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# The tip of the iceberg: genome wide marker analysis reveals hidden hybridization during invasion

**Running title: Hybridization of co-invaders**

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**Abstract**

Biological invasions are accelerating, and invasive species can have large economic impacts as well as severe consequences for biodiversity. During invasions, species can interact, potentially resulting in hybridization. Here, we examined two *Cakile* species, *C. edentula* and *C. maritima* (Brassicaceae), that co-occur and may hybridize during range expansion in separate regions of the globe. *Cakile edentula* invaded each location first, while *C. maritima* established later, apparently replacing the former. We assessed the evidence for hybridization in western North America and Australia, where both species have been introduced, and identified source populations with 4561 SNPs using Genotype-by-Sequencing. Our results indicate that *C. edentula* in Australia originated from one region of eastern North America while in western North America it is likely from multiple sources. *Cakile maritima* in Australia is derived from at least two different parts of Europe while the introduction in western North America is from one. Although morphological evidence of hybridization is generally limited to mixed species populations in Australia and virtually absent elsewhere, our genetic analysis revealed relatively high levels of hybridization in Australia (58% hybrids using Admixture) and supported the presence of hybrids in western North America (16% hybrids using Admixture) and New Zealand. Hybrids might be commonly overlooked in invaders, as identification based solely on morphological traits may represent only the tip of the iceberg. Our study reveals a repeated pattern of invasion, hybridization and apparent replacement of one species by another, which offers an opportunity to investigate the role of hybridization and introgression during invasion.

**Keywords: invasion, hybridization, *Cakile edentula*, *Cakile maritima*, Genotype-by-Sequencing (GBS), range expansion**

## 1 Introduction

Biogeographic barriers on a global, regional and local scale are often overcome by human activities, leading to biological invasions (Sax & Gaines, 2003; Simberloff, 2013; Vilatersana, Sanz, Galian, & Castells, 2016). Biological invasions can have a large economic impact (Hoffmann & Broadhurst, 2016; Pimentel, Zuniga, & Morrison, 2005), as well as severe negative consequences for biodiversity

and ecosystems (Sakai et al., 2001). Most long-distance introductions of invasive species in historic times are directly (e.g. ornamentals) or indirectly the result of anthropogenic activities (e.g. via ballast on ships) (Baker, 1974; Ruiz et al., 2000; Sakai et al., 2001). Invasions can also lead to novel interactions between species that previously had not co-occurred and, where there are no strong reproductive barriers, this may lead to instances of hybridization (Abbott, 1992; Ellstrand & Schierenbeck, 2000, Vallejo- Marín & Hiscock, 2016).

Rather than hybridization just being an incidental event, it could actually facilitate the success of invasive plant species, as invasive hybrid lineages can have increased fecundity and size (Hovick & Whitney, 2014). Various hypotheses have been proposed by which hybridization facilitates rapid range expansion (Bock et al., 2015; Ellstrand & Schierenbeck, 2000), including evolutionary novelty, increased genetic variation, heterosis, dumping genetic load (i.e. genetic rescue) (Ellstrand & Schierenbeck, 2000) and demographic rescue (Mesgaran et al., 2016). But convincing empirical data are limited. Hybridization is certainly not the sole evolutionary pathway to invasiveness, but can catalyze its evolution (Ellstrand & Schierenbeck, 2000). Not all of the potential consequences of hybridization are beneficial, however, and there can be significant costs associated with the phenomenon, such as outbreeding depression (Baack, Melo, Rieseberg, & Ortiz-Barrientos, 2015) and genetic swamping (Todesco et al., 2016). Our capacity to assess the role of hybridization during any particular invasion is hampered by the fact that it can be difficult to identify, especially when repeated backcrossing with one parental species has occurred rendering morphological identification difficult (Ward, Gaskin, & Wilson, 2008). However, genome-wide molecular markers can provide estimates of the extent of past hybridization and introgression across the genome (Payseur & Rieseberg, 2016).

On the beaches of Australia, the North Island of New Zealand and western North America a repeated pattern of invasion by two species of sea-rocket with contrasting mating systems (Barbour & Rodman, 1970; Cousens, Ades, Mesgaran, & Ohadi, 2013; Cousens & Cousens, 2011; Rodman, 1974, 1986) offers a rare opportunity to investigate the role of hybridization during invasion in distinct, geographically isolated regions. *Cakile edentula* (American sea-rocket), native to eastern North America, invaded each location first, while *Cakile maritima* (European sea-rocket) (Brassicaceae), native to Europe and northern Africa, arrived later. The invasion and replacement history in western North America and Australia are reviewed elsewhere (Barbour & Rodman, 1970; Cousens et al., 2013; Rodman, 1986), but we briefly outline it below.

In Australia, *C. edentula* was first recorded in Victoria in 1863 and subsequently spread along the coastline of Australia (Rodman, 1986). In 1897, *C. maritima* was recorded for the first time in Western Australia, and a second introduction into South Australia (1918: see Cousens et al., 2013; Ohadi et al., 2016) spread from there to the east (Heyligers, 1984; Rodman, 1986). In contrast to *C. edentula*, *C. maritima* seems still to be actively spreading in Australia and appears to have replaced *C. edentula* throughout much of its initial introduced range (Cousenes et al., 2013; Rodman, 1986). In western North America, a similar pattern of replacement occurred. *Cakile edentula* was found near San Francisco around 1880 (Barbour & Rodman, 1970), while *C. maritima* reached western North America by 1935 where it was found sympatric with *C. edentula* near San Francisco. The most recent published field study showed that *C. maritima* had replaced *C. edentula* throughout most of coastal California but not Oregon or Washington (Boyd & Barbour, 1993). In each case, there has been complete replacement of *C. edentula* by *C. maritima* over wide geographic areas (Barbour & Rodman, 1970; Cousens et al., 2013; Rodman, 1986), which was originally assumed to involve either direct or indirect competition (Rodman, 1986), although several additional mechanisms have been proposed such as disease (Bock, 2008; Cousens et al., 2013; Thrall, Young, & Burdon, 2000), coincidence (Cousens et al., 2013; Rodman, 1986), or greater lifetime fecundity of *C. maritima* (Boyd & Barbour, 1993). However, the mechanism of the replacement remains unclear.

*Cakile edentula* and *C. maritima* are closely related and cross-compatible (Li, Cousens, & Mesgaran, 2019; Mesgaran et al., 2016; Rodman, 1974). Both species are found in coastal strandline habitat, providing opportunities for hybridization in regions where they co-occur, but the species exhibit contrasting mating systems (Rodman, 1974). *Cakile edentula* (self-compatible) benefits from high levels of reproductive assurance as it is able to set seeds autonomously at high rates (Li, Mesgaran, Ades, & Cousens, 2020); one of Baker's (1965) ideal weed traits. In contrast, the establishment of *C. maritima* (self-incompatible) may be initially hindered (during both initial establishment as well as subsequent range expansion) by a lack of compatible mates limiting sexual reproduction and resulting in strong Allee effects. The apparent presence of hybrids, based on an intermediate leaf and fruit shape of both parental species, in some sites in Australia led Mesgaran et al., (2016) to develop a model for the interacting species, with the novel outcome that transient hybridization could overcome Allee effects in *C. maritima*. As a consequence, we hypothesized that past hybridization with *C. edentula* could be a common feature of *C. maritima's* establishment and range expansion in western North America, Australia and New Zealand.

