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A molecular survey of Ulva (Chlorophyta) in temperate Australia reveals enhanced levels of cosmopolitanism

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Keywords
Macroalgae;DNA barcoding;NIS ;tufA ;rbcL

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A molecular survey of *Ulva* (Chlorophyta) in temperate Australia reveals enhanced levels of cosmopolitanism[1]

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Running title: MOLECULAR SURVEY OF *ULVA* IN AUSTRALIA

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Abstract

The green algal genus *Ulva* includes a speciose group of marine macroalgae inhabiting shallow seas worldwide. Although algal blooms in Asia highlight the opportunistic nature of several 'nuisance' species, recent research clearly reveals important positive benefits of *Ulva*. Applied research requires accurate, reliable and rapid identification, however, identification of *Ulva* spp. has met with considerable difficulty. Consequently, many have turned to molecular markers to aid in taxonomy. Previous studies of plants and algae have relied heavily on ITS and *rbc*L. Recently, *tufA* has been presented as a suitable barcoding gene to facilitate species-level identification of green macroalgae and it is used here to explore the diversity of *Ulva* spp. in temperate Australia. Ninety *Ulva* specimens collected from 38 sites across five states were sequenced for this gene region with exemplars from each genetic group also sequenced for *rbc*L to test for congruence. Collections of Australian *Ulva* spp. were compared to samples from Asia and North America and exhibited trends consistent with recent studies in terms of species relationships. Results support an overwhelmingly cosmopolitan flora in temperate Australia that contrasts with other Australasian surveys of *Ulva* that report a greater number of endemics and new species. Four new records, as well as numerous range extensions for taxa already known from the country, are documented. Evidence for three non-indigenous *Ulva* species in temperate Australia is discussed.

Keywords: macroalgae, DNA barcoding, NIS, *tufA*, *rbc*L

Abbreviations: *rbc*L, large subunit of the RUBSICO gene; ITS, internal transcribed spacer; *tufA*, elongation factor *tufA*; ML, maximum likelihood; LRT (aLRT), (approximate) likelihood ratio test; SH-like, Shimodaira-Hasegawa-like; GTR, general-time reversible; BOLD, Barcode of Life Database; NIS,

non-indigenous species.

Introduction:

Marine species of the green algal genus *Ulva* Linnaeus in the division Chlorophyta are often conspicuous and locally dominant members of rocky intertidal and subtidal habitats worldwide. They have gained negative press owing to blooms in Asia (Hiraoka et al. 2004, Leliaert et al. 2009, Kong et al. 2011), but review of recent publications also points to the diverse range of positive applications. For example, *Ulva* spp. are regarded as key candidates for bioremediation applications (El-Sikaily et al. 2007), as keystone species in the emerging field of chemical ecology (Van Alstyne et al. 2007, Alstyne 2008), as important primary trophic producers (Horn 1983) and bio-indicators that respond to eutrophication pollution in the coastal zone (Kozhenkova et al. 2006). The bulk of these studies address chemical analysis or functional properties of macroalgae at the generic level, or without robust taxonomic identification. The potential problem with such an approach is highlighted by emerging research that has revealed that even closely related *Ulva* species exhibit unique ecophysiological and chemical characteristics (Eswaran et al. 2002, Michael 2009, Paulert et al. 2010, Winberg et al. 2011). Key attributes may not be similar (or even present) among morphologically similar or closely related species, may vary seasonally or across environmental gradients and differ in controlled laboratory settings versus in the wild. This presents implications for both the applied use of *Ulva* spp. for specific metabolites and in achieving an understanding of ecological processes. A best practice approach should strive to marry traits such as chemical constituents, physiological requirements and remediation potential directly with tested individuals of a confirmed strain.

Taxonomy is fundamental to all biological research, however, it is challenging and time-consuming work, especially for difficult taxa such as those in the genus *Ulva*. Previously, *Enteromorpha* (monostromatic

tubes) and *Ulva* (distromatic blades) were recognized as separate genera based on morphological characters, however, molecular evidence has not supported this distinction (Hayden et al. 2003). No longer recognized as reliable synapomorphies, a potential morphogenetic switch may explain shifts between these two states (Tan et al. 1999). The challenges of ecophenotypic or developmentally mediated variation at higher taxonomic levels also confound straightforward identification at lower taxonomic levels. For example, the reportedly cosmopolitan taxon *U. lactuca* L. is included as a species in approximately 30% of the peer-reviewed literature on *Ulva* internationally (1499 of 4854 studies searched in Scopus) and across Australia (235 of 682 studies). However, the validity of such a broad application of the name *U. lactuca* is questioned by several recent taxonomic studies. Heesch et al. (2009) only recovered *U. lactuca* infrequently in New Zealand from disturbed habitats, while Kraft et al. (2010) did not recover *U. lactuca* from extensive survey work in southern Australia. O'Kelly et al.'s (2010) newly sampled Hawaiian sequences provisionally identified as *U. lactuca* do not cluster with sequence data in Genbank for individuals identified as *U. lactuca* from Europe. Considering some of the key applications of *U. lactuca* in applied research (Hassan et al. 2011), not least of which are trigger indicators for environmental pollution (El-Sikaily et al. 2007), taxonomic clarification of this and other species in the genus *Ulva* is important.

