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Genetic analysis of the Linnaean *Ulva lactuca* (Ulvales, Chlorophyta) holotype and related type specimens reveals name misapplications, unexpected origins, and new synonymies

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Running title: Genetic analysis of Linnaean *Ulva*

Abstract

Current usage of the name *Ulva lactuca*, the generitype of *Ulva*, remains uncertain. Genetic analyses were performed on the *U. lactuca* Linnaean holotype, the *U. fasciata* epitype, the *U. fenestrata* holotype, the *U. lobata* lectotype, and the *U. stipitata* lectotype. The *U. lactuca* holotype is nearly identical in *rbc*L sequence to the *U. fasciata* epitype, a warm temperate to tropical species, rather than the cold temperate species to which the name *U. lactuca* has generally been applied. We hypothesize that the holotype specimen of *U. lactuca* came from the Indo-Pacific rather than northern Europe. Our analyses indicate that *U. fasciata* and *U. lobata* are heterotypic synonyms of *U. lactuca*. *Ulva fenestrata* is the earliest name for northern hemisphere, cold temperate Atlantic and Pacific species, with *U. stipitata* a junior synonym. DNA sequences from type specimens provide an unequivocal method for applying names to *Ulva* species.

Key index words: biogeography, *rbc*L, *Ulva fasciata, Ulva fenestrata*, *Ulva lobata*, *Ulva stipitata*

Abbreviations: *tuf*A, Elongation factor Tu

Carl Linnaeus (1707-1778) was the founder of modern taxonomic nomenclature. His most significant achievement was the universal system he proposed that designated every living plant and animal with a binomial Latin name, the genus and species (Stearn 1957, Jarvis 2007). The oldest legitimate plant names are those in *Species Plantarum* (Linnaeus 1753), with some 5,900 species, including the names of all major crop plants and many ornamental and medicinal plants (Jarvis 2007). Among these is the genus *Ulva*; the generitype species, *U. lactuca* (sea lettuce), is the most extensively studied and broadly distributed species of *Ulva* in the world (Guiry and Guiry 2018). For two and a half centuries, its reported distribution, based on the morpho-anatomical characters of thallus shape, cell number and shape, and pyrenoid number (Setchell and Gardner 1920) has expanded globally. However, identification is problematic because species of *Ulva* show a high degree of plasticity in these characters (Gayral 1959).

The first author to question the identification of the type specimen of *Ulva lactuca* was Papenfuss (1960). He published Yvonne Chamberlain's observations of the holotype specimen (LINN 1275-24 specimen #5; Fig. S1 in the Supporting Information) after re-suspension in water, writing "many fronds radiating from a central part ... the edge of the lower frond was dentate with quite large teeth. It was fairly narrow, sometimes had a darkish stripe up the middle...". Papenfuss concluded that the holotype was not typical of *U. lactuca* from Europe. Bliding (1968) disagreed, stating that it was probable that *U. lactuca* came from the Swedish west coast. The identity of *U. lactuca* became more perplexing in the age of molecular phylogenetics. Analyses using contemporary specimens collected from around the world showed that '*Ulva lactuca'* was polyphyletic, with specimens segregating into several distinct clades (Tan et al. 1999, Hayden and Waaland 2002, Shimada et al. 2003, Hayden and Waaland 2004). Currently, the genetic identity and origin of the holotype of *U. lactuca* remain unknown. Names placed into synonymy under *U. lactuca* and the nomenclatural identities of the distinct '*U. lactuca*' genetic clades require investigation.

