). An ambiguous reversal appeared in the *Tokyo Code* (Greuter & al., 1994), in which the proscription against living cultures was maintained verbatim (Art. 8.2) while an example was given of the name of a fungus for which a culture is "acceptable as a nomenclatural type as it is permanently preserved in a metabolically inactive state by lyophilization". The example was retained in the *St. Louis Code* (Greuter & al., 2000) while the exemption of fungi and algae from the proscription of type cultures was incorporated in the text of Art. 8.4 in the following words: "However, cultures of fungi and algae, if preserved in a metabolically inactive state (e.g., by lyophilization or deep-freezing), are acceptable as types." This provision remains unchanged in the current *Vienna Code* (McNeill & al., 2006).

For names of species or infraspecific taxa of microalgae published both before and after the adoption of the type method in botanical nomenclature, the type specimen or type collection is often a smear on a piece of paper or a liquid-preserved sample in a vial (usually dried up). The description and illustrations in the protologue provide the main basis for determining the application of a name. Nineteenth-century descriptions and illustrations are devoid of the critical analysis made possible by modern technology. The application of many such names merely follows tradition. Difficulties in linking names with taxa are discussed by Silva & Starr (1953). Taxonomists dealing with microalgae that produce durable structures have a clear advantage. In the case of diatoms, one or more frustules embedded in a mounting medium on a glass slide or piece of mica provides the information essential to anchoring a name to an organism. Whereas diatom taxonomy has traditionally been based on quantifiable characters exhibited by the siliceous frustule, the taxonomy of those microalgae that lack durable structures is ill-served by dead material, which rarely reveals ontogenetic or reproductive details. Students of microalgae have *de facto* relied on "type cultures" i.e., cultures derived from the original field-sample, ever since the methodology for isolation and perpetuation of strains was developed. Historically, uni-algal cultures derived from these original samples have been maintained as "authentic strains" by the culture collections ever since the methodology for isolation and perpetuation of strains was developed. Cultures are essential for molecular, metabolomic and proteomic studies and greatly facilitate traditional research.

The *Code* does not require microalgal type cultures to be deposited in a publicly accessible Biological Resource Centre (BRC). The scientific community can access live authentic strains (cultures derived from type material) that are lodged in one or more of the major culture collections, but without a guarantee that representatives of a given strain in all service centers are identical. In most cases the cultures have been maintained for prolonged periods by serial transfer. But only in very few cases has the constancy of a strain been tested. Müller & al. (2005) concluded that no genomic differences could be detected between duplicate strains of the same isolate (type strain of *Chlorella vulgaris* Beij.) that were maintained by continuous subculturing over many decades, or within those stored at ultra-low temperatures. This article aims to suggest a practical procedure that would allow the scientific community direct access to type material.

■ PROCEDURE TO CONSERVE MICROALGAL TYPE SPECIMENS

It is envisaged that the major algal culture collections affiliated to the World Federation for Culture Collections (http://www.wfcc.info/index.html), including the Culture Collection of Algae and Protozoa (CCAP) and Sammlung von Algenkulturen Göttingen (SAG), would have a major role to play in any scheme to cryopreserve type cultures. They have a long-term commitment for the *ex-situ* conservation of biodiversity and supply of cultures and their associated data to the scientific community. These cultures act as the basis of numerous scientific publications, for example in a recent issue of the *Journal of Phycology* (volume 45, issue 2) ten out of thirteen microalgal papers (77%) employed cultures from one, or more, of the major service collections. In addition to their curatorial and service roles, all the major collections have access to cryopreservation facilities and a suggested procedure of how these could be employed to conserve type material is outlined below.

Taxonomists would provide a holotype culture (The specimen/culture designated by the author as the nomenclatural type) to one of the service collections that have committed to participate in this procedure. In addition, subject to appropriate agreements and the involvement of appropriate taxonomic experts, existing authentic strains, or other cultures, could be re-designated as epitype cultures (specimen selected to serve as an interpretative type when the holotype, or previously designated neotype, or all original material associated with a validly published name cannot be identified for the purpose of precise application of the name of a taxon) [for recent examples involving clarification of, or establishment of a new, epitype see Pröschold & Silva (2007) and Jahn & al. (2008)]. These type cultures would then be cryopreserved employing standard procedures: (1) controlled rate 2-step cooling, employing a colligative cryoprotectant such as methanol, DMSO, or glycerol at 5%–10%; cooling using either a Controlled Rate Freezer (CRF); or a passive cooler, e.g., Mr Frosty (Day & Brand, 2005; Day, 2007); or (2) employing an encapsulation dehydration vitrification (EDV) based approach (Harding & al., 2008; Lukešová & al., 2008).