We used genome-wide markers derived from Genotype-by-Sequencing (GBS) to examine the invasion history of these two species in Australia and western North America and quantify the extent and distribution of hybridization. There have been several previous studies examining the population genetic structure of *C. edentula* and *C. maritima* in their native ranges in Europe (Clausing, Vickers, Kadereit, 2000; Kadereit, Arafeh, Somogyi, & Westberg, 2005; Westberg, 2005), Africa (Gandour, Hessini, & Abdelly, 2008), eastern and western North America (Gormally, Hamrick, & Donovan, 2011) as well as in the introduced range of Australia (Ohadi et al., 2016). However, no study of the invasion history on two continents has been attempted nor has the extent of hybridization across multiple introductions been quantified. Specifically, we aimed to (1) identify probable source regions (from Europe and eastern North America); (2) determine whether both recent and advanced generation hybrids occur in the introduced ranges and the extent of their geographic distribution; and (3) determine if the change in levels of species ancestry post-invasion reflects a chronosequence along the direction of invasion of *C. maritima*. We predicted that early generation hybrids should be present at the leading edge of *C. maritima's* invasion into *C. edentula*-occupied areas, but later generation backcrosses with *C. maritima* should be more common in areas closer to where *C. maritima* first established. This should contribute to a gradient in species ancestry whereby *C. maritima* ancestry will be dominant in hybrids near the invasion source, while *C. edentula* ancestry will be more prevalent in hybrids identified in areas recently invaded by *C. maritima*. We predicted high levels of *C. maritima* ancestry in hybrids near the invasion source because *C. maritima* phenotypes are now exclusively present in the regions surrounding the invasion source, and studies of pollinators suggest preferential visitation of both hybrids and *C. maritima* over *C. edentula* which should facilitate backcrossing to *C. maritima* (Mesgaran et al., 2016).

## 2 Methods

### 2.1 Study species

*Cakile maritima's* native range extends over a wide climatic range from northern Norway to northern Africa. Current taxonomy recognizes subsp. *maritima* (Mediterranean), subsp. *baltica* (Baltic), subsp. *integrifolia* (Atlantic coast), subsp. *islandica* (Northern Europe and Northwestern Russia) and subsp. *euxina* (Black Sea) (Marhold, 2011). This is paralleled in the western Atlantic by *C. edentula,* for which two subspecies are recognized in its native range (Rodman, 1974) subsp. *edentula* (Labrador to North Carolina) and subsp. *harperi* (North Carolina to Florida). Although *C. maritima* has a sporophytic self-incompatibility system, the level of self-incompatibility varies among plants (Thrall

et al., 2000). *Cakile edentula* is self-compatible and can set seed autonomously at a high rate (Barbour, 1970; Rodman, 1974), although field estimates are suggestive of intermediate levels of autonomous selfing (Li et al., 2020). Both species are diploid ($2n = 18$) (Rodman, 1974). Hybrids are readily produced through artificial pollination (Rodman, 1974) with either parent as the pollen donor when emasculated (Li et al., 2019; Mesgaran et al., 2016), although crosses are more successful when *C. edentula* acts as the pollen recipient, consistent with the SI x SC rule (Harrison & Darby, 1955).

### 2.2 Samples

Samples of *Cakile spp*. were obtained from the native ranges (Europe and northern Africa, eastern North America) and the two introduced ranges (Australasia, western North America). We collected four of the five subspecies (subsp. *baltica*, subsp. *maritima*, subsp. *integrifolia* and subsp. *islandica*) of *C. maritima*. In the native range of *C. edentula* we sampled only *C. edentula* subsp. *edentula* as this subspecies is most likely the source of invasions in Australia and western North America (Cousens et al., 2013; Rodman, 1974). We obtained 214 samples of *C. maritima*, 137 samples of *C. edentula*, 17 putative hybrids (identified by morphology in the field) and two *C. lanceolata* samples from 92 locations in total (Figure S1; Table 1 & S1). Most samples were our own field collections of silica dried leaf tissue although a few samples were purified DNA from colleagues. We collected our samples along a transect through a population, ensuring that individuals were at least 2 m apart to avoid sampling close relatives or the same individual and collected individuals randomly with respect to their putative species based on morphology.

### 2.3 DNA extraction and Genotype-by-sequencing

We performed DNA extractions from dried leaf material using a modified CCDB DNA Extraction Protocol following Whitlock, Hipperson, Mannarelli, and Burke (2008). DNA quantity was assessed using a QuBit broad-sensitivity DNA quantification system (Invitrogen, Carlsbad, CA, USA) and a double-digest GBS library preparation was carried out (using PstI-HF (NEB) and MspI (NEB) enzymes, see Supplementary Information for details). Sequencing (125bp PE) was conducted on an Illumina HiSeq2500 (McGill University and Genome Quebec Innovation Centre) on two lanes.

## 2.4 SNP calling

Quality statistics of raw reads were assessed though FastQC (http://hannonlab.cshl.edu/fastx_toolkit ) and the reads were demultiplexed using STACKS process_radtags (Catchen, Amores, Hohenlohe, Cresco, & Postlethwait, 2011). We removed adapter sequences and trimmed the reads using Sickle (Joshi & Fass, 2011) with a Q-score of ≥ 20 and read length of ≥ 20 base pair. FASTQ quality filter (http://hannonlab.cshl.edu/fastx_toolkit) was then used to filter for reads with a Q- score of 20 or greater for ≥ 90% of the read length. The filtered reads were aligned using the Burrows-Wheeler Aligner (BWA) (Li & Durbin, 2009) to a *C. maritima* draft genome. Early access to the draft genome was provided by S.I. Wright, University of Toronto (https://genome.jgi.doe.gov/portal/CakmarStandDraft/CakmarStandDraft.info.html, GenBank: MK637688.1). The reference genome is found in 26,153 scaffolds with a scaffold N50 of 85,425. We assessed if there was a bias when mapping the reads of *C. edentula* to the reference genome of *C. maritima* but found limited evidence for such a bias (see Supplementary Information, Figure S2 & S3).

We called variants with GATK HaplotypeCaller (Poplin et al., 2017). We refer to this as the *unfiltered dataset* (Rosinger et al., 2020). Using VCFtools (Danecek et al., 2011) we removed individuals with fewer than 25000 reads, removed indels and restricted individual genotypes to have a depth between 5- 100,000. Furthermore, we filtered for a minimum quality score of 20, a genotype quality of 20, and a minor allele frequency of 0.05. Subsequently, we kept only bi-allelic variants that were successfully genotyped in more than 50% of individuals and removed individuals that had more than 50% missing data. The above filtering steps resulted in 18,573 SNPs from 258 individuals. Additionally, we removed 121 SNPs which showed > 80% observed heterozygosity, because such high observed heterozygosity could be caused by paralogues. We refer to this as the *filtered dataset* (Rosinger et al., 2020), which had a mean coverage of 39.21 (minimum coverage 9.18, maximum coverage 504.73).

## 2.5 Genetic clustering

Population genetic structure was inferred using Admixture (Alexander, Novembre, & Lange, 2009). For Admixture and most of our analysis we thinned our *filtered dataset* for linkage using a single SNP per 1kb window, resulting in a reduction to 4561 SNPs from 257 individuals (excluding the outgroup *C. lanceolata*). We will refer to this as the *global thinned dataset*. We ran Admixture using the *global thinned dataset* with a major termination criterion of $1X10^{-9}$, 1000 bootstraps and ten-fold cross-validation for K=1-10, where K equals the number of genetic groups. The K that produced the lowest cross-validation error was selected as the best K value. We refer to this as the *unsupervised run*. All following analyses were conducted in R v.3.5.2 (R Core team, 2018) except where otherwise stated. The output of Admixture visualized with pophelper v.2.3.0 (Francis, 2017) and pie charts.

To complement the population clustering analysis and to provide further insight in the population differentiation, we conducted a principal component analysis (PCA) and an unrooted phylogenetic network analysis. Genetic differentiation between native and introduced populations was summarized in a PCA with an 95% confidence ellipse using the R package SNPRelate (Zheng et al., 2012), tidyverse (Wickham, Francois, Henry, & Müller, 2019) and car (Fox & Weisberg, 2019) on the *global thinned dataset*. We used SPLITSTREE5 (Huson & Bryant, 2006) to visualize the overall sample relatedness with an unrooted phylogenetic network. To do this, we created two datasets from our unfiltered dataset (see details in Supplementary Information); (1) a global dataset containing all samples (*global Splitstree dataset*) and (2) a native range dataset containing samples from Europe and eastern North America (*native range Splitstree dataset*).