Genetic analyses were carried out on: the holotype of *U. lactuca* (Fig. S1); the epitype of *U. fasciata* (UC 2050475; Fig. S2 in the Supporting Information) that supports the lectotype (designated herein: PC 0119343, Delile 1813: 297 [type locality: New Port, Alexandria, Egypt]; Fig. S3 in the Supporting Information); the holotype of *U. fenestrata* (Postels and Ruprecht 1840: 21, type locality: Kamchatka, Russia; Fig. S4 in the Supporting Information); the lectotype of *U. lobata* (= *Phycoseris lobata* Kützing 1847: 54, type locality: Chile; Fig. S5 in the Supporting Information); and the lectotype of *U. stipitata* Areschoug 1850: 411 (type locality: Bohuslän, Sweden, designated herein: Areschoug's Algae Scandinavicae exsiccatae quas adjectis Characeis distribuit BM013734109; Fig. S6 in the Supporting Information) to determine their taxonomic relationships. DNA from the *U. lactuca* holotype was extracted at the Royal Botanic Gardens, Kew using a CTAB protocol modified from Doyle and Doyle (1987). Amplifications and sequencing of short *rbc*L gene amplicons were performed at Queen's University Belfast using primers targeting four short segments of the *rbc*L gene (Appendix S1 in the Supporting Information), each approximately 100 bp. The *U. stipitata* lectotype and DNA from contemporary specimens called *U. lactuca* and *U. fasciata* were isolated using the DNeasy Plant Mini Kit following the manufacturer's instructions (Qiagen Ltd., Crawley, UK), and the PCR methods published by Shimada et al. (2003). DNA from the *U. fenestrata* holotype, the *U. lobata* lectotype and the *U. fasciata* epitype were extracted at Hartnell College from fragments approximately 5 x 5 mm in size following Lindstrom et al. (2011) and Hughey and Gabrielson (2012), except that the binding step was centrifuged at 4,000*g* for 3 min and the DNA was eluted after incubation for 7 min in 40 µL TAE. The paired-end library construction and sequencing were performed by myGenomics, LLC (Alpharetta, GA, USA). Organellar genomes of *U. lobata* were assembled using de novo and mapping methods, the default de novo settings in CLC Genomics Workbench 8.5 (®2016 CLC bio, a QIAGEN Company, Waltham, MA, USA), and MEGAHIT (Li et al. 2015), and by mapping contigs and reads against *Ulva* spp. published in GenBank with Geneious R8 (Biomatters Limited, Auckland, New Zealand). Genomes were annotated manually with Blastx and NCBI ORFfinder. Representative Ulvaceae *rbc*L gene sequences were downloaded from GenBank (Table S1 in the Supporting Information) and aligned, analyzed, and visualized using previous methods described in Gabrielson et al. 2018 (Appendix S1).

*rbc*L gene sequences from the *U. lactuca* holotype and the *U. stipitata* lectotype each aligned to 435 bp. High-throughput sequencing of the *U. fenestrata* holotype yielded complete *rbc*L and *tuf*A sequences and the complete plastid genome and nearly complete mitogenome for the *U. lobata* lectotype (Appendix S1). Maximum likelihood and Bayesian analyses of the four type *rbc*L gene sequences with *Ulva* spp. from around the world resolved the *U. lactuca* holotype in a well-supported

clade with the *U. lobata* lectotype and with predominantly subtropical sequences generated from the epitype and other specimens identified as *U. fasciata* from around the world (Fig. 1). The *U. lactuca* holotype sequence differed by 1 bp from two other sequences in the phylogram, one from New Caledonia and the other from Sri Lanka. *rbc*L sequences of the *U. fenestrata* holotype and *U. stipitata* lectotype were identical and positioned in a fully-supported clade with specimens from the north Pacific Ocean previously identified as *U. lactuca* and *U. fenestrata*, and with specimens from the north Atlantic Ocean previously identified as typical European *U. lactuca*. *rbc*L sequences from GenBank attributed to '*U. lobata'* shared a clade with the *U. expansa* holotype described from Monterey, California, USA.

Linnaeus's (1753) description of *U. lactuca* referred to earlier accounts by British and continental European authors, implying a European distribution. However, the geographical information Linnaeus originally provided, "in Oceano", suggested a much wider distribution when compared, for example, with "in Mari Europae" in the account of *U. latissima*. Linnaeus (1755) noted that *U. lactuca* was common on the western coast of Sweden. Botanists subsequently assumed that Sweden was the probable origin of Linnaeus' species concept and type specimen, and the name *U. lactuca* became associated with the commonest northern European sea lettuce species. Our *rbc*L gene sequences show that the *U. lactuca* holotype is the species currently called *U. fasciata* in the subtropics and *U. lobata* in the eastern Pacific Ocean (Fig. 1). We hypothesize that the holotype specimen was collected in the Indo-Pacific by one of Linnaeus's many colleagues and students who participated in early voyages of exploration and scientific discovery (see Jackson 1912 for a list). The current distribution of *U. lactuca*, confirmed by DNA sequences (Fig. 2; Table S2 in the Supporting Information), is the eastern (Australia) and northern (India) Indian Ocean, central (Hawai'i, USA) and temperate southeast (Chile, Peru), southwest (Australia), and northwest (South Korea, Japan) Pacific Ocean, warm temperate eastern (Azores) and western (Florida, USA) Atlantic Ocean and the eastern (Egypt, Israel) and western (Italy) Mediterranean Sea. The two sites in Italy may represent recent introductions as both have anthropogenic impacts, and *U. lactuca* has not been found elsewhere in the western Mediterranean (Miladi et al. 2018).