Molecular tools have proven useful at assisting in the identification process across the diversity of life (Savolainen et al. 2005) with ITS, *rbc*L and *mat*K markers commonly used to characterize relationships among flowering plants (Chase et al. 2005). For the purpose of identifying marine macroalgae, the plastid marker *rbc*L and the nuclear ITS region have proven useful (Coat et al. 1998, Malta et al. 1999, Hayden & Waaland 2004, Loughnane et al. 2008, O'Kelly et al. 2010) with studies from Australasia reflecting this trend in marker choice (Woolcott & King 1999, Shimada et al. 2003, Shimada et al. 2008, Heesch et al. 2009, Kraft et al. 2010). However, these markers are commonly hindered by poor amplification success and double bands indicative of divergent copies for the ITS region (Saunders & Kucera 2010)

while low levels of genetic diversity for *rbc*L have hampered species-level resolution amongst New Zealand Ulvaceae (Heesch et al. 2009). Near identical *rbc*L sequences in GenBank bearing a number of different species names, as well as distinct sequences often sharing the same name, only add to the confusion. Recent strides in marker development have, however, provided new options for rapid, and reliable species-level identifications. Targeted gene regions, often referred to as barcodes, are typically short (<800 bp) segments of protein-encoding organellar DNA. The COI-5P DNA barcode marker, for example, has been used to facilitate difficult taxonomic identification (Saunders 2005, McDevit & Saunders 2009), complete rapid floral surveys at multiple spatial scales (Sherwood et al. 2008), recognize cryptic species (Saunders & Kucera 2010) and uncover invasive species (Sherwood et al. 2008, Armstrong & Ball 2005) for red, Rhodophyta (Saunders 2005, Saunders 2009), and brown, Phaeophyceae (Kucera & Saunders 2008), macroalgae. The plastid marker *tufA* has been tested and proposed as a viable marker for species identification among Ulvaceae (Saunders & Kucera 2010) and is used here in combination with *rbc*L data to explore the diversity of *Ulva* spp. in Australia.

Difficulties with taxonomic identifications of the speciose genus *Ulva*, coupled with a paucity of recent marine macroalgal surveys, have resulted in a relatively weak state in our knowledge of both the diversity and distribution of this genus in Australasia. Rapid growth (Duan et al. 2011), good dispersal capabilities (Kong et al. 2011) and high fouling potential (Schaffelke et al. 2006) are all factors that have contributed to the notion that regional floras may be increasingly dominated by widespread or circumglobal *Ulva* spp. (Lopez et al. 2007), as opposed to endemics. However, Kraft et al. (2010) recently challenged the latter notion for *Ulva* spp. providing evidence that, although widespread species are certainly common components of temperate Australian macroalgal communities, an Australian signature is also apparent in that flora. This shifting balance between cosmopolitanism and endemism has been demonstrated for *Ulva* spp. in other remote Pacific locales as well as Hawaii (O'Kelly et al. 2010) and New Zealand (Heesch et al. 2009). Observations of highly cosmopolitan assemblages are often the first line of evidence for non-

indigenous species (NIS), with taxonomic and biogeographic research underpinning recognition of such taxa (Heesch et al. 2009). Given the high priority for biosecurity in Australia, coupled with the invasive track record of *Ulva*, such research is critical for identification and management of NIS species.

Considering the ecological, applied and biosecurity contexts and a need for certainty regarding *Ulva* taxonomy, the focus of this study was threefold: 1) utilize the *tufA* barcoding marker to determine how many genetic species groups occur for the genus *Ulva* in the temperate Australian flora*;* 2) generate *rbc*L data for a representative of each of the previous genetic groups for comparison to the wealth of *rbc*L data in GenBank for *Ulva* spp. (both to apply species names consistently, and as a secondary check of the *tufA* results); and 3) examine the biogeographic context and potential implications of the taxonomic findings and conduct a preliminary assessment of the potential for cosmopolitan species to be non-indigenous components of the temperate Australian flora.

Materials and Methods:

Collection information

Ulva samples were collected across five temperate Australian states as well as South Korea (Table 1: Appendix 1, see supporting information; Fig. 1), as part of collaborative work between two institutions: the Shoalhaven Marine and Freshwater Centre (SMFC) at the University of Wollongong in New South Wales, Australia and the Centre for Environmental and Molecular Algal Research (CEMAR) in New Brunswick, Canada. Voucher material of each sample, specifically algal presses, tissue samples, genomic extractions and photographs detailing key morphological characters (refer to BOLD project GULVA for sample by sample details of voucher locality as well as other information: www.boldsystems.org) are housed at their respective institutions.

Genomic extraction and PCR

Samples processed at SMFC (fresh, ethanol-preserved, frozen or preserved with RNA-later, sample ID designation LAK in Appendix 1) were rinsed with autoclaved seawater, patted dry and scraped to remove epiphytes if present. Thalli were then cut using a sterile razor blade and 100-150 mg of tissue was weighed for extraction. DNA was extracted using a Plant/Seed DNA kit (ZR-96 Zymo Research) following the manufacturer's recommendations, with the addition of an extra 250-500 µL of lysis solution during tissue maceration. The presence of genomic DNA was confirmed via gel verification (1-2% agarose). Samples processed at CEMAR (sample ID designation GWS in Appendix 1) followed published protocols (Saunders & Kucera 2010).