### 2.6 Hybrid identification

We used three different approaches to identify hybrids using genetic data:

(1) A *supervised run* of Admixture for K=2 using the *global thinned dataset*, by setting the samples from the two native ranges as reference individuals. Providing known ancestries allows the program to set some rows in the matrix Q to known constants and provides a more accurate estimation of the ancestries of the remaining individuals, and of the ancestral allele frequencies (Alexander et al., 2009). The other settings were retained from the *unsupervised run*. We refer to this as the *supervised run* and used this run to classify individuals by their Q-scores as hybrid, or pure species. We used the highest standard error from the Q scores, resulting in individuals classified as hybrids if $0.025 < Q > 0.975$ of their genome was assigned to the *C. edentula* cluster.

(2) We used the program NewHybrids (Anderson & Thompson, 2002) to identify early generation hybrids. It classifies their generation using a Bayesian model-based clustering framework to compute, by Markov chain Monte Carlo, the posterior probability that each individual belongs to each of the distinct first two generation hybrid classes (parental species, F1, F2, BC to species 1, BC to species 2). As the program is unable to deal with a large dataset, we restricted our data to 63 SNPs that showed fixed differences between the two species obtained from individuals classified as parental species using the *supervised run* of Admixture. Details of the settings used are provided in the Supplementary Information.

(3) We used the R package HIest (Fitzpatrick, 2012), which uses maximum likelihood to estimate ancestry and heterozygosity. For this package, we used the 471 loci that showed fixed differences between the individuals of the native ranges. Because it is possible that there is a low level of segregating variation within each species for these loci due to sampling error, particularly for SI *C. maritima* where the sample size is lower, we set the allele frequencies as 0.99 for *C. edentula* and 0.03 for *C maritima*. We also tested other SNP sets and allele frequencies. The details of the settings used and the hybrid assignments along with the results are provided in the Supplementary Information.

We tested for a chronosequence by assessing if there was a correlation between the distance of each population from the first entry point of *C. maritima* (Adelaide in Australia, San Francisco in western North America) and the level of *C. maritima* and *C. edentula* ancestry using a Spearman's rank correlation test in R using the ggpubr package (Kassambara, 2020). We used the ranked order of populations from this origin point along the coastline for each range. In Australia, we only used the south-east mainland individuals (see Supplementary Information for details). We tested the correlation between the Q value of the *C. edentula* cluster of the *supervised run* for each population and the rank order of the sampling locations along the coastline to the first entry point of *C. maritima*. We used individuals that were classified as hybrids by Admixture or all samples (including the parental species). We repeated this analysis using the S value from HIest and the hybrid classifications of this program.

Additionally, we used the program TreeMix (Pickrell & Pritchard, 2012) to identify evidence for hybridization in the introduced ranges using the *global thinned dataset* for which we constructed maximum likelihood trees and calculated the $f_3$ statistic (for details see Supplementary Information).

259

260 **2.7 Genetic diversity and differentiation**

261 Genetic diversity and differentiation within the two native ranges and two introduced ranges were
262 assessed for the 256 individuals (the New Zealand and *C. lanceolata* samples were excluded) using
263 the *global thinned dataset*. We calculated observed heterozygosity ($H_O$) and allelic richness ($A_R$)
264 with the diveRsity package (Keenan, McGinnity, Cross, Crozier, & Prodöhl, 2013). The 95%
265 confidence intervals of $A_R$ were calculated with 1000 bootstraps. We estimated differences in genetic
266 diversity between the species and ranges because we expected self-fertilization in *C. edentula* and
267 bottlenecks potentially experienced during introduction would reduce diversity. Because sampling at
268 individual locations was limited in the native ranges, we grouped individuals based on their range,
269 and their hybrid ancestry (pure parental or hybrid) using the *supervised run* Q-value assignments of
270 the *global thinned dataset* into eight groups. We used the Q value assignment of the *C. edentula*
271 cluster and the highest standard error (0.024) of the *supervised run* to classify individuals. To
272 determine regional differentiation we calculated Weir and Cockerham's (1984) pairwise $F_{ST}$ between
273 the above eight groups using the *global thinned dataset* with VCFtools (Danecek et al., 2011).
274 Additionally, we calculated the $F_{ST}$ for pure parental individuals, grouping individuals according to
275 their Admixture cluster from the *unsupervised run* and range (see Supplementary Information).

276

277 **3 Results**

278 **3.1 Genetic structuring and differentiation**

279 The Admixture analysis of the *unsupervised run* showed genetic structuring of *C. maritima*, *C.*
280 *edentula* and hybrids with an optimal K value of 8 (Figure 1 A, B & S4). Genetic structure was
281 present in the native range of *C. edentula,* where single samples from Lake Michigan and Rhode
282 Island constituted one group, samples from New Brunswick within the Gulf of St. Lawrence a second
283 group, samples from Newfoundland and Quebec (along the St Lawrence River) a third group and
284 samples from Nova Scotia a final group. As expected, for *C. maritima,* there were two main groups:
285 one group was largely from the Baltic and Atlantic coasts, which we term the "Atlantic" group
286 (comprising mainly the dark blue cluster, Figure 1 A, B) and a second admixed group was associated
287 with the Mediterranean, that we term the "Mediterranean" group (comprising mainly the light and

288 medium blue clusters, Figure 1 A, B). In Australia, several genetic clusters were identified. First, in
289 Queensland, New South Wales and Tasmania we identified pure *C. edentula* individuals. Second, for
290 populations along the west coast of Australia, we identified a *C. maritima* cluster associated with the
291 Atlantic coast in the native range. Third, in South Australia, genetic clusters associated with the
292 Mediterranean were found. In the south-east of Australia there was evidence of hybrids between *C.*
293 *maritima* and *C. edentula* (see below). In the introduced range of western North America, we
294 identified pure *C. edentula* along with pure *C. maritima* (Figure 1 A, B). A small number of samples
295 from Washington, Oregon and California showed evidence of hybridization (see below).

296 The PCA and SPLITSTREE5 analyses confirmed the findings of Admixture. There was clear
297 differentiation of *C. maritima* and *C. edentula* in the *global thinned dataset*. The first eigenvector
298 (EV) (Figure 2 A & S5 A) explained 33.17% of the variation and clearly delineated the species. The
299 *C. edentula* group showed less variation than the *C. maritima* group along the first two EVs. Two *C.*
300 *maritima* groupings were also evident with one representing *C. maritima* from Europe and Australia
301 (EV1<0, EV2<0) and the other representing exclusively *C. maritima* from western North America
302 (EV1<0, EV2>0). In the SPLITSTREE5 network, using the *global Splitstree dataset*, *C. edentula* (as
303 identified by the *supervised run*) formed a monophyletic group without admixture. *C. maritima*
304 samples were split into three groups (Figure 2 B, C): *C. maritima* (Mediterranean group), *C.*
305 *maritima* (Atlantic group) and *C. maritima* in western North America. Hybrids of the two species
306 were scattered in between the *C. maritima* groups or between the two-parental species along the
307 network. The additional native range SPLITSTREE5 analysis (Figure S6) mirrored this pattern but
308 provides clearer *C. edentula* grouping in the native range.

309 Pairwise $F_{ST}$ (Table S2) using the *global thinned dataset* revealed clear genetic differentiation
310 between the two-parental species originating from the native range ($F_{ST} > 0.527$). Within the
311 introduced ranges the pairwise $F_{ST}$ between the two species was similar to the comparison of the
312 native ranges. Hybrids identified using Admixture in the introduced ranges showed higher genetic
313 differentiation from *C. edentula* than from *C. maritima* (Table S2).

314

315 **3.2 Genetic diversity**

316 Population statistics revealed that in their native ranges, *C. edentula*, the self-compatible species, has
317 considerably less $H_O$ than *C. maritima* and the hybrids of the two species (Table S3). $A_R$ was

significantly reduced in *C. edentula* in comparison to *C. maritima,* the largely self-incompatible species. In the introduced ranges, no clear reduction of $H_O$ or $A_R$ was observed in either of the species. Hybrids of the two-species had higher $H_O$ and $A_R$ compared to both parental species.