These data show that the oldest available name for the European '*Ulva lactuca*' is *U. fenestrata* and that *U. stipitata* is a heterotypic synonym. GenBank accessions reported as '*Ulva lactuca*' from the northeastern (Loughnane et al. 2008) and northwestern (Guidone et al. 2013) Atlantic Ocean, northeastern (Hayden and Waaland 2004, Saunders and Kucera 2010), and northwestern (Shimada et al. 2003) Pacific Ocean are *U. fenestrata*; it is likely that this species was introduced to New Zealand. Specimens reported as '*U. lobata*' from the northeast Pacific (Hayden and Waaland 2002, 2004, Saunders and Kucera 2010) are *U. expansa* (Hughey et al. 2018). Based on *rbc*L and *tuf*A sequences, the current distribution of *U. expansa* is Vancouver Island, British Columbia, Canada, to San Simeon, California, USA. The correct name for specimens from other parts of the world that have been identified as '*U. lobata*' using morpho-anatomy cannot be determined without sequence data.

This investigation contributes to the short list of studies analyzing DNA from type material of species of *Ulva* (Mares et al. 2011, Hanyuda and Kawai 2018, Hughey et al. 2018, Suzuki et al. 2018). These results show that type specimens of *Ulva* contain valuable genetic information that can be used to address taxonomic, phylogenetic, biogeographic and genomic questions. Although destructive sampling of herbarium specimens, and in particular type specimens, is prohibited by some curators, our results justify this approach, demonstrating that the very small amounts of material required for sequencing can definitively resolve species problems that have confounded phycologists for decades to centuries (Hughey et al. 2014, Gabrielson et al. 2018). Finally, we recommend caution when

comparing sequences from field-collected specimens with those in GenBank as a tool for identification, since many of the names applied to GenBank accessions have not been confirmed by sequences from type material.

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FIG. 1. RaxML phylogram of *rbc*L sequences showing evolutionary relationships of type materials of *Ulva lactuca*, *Ulva fasciata*, *Ulva fenestrata*, *Ulva lobata* and *Ulva stipitata*. Bootstrap support based on 1,000 replicates/Bayesian posterior probabilities cited at nodes. * represents bootstrap support of 100% or posterior probability of 1.0. -- indicates less than 80% bootstrap support or less than 0.80 posterior probability.

FIG. 2. Current distributions of *Ulva lactuca* and *Ulva fenestrata* based on *rbc*L and *tuf*A DNA sequences. Refer to Table S2 for code numbers, voucher, collection, GenBank information, and publication sources for specimens used to construct this figure.

TABLE S1. Information for GenBank sequences used in the *rbc*L phylogenetic analysis of *Ulva* species.

TABLE S2. Voucher, collection, GenBank information, and publication source for specimens used to construct the *Ulva lactuca* and *Ulva fenestrata* distributional map depicted in Figure 2. Blue colored font corresponds to *tuf*A sequences.

FIG. S1. Holotype specimen of *Ulva lactuca* (LINN 1275-24, #5) from which portions of the *rbc*L marker were sequenced.

FIG. S2. Designated epitype (UC 2050475) collected at the type locality (New Port, Alexandria, Egypt) of *Ulva fasciata*. See Table S1 for collection details. The epitype supports the lectotype (PC 0119343) of *Ulva fasciata* (see Fig. S3). The embargo on loans from PC necessitated the designation of an epitype. In the future, we hope that DNA sequences can be obtained from the lectotype specimen that obviate the need for an epitype.

FIG. S3. A. Image of *Ulva fasciata* from Delile (1826, Plate 58, fig. 5). B. Designated lectotype is right hand specimen on sheet PC 0119343 that best matches the Delile (1826) illustration. Specimens on sheet were collected at New Port, Alexandria, Egypt, the type locality. Due to the embargo on loans from PC, we were unable to obtain a fragment from the lectotype specimen for genetic analysis.

FIG. S4. Photograph of *Ulva fenestrata* holotype specimen and its label in LE along with a fragment from the holotype (UBC A57002) sent to C. Tanner from K. L. Vinogradova in 1976. A fragment about 5 mm long from the far right of the holotype fragment was removed for DNA extraction, amplification and sequencing.

FIG. S5. Lectotype (L 0054996) of *Phycoseris lobata* Kützing, basionym of *Ulva lobata* (Kützing) Harvey.

FIG. S6. Lectotype (BM 013734109) of *Ulva stipitata*, #224 in Areschoug's (1864) Algae Scandinavicae exsiccatae quas adjectis Characeis distribuit.

APPENDIX S1. PCR and sequencing primers and the phylogenetic analysis.