The primers GtufAR 5'-CCTTCNCGAATMGCRAAWCGC-3' and tufGF4 5'-GGNGCNGCNCAAATGGAYGG-3' were used to amplify the *tufA* region (Saunders & Kucera, 2010). PCR cocktails included 2 µL of template, 5 µL of 10X buffer, 5 µL 10mM dNTPs, 2 µL of 10 mM solution of each primer, 0.2 µL TAQ, brought up to a total volume of 50 µL with sterile water (sH_2O). Reactions were run for 38 cycles with the following parameters: an initial 4 min denaturation at 94°C; further denaturation at 94°C for 1 min, annealing at 45°C for 30 s and extension at 72°C for 1 min, followed by final elongation at 72°C for 7 min (Saunders & Kucera, 2010). The PCR product was electrophoresed, stained, and photodocumented. Multiple PCR products indicated by double bands were subjected to increased annealing temperatures (50°C) during subsequent rounds. At SMFC successful PCR products were cleaned for cycle sequencing using QIAquick PCR Purification Kit (Qiagen) following manufacturer's recommendations and then visualized. The Australian Genome Research Facility (AGRF, Westmeade, Sydney)

generated sequences using an AB3730xl automatic sequencer. At CEMAR (Appendix 1) *tufA* data were generated as published (Saunders & Kucera, 2010). All newly generated *rbc*L-3P data (Appendix 1) were generated at CEMAR following published protocols (Saunders & Kucera 2010).

Alignment and Molecular analysis

The *tufA* and *rbc*L data were easy to align due to an absence of indels. Newly generated *tufA* sequences were added to previously published data (Saunders & Kucera 2010;Appendix 1) and included representative species of the genera *Ulva*, *Umbraulva* and *Ulvaria*. Newly generated *rbc*L data were similarly added to those of Saunders and Kucera (2010) with additional data from GenBank (see accession numbers included directly in Fig. 3). Both gene alignments were subjected to maximum likelihood (ML) analyses using the phylogeny.fr online software (Dereeper *et al.*, 2008) as implemented in PhyML v 3.0 aLRT (Guindon & Gascuel 2003) with a GTR + I + G model. Tree robustness was assessed using the bootstrap (100) or approximate LRT (aLRT) (SH-like) test (Anisimova & Gascuel 2006) and initial annotation and editing of ML trees was carried out in TreeDyn v. 198.3 (Chevenet et al. 2006).

Sequence identity

Both previously published (Saunders & Kucera 2010) and newly generated *rbc*L data (Appendix 1) were blasted in GenBank and subjected to comparative phylogenetic analysis of *rbc*L data from Kraft et al. (2010) to achieve consistent use of nomenclature (Table 1). Australian collections with *tufA* sequences

that fell into well-supported monophyletic groups with previously published North American taxa (Saunders & Kucera 2010) were assigned to that species (refer to Table 1 for rationale on species by species basis).

Genetic distances

Uncorrected p-distances were generated from the *tufA* alignment using Mesquite v 2.74 (build 550; Maddison & Maddison, 2010). Distances were exported to Microsoft Excel for Mac 2008 version 12.1.0 (Microsoft Corporation) to facilitate intra- and interspecific comparisons and estimate barcode gaps.

NIS criteria

A number of criteria specific to *Ulva* have been presented recently to assist in recognition of potential NIS (Heesch et al. 2009). Two key criteria for assessment are: 1) number of collections and type of sites where an entity was observed; and 2) the genetic distance among sequences from a test area compared with the genetic distance among sequences from overseas. These criteria were followed in this study and data were scored based on collection information for eleven species of *Ulva* that had distribution in Australia (www.boldsystems.org; Table 2). For the first criterion, evidence for an NIS species was suggested if a species was found in a disturbed site on artificial substrate in close proximity to a large port or harbour. For the second criterion, genetic diversity was expected to be lower in a population that had recently colonized an area compared with a population that had not recently colonized. Two pools of genetic distance data (Kimura 2-parameter) were generated and compared for the *tufA* gene region. This included one pool from the test area (within Australia) and one pool globally (within and outside Australia). The pool of genetic data that exhibited the lower diversity was inferred to represent evidence

of a recent colonization, suggestive of a NIS. Neighbor-joining trees were constructed from global pools of genetic distance data for each species to visualize trends. As available, *rbc*L data were also queried for diversity both within Australia and globally. Genetic analyses were carried out through the BOLD website (www.boldsystems.org). These two criteria were then considered together to assess NIS status.

Results:

Molecular analyses

One hundred and ten new *tufA* sequences were added to sixty-nine previously published records from North America of *Ulva*, *Ulvaria obscura* as an outgroup, as well as representatives from the genus *Umbraulva* (Appendix 1). The full dataset of 179 specimens (Appendix 1) and 807 bp was subjected to ML analyses to assign our collections to genetic species groups (Table 1). Identical *tufA* sequences were culled and representative sequences highlighting genetic diversity were retained for final analysis of 45 taxa (Fig. 2 and supporting information). Eight newly determined *rbc*L sequences (Appendix 1) were similarly added to published data for a final alignment of 108 taxa and 744 bp that was also subjected to ML analysis with the aim of matching our genetic groups to names currently recorded in Kraft et al. (2010) as well as GenBank (Table 1). Final *rbc*L analyses were conducted on a smaller dataset with 83 taxa that illustrated taxonomic identity and demonstrated corroboration with *tufA* (Fig. 3).

tufA results

The *tufA* gene region resolved 19 lineages from the genus *Ulva* with biogeographic sampling focused on 41 sites at mainland or insular Australia (Table 1; Fig. 1). These lineages were

distributed across two groups, UI and UII (Fig. 2A). UI was split into two major subgroups: 1) *U. procera*, *U. prolifera, U. linza*, *U. stenophylla, U. torta, U. flexuosa* and *U. californica* and 2) *U. gigantea* sister to *U. fasciata*, *U. ohnoi*, unidentified *U.* sp. 5GWS and *U. laetevirens* (Fig. 2A). No Australian representatives grouped with North American samples of *U. prolifera, U. linza, U. stenophylla* or *U.* sp. 5GWS. UII included two well-supported lineages: 1) *U. howensis* + *U. compressa* sister to *U. intestinalis* and 2) *U. lobata*, *U. lactuca* and *U. australis*. No Australian representatives of either *U. lobata* or *U. lactuca* were identified in this study. The outgroup *Ulvaria obscura* was distantly related to all sampled *Ulva* as well as the two included *Umbraulva* lineages, *Umbraulva* sp.1AUS and *Umbraulva japonica* that fell sister to *Ulva* (Fig. 2A).