### 3.3 Hybrid classification

The three approaches classified different proportions of individuals as hybrids, as expected due to their ability to detect recent hybrids (NewHybrid, HIest), versus hybrid ancestry (Admixture, HIest). All hybrids identified by NewHybrids were also identified as hybrids with HIest and Admixture (Table S4 & S5). The fourteen putative hybrids included in the samples as a result of morphological identification were assigned by all analyses as hybrids, providing evidence of the accuracy of the assignments. Furthermore, the NewHybrid analysis confirmed that these hybrids were likely the product of the first two generations of interbreeding. NewHybrids analysis revealed 19 hybrids (Figure 3; Table S4) with 17 hybrids in Australia (13.49%), one in western North America (1.47%) and one in New Zealand. In Australia, F1 and F2 hybrids were detected in the current sympatric zones where individuals with both species' phenotypic traits were clearly identifiable in the populations. Hybrids (Figure S5 B) grouped in the PCA according to their generation, with F1 and F2 hybrids grouped between the parental species, and backcrosses grouped closer to species they backcrossed to. In this same PCA the advanced generation hybrids identified with the *supervised run* of Admixture as well as HIest frequently grouped with *C. maritima,* suggestive of further backcrossing to that species.

Classification of hybrids using the *supervised run* of Admixture revealed 73 hybrids in Australia (57.94%) from 15 locations, 11 hybrids in western North America (16.18%) from five locations and one hybrid from New Zealand (Figure 1; Table S4). In western North America hybrids were found in each of two locations in California and Oregon and in one location in Washington.

All Admixture hybrids were also identified as hybrids in HIest and the ancestry assignments were highly correlated between the programs (Figure S7). When the 471 loci that are fixed between native range samples were used, and we allowed for a low level of polymorphism within each species (0.99 *C. edentula*, 0.03 *C. maritima*), a larger number of hybrids were identified using HIest than Admixture (138 versus 85, Table S6). Changing the allele frequencies and SNP set impacted the number of hybrids identified (see Supplementary Information), but this only influenced the

348 classification of individuals with an apparent low level of ancestry from the alternate species. In all
349 the runs, advanced generation hybrids were identified in this analysis with many in regions where *C.*
350 *maritima* has not been recorded for many decades, but also in the current sympatric zone (New South
351 Wales, Queensland and Tasmania).

352 We then examined if patterns of ancestry in Australia and western North America reflected the likely
353 invasion route of *C. maritima*. Specifically, we tested if low levels of *C. edentula* ancestry were
354 found in areas where *C. maritima* first arrived, and if high levels of *C. edentula* ancestry were found
355 in regions *C. maritima* has more recently invaded and where *C. edentula* is still present. Using the
356 supervised Admixture analysis, the mean *C. edentula* ancestry of hybrids at each location was
357 correlated with the ranked distance from where *C. maritima* first arrived in south-eastern mainland
358 Australia ($\rho = 0.82$, $p < 0.01$) (Table 2). This pattern was also significant when testing across all
359 samples, including individuals identified as parental species ($\rho = 0.89$, $p < 0.05$). However, in
360 western North America, although the direction of the correlation was as predicted, a geographic
361 pattern in ancestry was only significant when using locations north of San Francisco as well as
362 parental and hybrid individuals ($\rho = 0.72$, $p < 0.05$). The same pattern of significance was found
363 when using the results of the HIest (Figure 4 & 5 & S8; Table 2).

364 We used TreeMix to assess geneflow between *C. edentula* and *C. maritima* within each introduced
365 range. The maximum likelihood tree in both invasive ranges showed bidirectional gene flow (Figure
366 6). In Australia geneflow occurred from the *C. edentula* branch into Australian *C. maritima*
367 (Mediterranean); a migration event also occurred from this group into the *C. edentula* branch (Figure
368 6 B). In western North America the same pattern occurs. There is evidence of a migration event from
369 the *C. edentula* branch into western North American *C. maritima* as well as a migration event from
370 the western North American *C. maritima* branch into the western North American *C. edentula*
371 (Figure 6 A). The $f_3$ statistic of TreeMix (Table 3) confirmed that the hybrids (identified by the
372 *supervised* Admixture *run*) in the introduced range are admixed from the *C. edentula* and *C.*
373 *maritima* parental individuals within both introduced ranges (Australia $f_3 = -0.006$, $Z = -31.97$;
374 western North America $f_3 = -0.005$, $Z = -23.22$).

375

376 **4 Discussion**

Our analysis sheds light on the origin and extent of hybridization of two introduced species in two separate invasions, which experienced a parallel pattern of invasion and apparent replacement of one species by another. Except at places where the two species are currently sympatric and new hybrids are still being formed, it would be difficult to determine morphologically that hybridization has ever taken place, since backcrossing soon hides its phenotypic evidence. *Cakile maritima* is highly variable within and between populations in its native range and hybrids in the introduced range could easily be overlooked (e.g. Cousens et al., 2013) without the use of molecular methods. However, our analysis identified extensive hybrid ancestry in the introductions, particularly in Australia. It is therefore an intriguing possibility that hybridization may be commonly overlooked in a much wider range of invasive taxa, especially where morphological trait indicators of hybridization are more cryptic. Alien floras commonly include many congeneric species whose capacity for interbreeding is yet to be established. While previous authors (Ellstrand & Schierenbeck, 2000) have raised our attention to obvious hybrid species and allopolyploids, perhaps the impacts of hybridization are often more insidious. It is thus important – though not an easy task – to determine in future the extent to which such non-apparent introgression has been beneficial during invasion.

### 4.1 Native range patterns

One of our primary goals was to identify the source regions for the invasions for each species and our analysis provided evidence of geographic structuring in the *C. edentula* native range, at a much finer grain than currently recognized taxonomically (Figure 1). Samples from Quebec, Newfoundland, Nova Scotia and New Brunswick contain separate Admixture clusters, likely within *C. edentula* subsp. *edentula* var. *edentula* as this subspecies is the only one described in this region of the North American Atlantic coast (Rodman, 1974). Two single samples from Lake Michigan and Rhode Island grouped together in one cluster of the Admixture analysis; those samples might belong to the Atlantic coast variety of *C. edentula* subsp. *edentula* var. *edentula* as it is known to have invaded Lake Michigan in historical times (Huebner, 2009; Rodman, 1974), where it now coexists with the Great Lakes endemic var. *lacustris*. A second possibility, suggested by Gormally et al., (2011), but without morphological evidence, is that var. *lacustris* has dispersed to the Atlantic. Genetically distinct regional variation is not surprising, as the directions of currents and the influences of geological features on seed dispersal can be highly predictable (Lapointe, 2000). Similar conclusions have been reached in the Mediterranean by Westberg (2005) and Gandour et al. (2008). *Cakile edentula* subsp. *harperi* occurs in areas south of the populations sampled in our study (Rodman, 1974), but comprehensive studies of herbarium samples by Rodman (1974) and Cousens et al.,

409 (2013) have found no morphological evidence that subsp. *harperi* has been introduced anywhere
410 outside its native range.

411 Our analyses revealed clustering of *C. maritima* in its native Europe largely consistent with the
412 accepted taxonomic distributions (Ball, 1964; Marhold, 2011; Rodman, 1974) as well as one previous
413 population genetic analysis (Clausing et al., 2000). Other genetic studies with greater sampling
414 intensity, however, showed more differentiation on a local level (Kadereit et al., 2005; Westberg,
415 2005). The absence of fine-grain local differentiation in our study might be driven by the limited
416 number of native range samples for this species and restricted sampling of the Baltic area.

417 *Cakile edentula* showed lower genetic diversity than *C. maritima* in their native ranges as measured
418 by $A_R$ and $H_O$ (Table S3) and showed less variation along the EVs and in the SPLITSTREE network
419 analysis (Figure 2). Higher selfing rates in *C. edentula* would be expected to reduce the effective
420 population size compared to the largely self-incompatible *C. maritima* (Pollak, 1987).