In all cases procedures involve the cryopreservation of a single batch of cryovials, with the algal suspension taken directly from the holotype, or epitype, culture. The number of vials will depend on the capacity of the cooling system, for a CRF 50–100 and for the use of a passive cooler, e.g., Mr Frosty™, 3× Mr Frosty™ units would be employed, i.e., 54 vials would be cryopreserved in a single batch. For EDV-cooled samples batch sizes of 50–100 should be employed. The batch of cryovials is subsequently designated as either holotype (for new species that was cryopreserved on receipt), neotype (selected to serve as nomenclatural type for an existing species where the specimen or illustration no longer exists/ has been lost), or epitype, cultures depending on the status of the original culture (Fig. 1). Storage should be in either vapour, or liquid, phase liquid nitrogen, or in an equivalent ultra-cold electric freezer, with a maximum storage temperature –140°C (Grout, 1995). Cryovials from any individual batch should be held in more than one cryostat/ refrigerator and ideally on more than one site to minimise the possibility of catastrophic loss of the sample (see also below).

It is suggested that individual cryovials of holotype samples could be thawed as described previously (Day & Brand, 2005; Day, 2007), cultures re-grown under standard conditions (Lorenz & al., 2005). These would be designated as ex-holotype/ex-epitype cultures for distribution to taxonomists, or other interested scientists. In addition, if cryopreserved stocks were low the culture could be re-cryopreserved to replenish stocks. Cultures would be distributed with a detailed Material Transfer Agreement (MTA) and could not after subsequent transfers be considered to be holotypic or epitypic material, as it is no longer held in a metabolically inactive state.

Quality control (QC). — Viability assessment is a key component of the procedure. There is no evidence for genotypic change, or active selection of a cryo-tolerant sub-population in algae (Müller & al., 2005, 2007). However, problems have been noted when non-clonal fungal cultures were cryopreserved (Pearson & al., 1990), or sub-optimal preservation

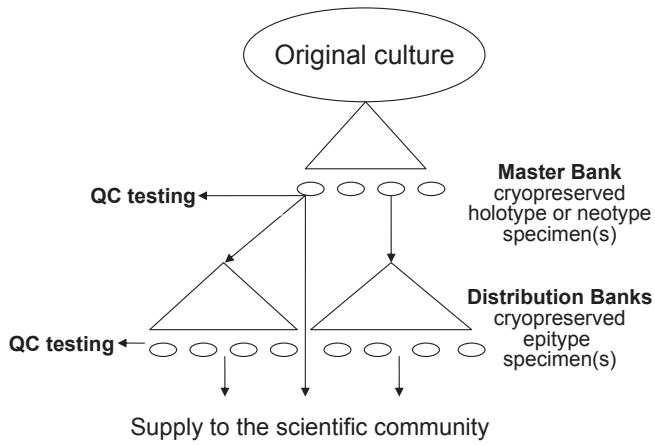

Fig. 1. Schematic of conservation and supply of cryopreserved type specimens.

protocols were used (Ryan & al., 2003). Viability assessment must employ a method that allows the detection of metabolic activity, and/or the capacity to reproduce, of the cells that have undergone the cryopreservation process. Vital staining employing fluorescene diacetate, or re-growth of cells are the most practicable approaches (Fleck & al., 2006; Day & al., 2007). It is advised that there should be a minimum acceptable viability level (~30%); therefore, taxa with lower post-thaw viability levels should only be maintained as ex-type cultures, or authentic strains. In addition, it is important that any methodology should be "robust" and that where both holotype and epitype cultures are held in a cryopreserved state that viability levels of both batches should be comparable (< 20% difference in viability). In addition, where practicable whole-genome fingerprinting (e.g., AFLP; Müller & al., 2005) methods should be applied, prior to and after cryopreservation. There should be no significant differences in genotype, or phenotype between control and post-thaw cultures (Fig. 2). Realistically this is currently problematic to achieve, may require further development for some taxa, and is not technically feasible for non-axenic material. The above is a long-term objective, which as routine genetic barcoding and/or whole-genome genotyping methods are developed will become a practicable option. We feel in the future it will be possible to perform stability assessments on most, if not all, type strains.