Genetic distances were generated for 15 lineages that had Australian representation or were potentially new taxa (Fig. 2B). Within the UI group of *Ulva*, *U. procera* exhibited the most within species divergence (0.78%) and also had the largest sample size. Three lineages, *Ulva fasciata*, *U.* sp.5GWS and *U.* sp.10GWS, showed no genetic variability, however the latter taxon exhibited the highest interspecific distance in UI. *Ulva flexuosa, U. laetevirens* and *U. ohnoi* all exhibited the same levels of within species genetic divergence (0-0.13%). Little within species divergence again characterized genetic distance data in the UII subgroup. *Ulva compressa* exhibited the highest level of intraspecific diversity observed (1.03%), more than four times that of next most diverse taxon (Fig. 2B). *Ulva compressa* showed comparable levels of sample and geographic distribution to *U. intestinalis,* but possessed almost eight times the diversity. Samples from Bendalong and Lord Howe Island (both NSW) were the most divergent populations tested in *U. compressa*. *Ulva australis* had the highest sample size (41) and a huge geographic spread,

yet only exhibited a fraction of the diversity (1/4 or 25%) to that of *U. compressa*. This contrasts with the relatively high level of nearest neighbor interspecific distance observed between *U. australis* and its closest sister. *Ulva howensis* exhibited no within species divergence. The divergent *Umbraulva* sp. 1AUS exhibited the same level of within species divergence (0.13%) as *U. flexuosa, U. laetevirens, U. ohnoi* and *U. intestinalis*. Like *Ulva* sp. 10GWS and *U. australis*, *Umbraulva* sp.1AUS showed high levels of interspecific genetic distances. The barcode gap criterion was met with all taxa exhibiting less intraspecific than interspecific diversity (Fig. 2B).

The purpose of including *rbc*L data from Genbank was twofold: 1) it provided scaffolding that permitted identification of *Ulva* species, which in some instances pointed to taxonomic inconsistencies (Table 1; Figs. 2 and 3); and 2) it served to corroborate lineages resolved by *tufA* (compare Fig. 3 with Fig. 2). Although agreement between the two topologies was high, slight discrepancies arose between *rbc*L and *tufA* trees as a result of the inclusion of additional species for the former gene region compared with the latter. For example, *U. rotundata*, *U. stenophylloides*, *U. tanneri* and *U. brisbanensis* have been sequenced for *rbc*L, but not *tufA* (Kraft et al. 2010).

NIS assessment

Eleven species of Australian *Ulva* were scored for two published criteria (Heesch et al. 2009) to establish potential NIS status in Australia. Three species are considered likely to have been introduced to Australia from overseas: *U. procera*, *U. torta* (UI subgroup) and *U. australis* (UII subgroup; Table 2). Inference was limited in most species due to low sample size and/or missing

data.

Discussion:

Diversity, Taxonomy and Distribution

The *tufA* gene region delineated nineteen lineages of *Ulva* (Fig. 2). Of these, six did not include Australian representatives, viz., *Ulva gigantea, U. lactuca, U. linza, U. lobata, U. prolifera* and *U. stenophylla* (based on *tufA* sequences from non-Australian collections available in GenBank, Appendix 1). Two of the remaining lineages, *Ulva* sp. 5GWS and *Ulva* sp. 10GWS, did not have *tufA* or *rbc*L matches in Genbank indicating that they represent previously unsampled, possibly new, species. All of the remaining Australian collections fell into 11 groups for which we were able to assign names based on comparisons to data in Genbank.

Aligning our results with Kraft et al. (2010) indicates general congruence but also the need for taxonomic reconsideration of two species (Table 1, Appendix 1). The newly described Australian endemic *U. clathratioides* was genetically assignable to a species that was widely distributed in Canadian waters (Saunders & Kucera 2010) and loosely identified on morphological grounds as *U. torta*. It is clear that this genetic group is not endemic to Australia and synonymy of *U. clathratioides* with the reportedly globally distributed *U. torta* will be necessary if the morphological indications hold. Similarly, plants identified as *U. howensis* in our study had 100% sequence similarity (*rbc*L) to the newly described *U. proliferoides* (Kraft et al. 2010) nomenclatural priority going to the former (Table 1). We did not find sequence misidentification discussed by Couceiro et al. (2011) with the two sequences (EU933954 Voucher code 031 and EU933957 Voucher code 034) identified as *U. australis* by Kraft et al. (2010).