421 **4.2 Introduced range patterns**

422 **4.2.1 Australia and New Zealand**

423 Although *C. edentula* has now disappeared from much of its original introduced range in Australia,
424 some pure *C. edentula* populations still remain. Our analyses show that they likely originate from
425 populations located in Nova Scotia as they contained an Admixture cluster found exclusively in this
426 region of the native range and showed the lowest genetic differentiation from this region (Figure 1;
427 Table S7). *Cakile edentula's* $A_R$ and $H_O$ did not change considerably in Australia compared to the
428 native range (Table S3), which is inconsistent with a strong invasion bottleneck. The genetic
429 structure of the Australian *C. maritima* samples is consistent with a history of multiple introductions.
430 This is in accordance with previous morphological and genetic studies of invasion history in
431 Australia (Cousens et al., 2013; Ohadi et al., 2016; Rodman, 1976, 1986). In particular, the cluster
432 associated with the Atlantic European group is found in western Australia, while a Mediterranean
433 cluster predominates in southern and eastern Australia (Figure 1; Table S8). Similarly, analysis of
434 microsatellite markers indicated that that western and south-eastern populations of *C. maritima* in
435 Australia were genetically distinct and most likely resulted from independent introductions with
436 severely limited gene flow from west to east (Ohadi et al., 2016). Finally, Australian *C. maritima*
437 showed higher $A_R$ and $H_O$ values than its native range, consistent with admixture of multiple source
438 populations and/or hybridization with *C. edentula.* Many successful invasions are sourced from

multiple introductions (e.g., Vallejo- Marín et al., 2020; van Boheemen et al. 2017) and both hybridization and multiple introductions and admixture may spur successful invasions (Ellstrand & Schierenbeck, 2006; Dlugosch & Parker, 2008; Hodgins, Bock, Rieseberg, 2018).

Our data provides substantial evidence for extensive hybridization in Australia between the two species. TreeMix supported bidirectional gene flow between the parental species (identified morphologically) (Figure 6). This was confirmed by the Admixture global analysis (Figure 1), the PCA and Splitstree analysis, as many Australian samples fell in-between the native range samples of both species (Figure 2), and the $f_3$ test (Table 3). Further support is provided by three separate analyses which specifically detect hybrid individuals (Figure 1 & 3 & 4 & 5 & S8; Table S4). As expected, Australian hybrids (*supervised* Admixture *run*) had higher genetic diversity than both parental species (Table S3). Furthermore, the pattern of hybrid ancestry was geographically structured and reflected the historical invasion route of *C. maritima* in south-eastern Australia. This pattern was consistent across two separate approaches (*supervised* Admixture *run*, HIest) to identify hybrid ancestry (Figure 1 & 4; Table 2). NewHybrids confirmed the presence of a small number of early generation hybrids (within two generations) where both species still co-occur and some mixed populations show pure genotypes of both parental species and early generation hybrids, demonstrating on-going hybridization of the two taxa (Figure 3). In areas where *C. edentula* still persists, backcrossing to *C. edentula* has also occurred, but is rare, and recent backcrosses to *C. maritima* appear to be more common. In those parts of Australia where *C. maritima* has already appeared to have replaced *C. edentula* (i.e., where no *C. edentula* phenotypes remain; Cousens et al., 2013; Rodman, 1986), evidence is consistent with past hybridization between the species and repeated backcrossing to *C. maritima* (Figure 1 & 4 & 6). In areas of Western Australia, where *C. edentula* has never been identified, evidence of hybridization with *C. edentula* was also found, confirming a previous observation by Ohadi et al., (2016). The sample from New Zealand was identified as a hybrid where the same replacement of *C. edentula* by *C. maritima* has also taken place (Cousens & Cousens, 2011).

#### 4.2.2 Western North America

Our results revealed that *C. edentula* in western North America most likely originated from two sources in eastern North America. We also found that western North American *C. maritima* potentially originated from the Mediterranean region, as *C. maritima* in western North America contained the same Admixture clusters as the Mediterranean and showed the lowest differentiation

from this region (Figure 1; Table S7 & S8). However, these populations were genetically distinct (Figure 2 & S5) suggesting the possibility of an unknown source for this invasion, or the impact of an invasion bottleneck. *Cakile edentula* and *C. maritima* in western North America showed, as in Australia, no reduction of $H_O$ and $A_R$, which may reflect the impacts of undetected hybridization, large founding populations, or multiple introductions.

Like Australia, hybridization was identified between the two species in western North America, although the proportion of hybrids was less (e.g., 58% versus 16% using the *supervised Admixture run*). TreeMix identified bidirectional gene flow between the species in western North America (Figure 6; Table 3), and evidence consistent with hybridization was apparent in the global Admixture analysis (Figure 1), the PCA and Splitstree analysis (Figure 2). Furthermore, we employed three independent methods to specifically identify hybrid individuals and their likely generation. From this we identified 11 hybrid samples (all 11 were identified by both HIest and Admixture and one as an F2 by NewHybrids) from five locations in western North America. Specimens of hybrids based on morphological identification are largely unknown for this region, either in herbaria or in the field (Rodman, 1974). But more recently, Cody and Cody (2004) reported a small percentage of hybrids in a population from British Columbia. Although the fitness and demographic consequences of hybridization during introduction require further investigation, the lower incidence of hybrids in western North America compared to Australia suggests that hybridization could have facilitated the establishment and rapid spread of *C. maritima* to a greater degree in Australia. In support of this hypothesis, the complete replacement of *C. edentula* by *C. maritima* phenotypes has not progressed as far north in western North America compared to Australia, where few northern populations of *C. edentula* remain. Indeed, although the introduction of *C. maritima* in western North America is more recent than Australia, migration rates for this species based on herbarium records are much lower in western North America (Barbour & Rodman, 1970; Rodman, 1986). However, the mechanism driving differences in hybridization rates in western North America compared to Australia is unclear and requires further investigation.

### 4.3 Hybrid identification and significance

The pattern of invasion first by *C. edentula*, then by *C. maritima*, has been repeated in three regions. Prior to this study, hybrids were known only from Australia. However, we also identified clear evidence of hybridization in western North America and in New Zealand. Hybrids between the two species can be produced readily by handcrossing (e.g. Li et al., 2019; Mesgaran et al., 2016; Rodman,

1974) and our data demonstrate that recent and advanced generation hybrids are at least partially fertile in natural populations. Our results show backcrossing to both parental species, although backcrossing to *C. maritima* was much more frequent. This pattern of biased backcrossing towards *C. maritima* was predicted based on field observations of pollinator visitations (Mesgaran et al., 2016), the morphological replacement of *C. edentula* by *C. maritima,* and previous genetic studies (Mesgaran et al., 2016; Ohadi et al., 2016). It is also consistent with expected mating asymmetries between these species and their hybrids caused by the inheritance of the self-incompatibility system and traits associated with pollinator attraction in hybrids (Li et al., 2019). In artificial crosses, early generation hybrids inherited mostly (but not exclusively) self-incompatibility, as well as larger floral displays, similar to *C. maritima* (Li et al., 2019). This suggests that F1 hybrids will often need to rely on outcrossing, and that larger floral displays should facilitate this. Consequently, these traits in the hybrids should further contribute to backcrossing to the self-incompatible parent (*C. maritima*). A similar asymmetric pattern of species ancestry has been identified in hybrids of other species with such differences in mating system (Brandvain, Kenney, Flagel, Coop, & Sweigart, 2014; Pickup et al., 2019; Ruhsam, Hollingsworth, & Ennos, 2011).

Our identification of advanced generation backcrosses to *C. maritima* means that portions of the *C. edentula* genome have been retained in a largely *C. maritima* background (i.e. introgression), long after morphological evidence of hybridization has gone from a population. The role of selection and neutral evolutionary processes in governing patterns of introgression across the genome, however, remains to be investigated in this system. Theory suggests that regions of the genome that are not introgressed will harbour incompatibilities or a high number of additive deleterious alleles in the introgressing species (Harris & Nielsen, 2016; Juric, Aeschbacher, & Coop 2016). A greater fixation rate of weakly deleterious alleles is predicted in the *C. edentula* due to its higher level of inbreeding, and indeed, the low levels of genetic variability in this species relative to *C. maritima* support a lower effective population size in this species. Selection against a higher genetic load originating from *C. edentula* in hybrids should more rapidly lead to the reconstitution of a *C. maritima* genome following transient hybridization during range expansion. In line with the expectation of selection against selfing ancestry in outcrossers, in *Mimulus gutattus* (outcrossing) genomic regions with high recombination rates have reduced levels of ancestry from the selfing species *Mimulus nasutus* (Brandvain et al., 2014). However, several remarkable examples in plants have demonstrated the infusion of favorable alleles via hybridization (adaptive introgression), including the transfer of herbivore resistance in *Helianthus* (Whitney, Randell, & Rieseberg, 2006). Indeed, Cody and Cody

(2004) proposed the intriguing possibility of adaptive introgression in the *Cakile* system but this remains to be investigated. Our identification of replicated patterns of hybridization, replacement and invasion in *Cakile* provide an exciting opportunity for further investigation of the beneficial and detrimental consequences of hybridization during range expansion.