Conservation and supply. — Cryopreserved samples should be held in publicly accessible service culture collections. Material should be held in at least two refrigerators (cryostats) at any site and if possible at more than one service collection. Technically this is relatively easy to manage within Europe, or North America where liquid nitrogen–charged dry-shipper will allow samples to be exchanged without compromising the storage temperature (Bielanski, 2005). Holotype or epitype samples should be distributed to the research community and could, if requested, be supplied in a cryopreserved state. Because of their scientific value and relatively restricted number, holotype samples should be supplied at the discretion of the collections' curator to be employed in significant/major taxonomic studies. However, because of the possibility to regenerate additional epitype samples (Fig. 1), these could be distributed without restriction. As at present, there would be no constraints on the supply of ex-type authentic strains.

Additional considerations. — Many microalgae are recalcitrant to current cryopreservation methodologies, so it will not be possible to cryopreserve either holotype, or epitype, samples. For these organisms we suggest that a DNA extraction and storage procedure is undertaken and then, when appropriate cryopreservation methodologies are subsequently developed, authentic strains could be re-designated as epitype material, cryopreserved and as an additional QC step the DNA compared to the original extracted, banked DNA. DNA can be extracted using standard procedures and batches (50–100) dried, these can then be held at indefinitely at –80°C, or even room temperature (Duncan & al., 2003). As with cryopreserved material, samples should be held in more than one refrigerator, ideally at more than one site. Supply of samples should be on the same basis as live whole cell samples.

All of the above have cost implications, not least to the service collections who would commit to cryopreserving holotype specimens, supplying ex-type, or epitype, specimens to the scientific community as well as maintaining/curating and enhancing the bioinformatics data associated with these specimens. The authors suggest that, with the potential exception of commercial organisations, the depositors should not pay for this service. Globally most countries are signatories to the Convention on Biological Diversity (CBD) (http://www.cbd.int/) and as such are committed to the *ex-situ* conservation of biodiversity. We suggest that appropriate local governmental, regional (e.g., European Union) and international (e.g., UNESCO) funding should be sought to facilitate the cryopreservation of type material and ensure its long-term curation.

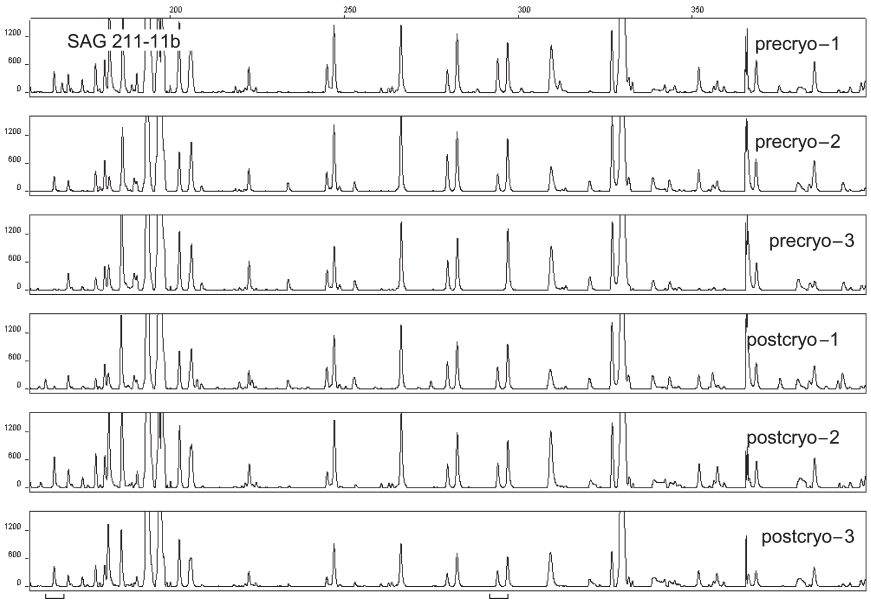

Fig. 2. Quality control: AFLP pattern of *Chlorella vulgaris* Beij. SAG 211-11b prior to (precryo 1–3) and after (postcryo 1–3) cryopreservation. AFLP electropherograms of SAG 211-11b demonstrating identical AFLP patterns before and after cryopreservation. Primer combination EcoRI+C/MseI +C. Vertical scales, relative fluorescent units; horizontal scales, size of fragment in nucleotides. From Müller (2005); for full methodology see Müller & al. (2005).

■ ACKNOWLEDGEMENTS

JGD and TP acknowledge funding from NERC Oceans 2025 (NF3–CCAP).

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