Several new records for *Ulva* spp. in Australia were uncovered at the national, state and local levels (Appendix 2, see supporting information). At the national level, four new genetic groups were identified and tentatively (Table 1) assigned to the species: 1) *Ulva californica*; 2) *U. ohnoi;* 3) *U. procera;* and 4) *U. torta*. Two additional taxa, *U.* sp. 10GWS and *Umbraulva* sp.1AUS, were unique relative to *rbc*L and *tufA* data in Genbank and may represent new species, for now they are certainly new records to Australia. *Ulva* sp. 10GWS was also sampled from eastern Canada, while *Umbraulva* sp.1AUS is thus far known only from Australia (Lord Howe Island, mainland NSW and WA). Sixteen range extensions were uncovered among Australian states (Appendix 2). The Lord Howe Island (NSW) endemic *Ulva howensis* was collected not only from mainland NSW, but also Western Australia (Appendix 1) and includes published records from Victoria with the synonymy of *U. proliferoides* (discussed above). Of note is the absence of *Ulva lactuca* from our survey, an observation consistent with Kraft et al. (2010), and one that reinforces the notion that reports of *U. lactuca* in Australia are based on misidentifications (although it is critical to acknowledge that the identity of *U. lactuca* requires further investigation, our usage here matching that of Kraft et al. (2010), but in conflict with that of O'Kelly et al. (2010), the former selected here simply to establish consistency in the literature for Australian records of this genus).

Shifting the balance from endemism to cosmopolitanism

Recent molecular surveys of *Ulva* in remote regions of the Pacific Ocean have been conducted in an effort to inventory floras (Heesch et al. 2009, Kraft et al. 2010, O'Kelly et al. 2010). Focusing exclusively on *Ulva* spp. that were collected and sequenced for *tufA* with representation both within and outside of Australia, 10/11 taxa (10/11 or 91%) showed high sequence similarity to representative species sampled from outside Australia (Table 1; Appendix 2). There is little evidence in this study of endemicity for the genus *Ulva* in the Australian flora excepting *U. howensis*. Even *U. howensis*, previously considered

endemic to Lord Howe Island, has been shown through our work to have a wider distribution in Australia than was previously thought.

Geographic structuring within species offers the potential for additional insight into a possible Australian signature. If Australian samples exhibit reciprocal monophyly with other Australian samples and not with samples from outside Australia, this potentially is evidence of incipient species. An emerging Australian signature is evident only in one strongly supported lineage within *U. compressa* from NSW, including Lord Howe Island (94% support in phylogenetic analyses; Fig. 2). More pervasive is the trend of Australian taxa falling with included representatives from outside Australia and not in discrete groups of solely Australian individuals (Fig. 2). Cosmopolitanism characterizes trends in the bulk of *U. compressa, U. intestinalis* and even *U. australis,* where sample sizes are relatively high, but as well for moderately sampled taxa such as *U. torta* and *U. procera*. This intermingling of geographic samples supports the notion that the bulk of sampled Australian taxa screened in this study are members of widely distributed species. This finding is only strengthened by consideration of the *rbc*L genetic data, which is a more conservative and thus less variable marker than *tufA*, and still points to multiple instances of widely sampled taxa with minor genetic differentiation (Fig. 3). Indeed, *U. stenophylla, U. prolifera. U. lactuca, U. australis, U. fasciata* and *U. tanneri* show very little variation across large geographic distances for included *rbc*L sequence data.

These results contrast with the findings of other recent molecular studies of regional floras from the remote Pacific that report higher incidences of endemism in the genus *Ulva* (Heesch et al. 2009, Kraft et al. 2010, O'Kelly et al. 2010). In particular is the contrast with Kraft et al., of interest given the overlapping geography between the two studies in temperate Australia. Our study yields 11 cosmopolitan/widely distributed taxa and 1 endemic while Kraft et al. (2010) reported six cosmopolitan

species, two subspecies and described four new species endemic to Australia, including: 1) *U. brisbanensis*, 2) *U. stenophylloides,* 3) *U. clathratioides* and 4) *U. proliferoides.* However, *U. stenophylloides* exhibits close match to *U*. sp1 CHE AY255871 from Chile, while results from this study suggest that *U. clathratioides* could be widely distributed *U. torta* and *U. proliferoides* is likely a synonym of *U. howensis* (Table 1; Fig. 3). These proposed amendments cast doubt on the level of endemicity outlined by Kraft et al. (2010). If these proposed amendments hold up following additional verification, instead of contributing to a growing Australian signature, these records could instead represent gathering evidence of the cosmopolitan nature of *Ulva* spp.

Indications of putative NIS?

The presence of a number of wide-ranging *Ulva* spp. in temperate Australia could be the result of natural transport, anthropogenic transport (and therefore NIS) or both. To distinguish between these alternatives, a number of criteria specific to *Ulva* have been proposed by Heesch et al. (2009), with an NIS designation given to a taxon both found in highly modified environments and/or areas with frequent vessel traffic and exhibiting high genetic similarity with samples from overseas. These criteria were adhered to in this study with the following clarifications. In regards to Criterion 1, although restriction of a taxon to a highly modified site or to areas with frequent vessel traffic is a solid indicator for geographical recognition of NIS, the reciprocal does not necessarily hold. Native species are not the only taxa to inhabit pristine sites and introduced species can occur widely, including at such sites. For example, *Sargassum muticum* is introduced to British Columbia, Canada but is found in a wide range of habitats, including pristine areas (Cheang et al. 2010). In regards to Criterion 2, test area genetic diversity 'pools' were compared with global genetic diversity 'pools' where possible. If a given taxon exhibited higher global diversity than in the putative introduced or test range (= Australia), then this was considered an indication of NIS.

Application of these criteria to eleven *Ulva* spp. in this study resulted in three taxa, *U. procera, U. torta* and *U. australis,* considered likely NIS introduced to Australia from overseas. These species were collected from a diversity of habitat types and settings and exhibited zero or less genetic diversity within Australia in comparison to a global diversity pool (Table 2).