## 5 Conclusion

Here we confirm that, particularly in Australia, the apparent replacement of *C. edentula* by *C. maritima* is not complete and remnants of the *C. edentula* genome are evident in contemporary *C. maritima* populations. Furthermore, it appears that both early and later generation hybrids are at least partially fertile in natural populations and that there is a higher frequency of backcrossing to *C. maritima*. The patterns of hybridization we identified is consistent with the hypothesis that mating among these cross-compatible invaders has facilitated the establishment of the self-incompatible *C. maritima* whose range expansion may otherwise be limited due to Allee effects, as has been observed in other potential self-incompatible invaders (Uesugi, Baker, de Silva, Nurkowski, & Hodgins, 2020). The evolutionary consequence of hybridization for both species remains unclear, as is its role, if any, in the rapid expansion of one invader at the expense of another.

## 6 Author Contributions

KH, RC and LR conceived of and designed the study. KH, KN and RC carried out sampling. KN conducted the molecular laboratory work. HR carried out the bioinformatics analyses with significant input from AG, PB and KH. AG, KH, LR, PB, RC and HR contributed to the writing and approved the final manuscript.

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## 9 Data Availability Statement

Sequence data are available at the National Center for Biotechnology Information Sequence Read Archive under Bioproject PRJNA637114. Unfiltered dataset available on https://bridges.monash.edu/articles/dataset/GBSCAK_vcf_gz/12526220/1; filtered dataset on https://bridges.monash.edu/articles/dataset/filtered_dataset_GBS_Cakile/12996854. Scripts are available on https://github.com/HannaRos/Cakile-GBS-scripts.

## 10 Data reference


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**Figures and figure captions**

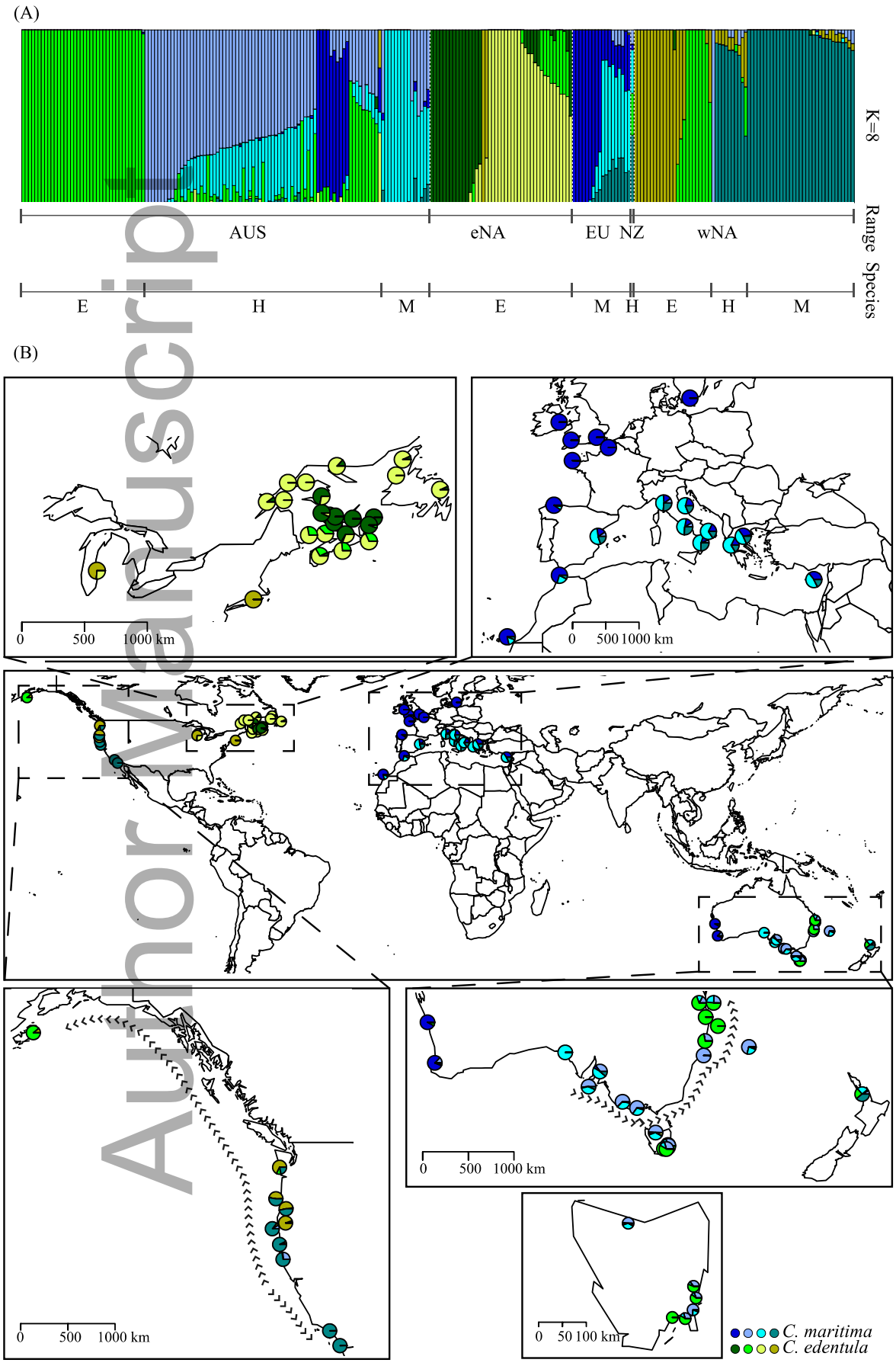




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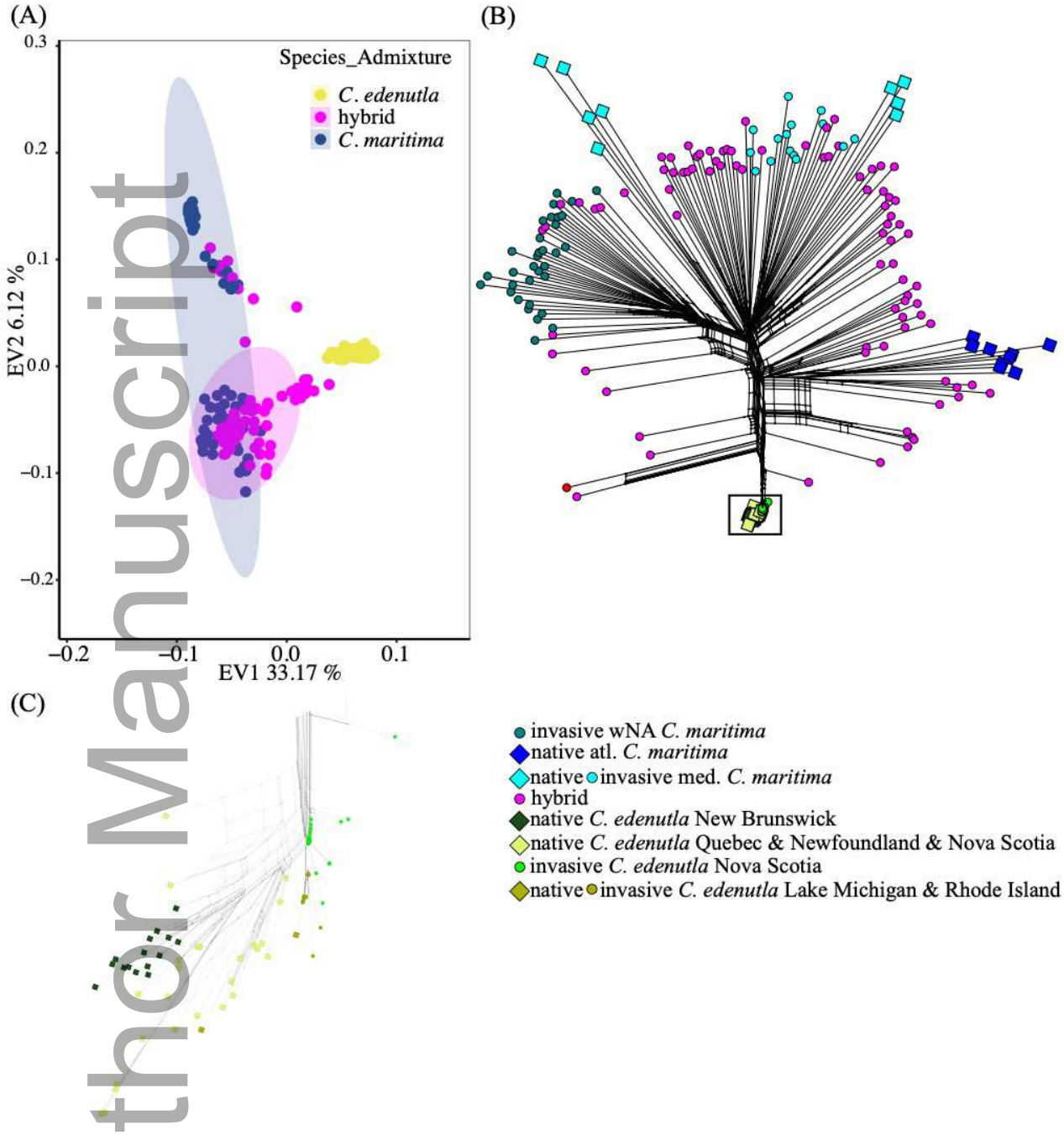
Figure 1 Admixture results of the *unsupervised run* of the *global thinned dataset*. (A) A distruct plot for K=8. Individuals are ordered according to their cluster association of the *supervised run*. AUS=Australia, eNA= eastern North America, EU= Europe and northern Africa, NZ= New Zealand, wNA=western North America. E= *C. edentula*, M= *C. maritima*, H= Hybrids. (B) Population pie charts for K=8, Admixture proportions for each population are displayed. A global map is displayed as well as close ups of western North America, Europe, the Australian mainland and Tasmania. Colours correspond to the clusters in the distruct plot. Arrows indicates direction of invasion and direction of Spearman test.




784

785 Figure 2 (A) Principal component analysis of the *global thinned dataset*. First two eigenvectors are
786 presented. Individuals are coloured according to their species and hybrid status based on the
787 *supervised run* of Admixture. Ellipses indicate the 95% confidence range of the cluster. (B) Splitstree
788 network of the *global Splitstree dataset*. Individuals are coloured according to their predominant
789 cluster of the *unsupervised run* of Admixture cluster (K=8 of the *global thinned dataset*), with
790 hybrids identified using the *supervised* run. The shapes indicate native vs. invasive range.

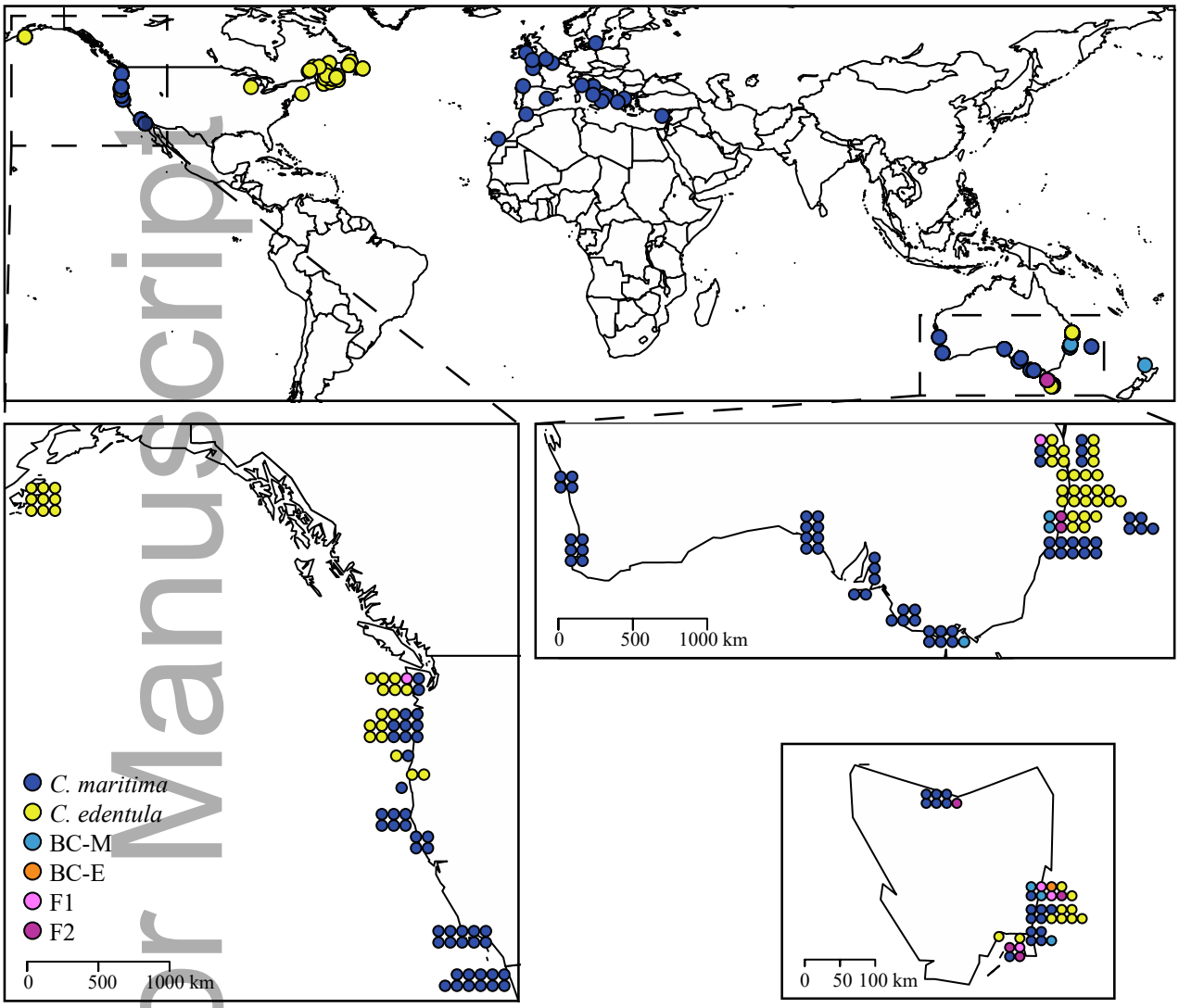
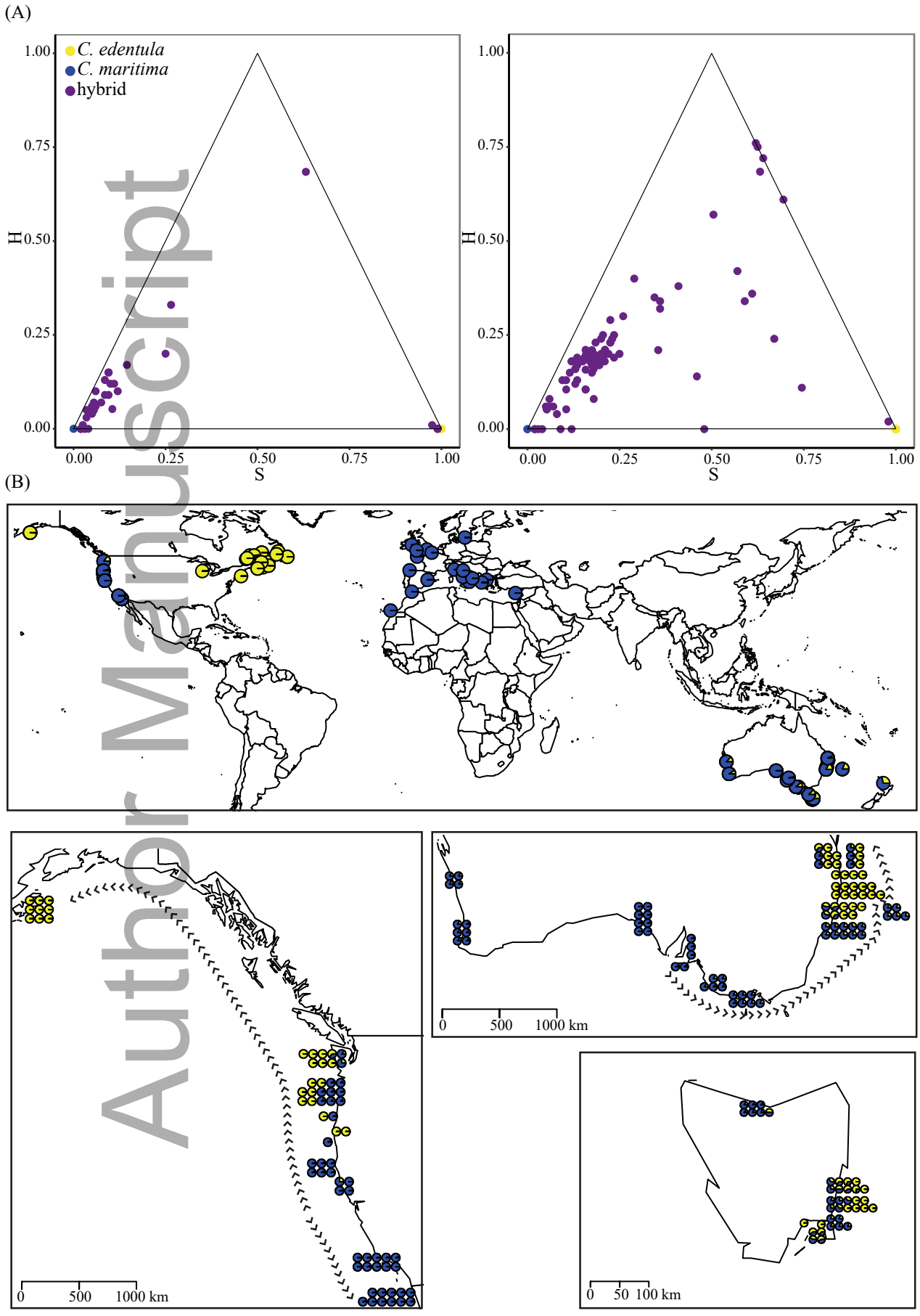