Conflicting trends complicated straightforward NIS inference for several species of Australian *Ulva*. For *U. intestinalis*, *U. fasciata, U. flexuosa, U. laetevirens* and *U. ohnoi* additional sampling, either from overseas or within Australia, would clarify preliminary patterns indicated here. For example, *U. intestinalis* exhibits no intra-Australian diversity, higher global diversity for both *tufA* and *rbc*L and sampling sites in good proximity to port towns. However, the argument against NIS status is that the observed diversity within Australia is based on only two samples (albeit over wide geographic distances, TAS and NSW) compared to 68 overseas samples and that both Australian samples were collected from open coast areas on natural substrates. Although *Ulva californica* was identified as a likely NIS to New Zealand (Heesch et al. 2009), it was only given possible NIS status in this study, as the small sample size (n=1) negated a test area genetic diversity assessment at this time. Status of *Ulva howensis* was clearcut, as this species has a restricted distribution to southern Australia and is most likely an Australian endemic. *Ulva compressa* was found broadly and exhibited higher diversity within than outside Australia, suggestive of introduction from Australia to rest of the world. Data from *rbc*L were consistent with *tufA* trends for this species and exhibited no genetic diversity between samples collected from the Pacific and Atlantic seaboards of Canada. Querying additional studies that utilize other molecular markers (e.g. Kraft et al. 2010 for Australian *Ulva* using *rbc*L and ITS), as well as increasing sample sizes for all species, would improve the preliminary NIS assessments discussed here. However, as evident from this study and others, a solid taxonomic foundation needs to be in place for *Ulva* in order to facilitate meaningful comparisons (Table 1).

Conclusions

The recently identified green algal barcode marker *tufA* delineated twenty-two lineages of *Ulva*, reliably discriminated between closely related Australian species and indicated low levels of genetic differentiation between tested Australian species and overseas representatives. In an effort to achieve nomenclatural consistency, individual *rbc*L sequences were generated for species delineated by our *tufA* work and then analyzed together with *rbc*L data from Kraft et al. (2010). Identifications were broadly congruent except for two new species designations that we reinterpret as likely members of widespread species. In contrast with recent work on *Ulva* spp. in the region, our findings provide little evidence of endemism in the genus *Ulva* in temperate Australia. As widespread species are the most likely candidates for NIS, analyses based on ecological and genetic data were conducted to ascertain if any of the tested Australian *Ulva* species were also potential NIS. Three widespread *Ulva* species were found to be putative NIS in Australia.

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Fig. 1. Collection localities for Australian taxa included in this study (numbers cross reference to Table 1 and Appendix 1).

Fig. 2. A. ML phylogram of representative Australian *Ulva* to highlight genetic diversity of *tufA* sequence data. Genbank number reported as well as total sequences in brackets. Bootstrap (100) support values above branches. B. Table of intraspecific and interspecific distances.

Fig. 3. ML phylogram of *Ulva rbc*L sequence data. SH-like support values above branches. Data generated from this study (JN GenBank prefix) and from Saunders & Kucera (2010) (HQ GenBank prefix). Names have been retained on branches if not in agreement with bulk of identified taxa.

Table 1. Name usage, species authority, rationale, distribution and Australian collection information for species in this study. Name usage was bas Kraft et al. (2010) except as indicated. Distribution notes are based on our own collection records, as well as top 5 hits (98% or greater) in BLAST for *rbc* L data (Appendix 1). A, I, P indicate Atlantic, Indian and Pacific ocean, respectively and N refers to total number of sequences.

Name used	Rationale	Global Distribution	N	Australian Collection sites[A,B]
Ulva australis Areschoug 1854: 370	Consistent with usage in Kraft et al. (2010) *Ulva australis* and *U. pertusa* Kjellm. are conspecific based on our genetic data. *Ulva australis* , the older name, has priority. New South Wales collections field identified as *U. ranunculata* Kraft & A. Millar fall in this group as well.	Australia (I,P), North America and Europe (A,P) and South Korea.	41	TAS: 1-7 VIC: 9,10 NSW: 11, 22, 33-35 SA: 32 WA: 12,13,17,18
Ulva californica Wille in F.S. Collins, Holden & Setchell 1899: no. 611	Included in the Kraft et al. (2010) *U. stipitata* var. *linzoides* L.G.Kraft, Kraft & R.F.Waller complex (which is clearly composed of multiple closely related species). This name appears to be the best match based on data in Genbank.	Australian (P), North America (A,P)	2	VIC: 41
Ulva compressa Linnaeus 1753: 1163	Equivalent to the Kraft et al. (2010) *U. compressa* complex. Considerable names have been applied to allied sequences in Genbank - confirmation of true species name necessary.	Australia (P), North America (A,P).	28	TAS: 2, 4 VIC: 41 NSW: 21, 26, 33-6, 38-9 SA: 32
Ulva fasciata Delile 1813: 297 [pl. 58: fig. 5, 1826]	Consistent with usage in Kraft et al. (2010).	Australia (P), North America and Asia (P), Europe (A)	8	NSW: 22, 24, 26, 28, 31, 38, 40
Ulva flexuosa Wulfen 1803: xxii, 1	Included in the Kraft et al. (2010) *U. stipitata* var *linzoides* complex (clearly multiple closely related species). GWS originally keyed many of these collections to *U. prolifera* . Considerable names applied to allied sequences in Genbank - confirmation of true species name necessary.	Australia (I, P), North America (A,P), South Korea.	4	NSW: 35 WA: 18
Ulva gigantea (Kützing) Bliding	Consistent with usage in Kraft et al. (2010).	North America and Europe (A).	1	N/A
Ulva howensis (A.H.S.Lucas) Kraft 2007: 37, 319, fig. 14 A-I	100% *rbc* L match to newly described *U. proliferoides* of Kraft et al. (2010). These likely represent a single species for which the older name, *U. howensis* , is applied.	Australia (I,P)	9	NSW: 22, 35, 36, 37 SA: 32 WA: 13
Ulva intestinalis Linnaeus 1753: 1163	Consistent with usage in Kraft et al. (2010).	Australia (P), North America (A,P).	26	TAS: 5 NSW: 29
Ulva lactuca Linnaeus 1753: 1163	Consistent with usage in Kraft et al. (2010).	North America (A,P).	1	N/A
Ulva laetevirens Areschoug 1854: 370	Consistent with usage in Kraft et al. (2010).	Australia (I,P), North America (A), Europe (A) and Japan (P) as *U. armoricana* (100% match).	8	TAS: 6, 8 VIC: 10 WA: 13, 14, 19 NSW: 36
Ulva linza Linnaeus 1753: 1163	Consistent with *U. linza* USA in Kraft et al. (2010). All of our collections in this group are Pacific only, but the type locality is reported as England - confirmation of true species name necessary.	North America (P).	1	N/A
Ulva lobata (Kützing) Harvey 1855: 265	Consistent with usage in Kraft et al. (2010).	North America (P).	1	N/A
Ulva ohnoi Hiraoka & Shimada 2004: 17, figs 1-25	Consistent with data in Genbank and falls with *U. laetevirens* group of Kraft et al. 2010.	Australia (I,P) and Japan (P)	8	WA: 15, 16 NSW: 22-23, 30, 37, 39
Ulva procera (K.Ahlner) Hayden, Blomster, Maggs, P.C.Silva, M.J.Stanhope & J.R.Waaland 2003: 290	The *U. procera* FIN/ *U. linza* JPN cluster from Kraft et al. (2010). Based on both genes used here, there are actually 3-5 genetic groups in this cluster. Further, our plants in this group range from full blades, to linza-like blades to tubes. Impossible to use gross morphology for identifications, which likely explains the multiple names in Genbank - confirmation of true species name(s) necessary.	Australia (P), North America (A,P).	10	TAS: 4 VIC: 41
Ulva prolifera O.F.Müller 1778: 7, pl. DCCLXIII: fig. 1	Matches *U. prolifera* GBR in Kraft et al. (2010).	North America (A,P).	1	N/A
Ulva stenophylla Setchell & N.L.Gardner 1920: 282, pl. 26: fig. 2; pl.	Data in Genbank for both *U. stenophylla* (AY255874) and *U. taeniata* (Setchell) Setchell & N.L.Gardner (AY255874) match this group. The latter are incorrect and we apply the former name to this group (also see	North America (P).	1	N/A
Ulva torta (Mertens) Trevisan 1841: 480	Identification loosely based on morphology. Type from Europe, but we have collected this species from many locations. Genetic match to the new *U. clathratioides* L.G.Kraft, Kraft & R.F.Waller in Kraft et al. (2010). If our identification is correct, the new 'endemic' would have to be subsumed into the global (introduced to Australia?) *U. torta* .	Australia (P), North America (A,P).	7	TAS: 2 NSW: 36, 37 SA: 32
Ulva sp. 1GWS	Unique genetic group relative to all available data in Genbank.	North America (P).	1	N/A (*rbc* L data only)
Ulva sp. 5GWS	Unique genetic group relative to all available data in Genbank.	North America (P).	3	N/A
Ulva sp. 10AUS	Unique genetic group relative to all available data in Genbank.	Australia (P), North America (A).	2	TAS: 4
Ulvaria obscura (Kützing) P.Gayral ex C.Bliding 1969: 574	Consistent with morphology and data in Genbank.	North America (A,P).	1	N/A
Umbraulva japonica (Holmes) Bae & I.K.Lee 2001: 230	Consistent with morphology and data in Genbank.	South Korea (P).	3	N/A
Umbraulva sp. 1AUS	Unique genetic group relative to all available data in Genbank. Closest match *Umbraulva* spp.	Australia (I,P).	13	NSW: 20, 22, 23, 25, 27 WA: 17

[A]N/A refers to taxa from outside Australia.

[B]Numbers cross-reference to Appendix 1 and Figure 1.

Table 2. Likelihood of introduction to Australia of taxa collected and identified in this study. Maximal genetic distance data were generated at the BOLD website using Kimura 2-parameter distance estimates. Predictions are for likelihood that species was introduced to Australia.

Species	Types of Sites[A]	N[B]	Australian genetic diversity for *tufA*.	N[C] (tufA; rbcL).	Global genetic diversity (*tufA*; *rbc*L).	NIS assessment
UI Subgroup						
Ulva californica	HE, A	1	No data	14; 5	0.389; 0.27	**Possible**. Genetic sampling (n=1) insufficient to assess test area diversity for comparison to overseas. No diversity within 5 samples from overseas (B.C. Canada), instead all diversity between Australia and Canada. Habitat data suggest NIS.
Ulva fasciata	OC, N	8	0	No data	No data	**Possible.** Complete lack of diversity within Australia (samples from mainland NSW and Lord Howe Island) makes introduction plausible, but global genetic reference pool lacking. Habitat data do not suggest NIS.
Ulva flexuosa	OC and HE, N and A	2	0	7	0.13	**Possible**. Complete lack of genetic diversity within Australia (between WA and NSW), between two coasts of Canada and between Canada and South Korea. Only diversity between Australia and Canada + South Korea. More samples needed. Habitat data are not inconsistent with NIS and one site (Swan River, WA) highly disturbed.
Ulva laetevirens	OC and HE, N and A	7	0.129	9	0.129	**Unlikely**. Test area diversity driving global diversity. Two groups, one solely Australian (TAS and WA) and other Canada, NSW and WA. No intragroup diversity, only intergroup diversity. More samples needed, but present trends suggestive of introduction from Australia. Found broadly.
Ulva ohnoi	OC, N	8	0.129	No data	No data	**Uncertain**. No genetic data from overseas samples for comparison. Habitat data do not suggest NIS.
Ulva procera	HE, A	2	0.78	44; 8	1.185; 0.136	**Likely**. Higher diversity in global pool than test pool for both *tufA* and *rbc*L makes introduction to Australia likely. Genetic data consistent with dual introduction to Australia from overseas, one to VIC and one to TAS, but more Australian samples needed to confirm pattern. Habitat data from VIC and TAS suggest NIS.
Ulva torta	OC and HE, N and A	4	0.129	19; 2	0.259; 0.136	**Likely**. Diversity within Australia half that found globally for *tufA* makes introduction to Australia likely. Levels for 2 overseas sequences of *rbc*L higher than *tufA* genetic diversity from Australia also supportive of NIS status. Habitat data are not inconsistent with NIS.
UII Subgroup						
Ulva australis	OC and HE, N and A	30	0	44; 8	0.259; 0.136	**Likely**. Higher diversity in global pool than test pool for both *tufA* and *rbc*L makes introduction to Australia likely. Habitat data are not inconsistent with NIS.
Ulva compressa	OC and HE, N and A	15	1.043	38; 13	1.043; 0	**Unlikely**. Test area diversity driving global diversity. Genetic data consistent with introduction from Australia to rest of world. No diversity between B.C. Canada (Pacific) and Bay of Fundy (Atlantic) for *rbc*L. Found broadly.
Ulva howensis	OC, N	9	0	; 4	; 0	**Unlikely.** No genetic diversity from Australian samples for *tufA* or *rbc*L. Not found outside Australia. Likely Australian endemic.
Ulva intestinalis	OC, N	2	0	70; 23	0.134; 0.411	**Possible**. Higher diversity in global pool than test pool for both *tufA* and *rbc*L. More Australian samples necessary. Habitat data inconsistent with NIS, but both sample sites (Devonport, TAS Spirit of Tasmania dock and Coledale, NSW 20 km north of Port Kembla and 64 km south of Sydney) in somewhat close proximity to ports.

[A]Open coast (OC) or harbour/embayment (HE) and natural (N) or artificial (A) reported where known.
[B]Number of individuals sampled from Australia
[C]Number of individuals sampled globally including Australia

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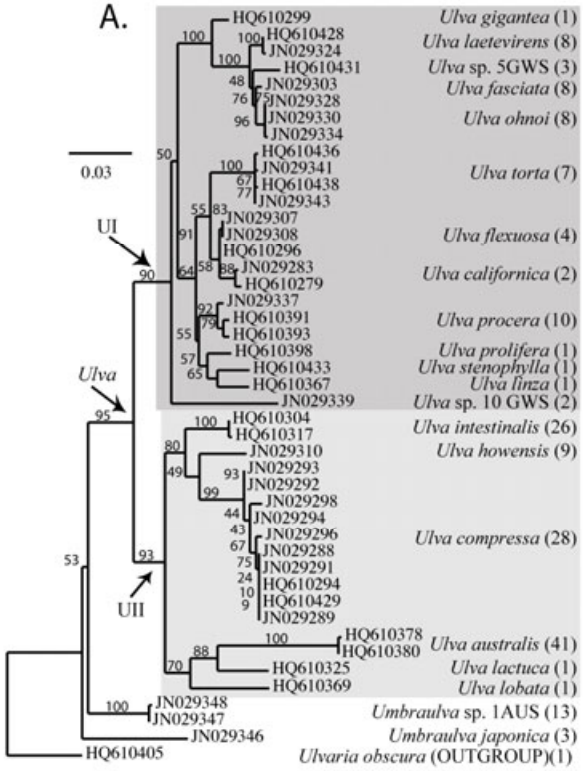
A.



B.

Species	Intraspecific Min(%)	Intraspecific Max(%)	Interspecific[A] Min(%)	Barcode Gap[B]
UI Subgroup				
Ulva californica	0.00	0.39	0.9	0.51
Ulva fasciata	0.00	0.00	0.65	0.65
Ulva flexuosa	0.00	0.13	0.78	0.65
Ulva laetevirens	0.00	0.13	1.16	1.03
Ulva ohnoi	0.00	0.13	0.65	0.52
Ulva procera	0.00	0.78	1.81	1.03
Ulva torta	0.00	0.26	2.07	1.81
Ulva sp. 5GWS	0.00	0.00	1.55	1.55
Ulva sp. 10GWS	0.00	0.00	4.91	4.91
UII Subgroup				
Ulva australis	0.00	0.26	5.56	5.30
Ulva compressa	0.00	1.03	3.23	2.20
Ulva howensis	0.00	0.00	3.23	3.23
Ulva intestinalis	0.00	0.13	3.49	3.36
OUTGROUP				
Umbraulva sp. 1AUS	0.00	0.13	5.22	5.09

[A]Interspecific minimum estimated for most closely related species based on phylogenetic results from this study

[B]Difference between minimum and maximum intraspecific distances must not exceed minimum interspecific distance and yield positive number.