Figure 3 Geographic distribution of the hybrid assignment test by NewHybrid. Individuals are coloured according to their NewHybrid classification. A global map and close-ups of western North America, the Australian mainland and Tasmania are presented. BC-E= backcross to *C. edentula*, BC-M= backcross to *C. maritima.*






798

799 Figure 4 Results of a hybridization assignment test implemented by HIest using 471 SNPs (0.99,
800 0.03). (A) Association of ancestry index (S) and interclass heterozygosity (H) are given for western
801 North America (left) and Australia (right). Individuals are coloured according to their HIest
802 classification. For hybrids the continuous model was a better fit than the hybrid classes. (B) The
803 geographic distribution of individuals and their S index; yellow= *C. edentula* proportion, blue= *C.*
804 *maritima* proportion. A global map and close-ups of western North America, the Australian mainland
805 and Tasmania are presented. Arrows indicates direction of invasion and direction of Spearman's test.

806

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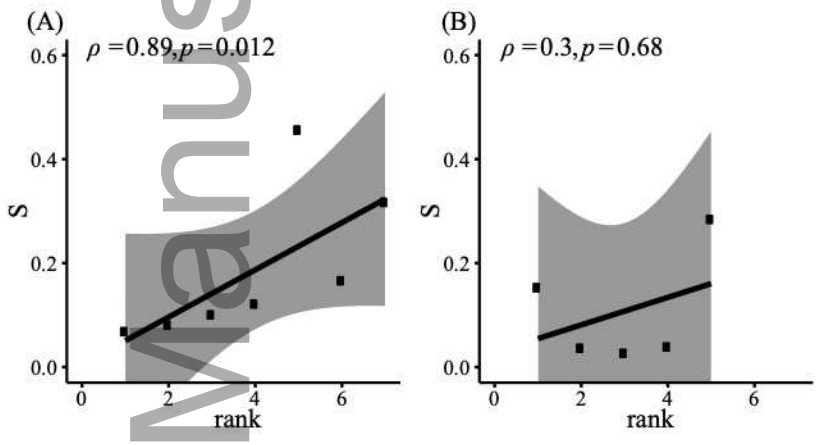
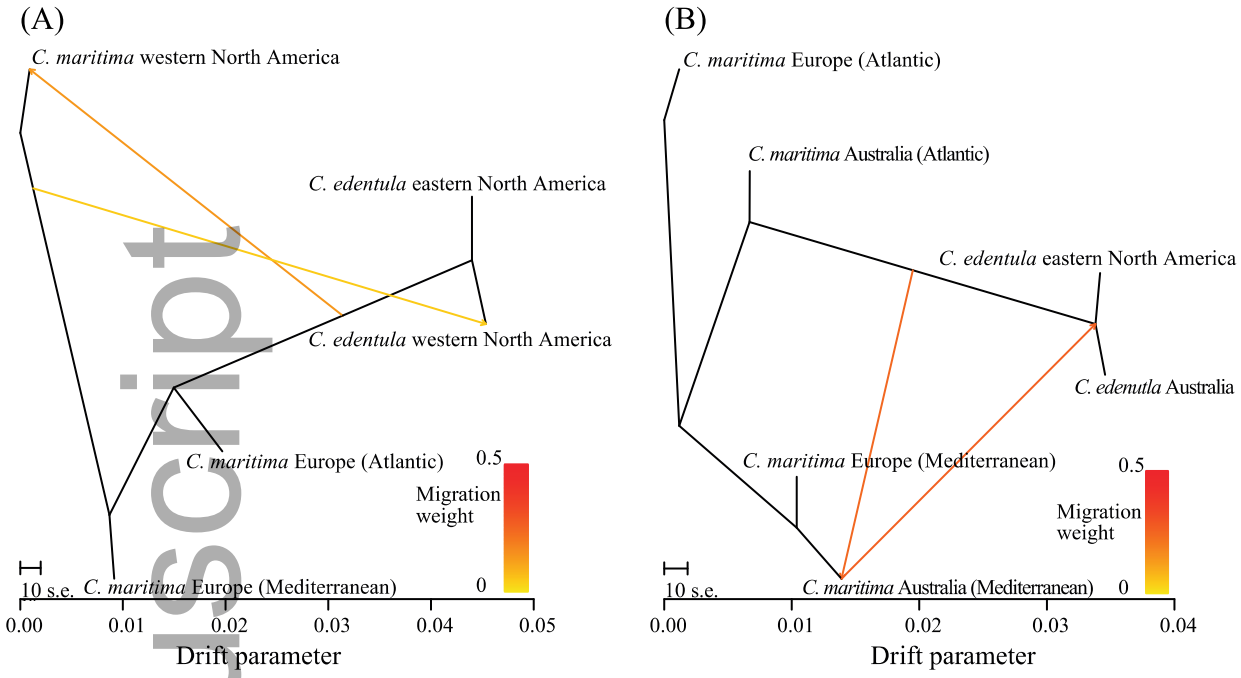



808

809 Figure 5 Results of the Spearman correlation test displayed (Table 2). The associations between
810 population mean Q values of hybrids identified using the *supervised* Admixture *run* and the ranked
811 order of populations from the first entry point of *C. maritima* (A) in eastern Australia and (B) western
812 North America.

813

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815

816 Figure 6 Maximum likelihood trees with two migration events generated by TreeMix. Native ranges
817 and (A) western North America, (B) Australia. Individuals are grouped by species (identified
818 morphologically), likely subspecies and geographic origin.

819 Table 1 Number of individuals and sampling locations as well as mean number of individuals sampled per sampling location in each range is
820 presented.

821

| **Range** | Phenotype | **Number of individuals** | **Number of sampling locations** | **Mean number of individuals sampled per sampling location** |
|---|---|---|---|---|
| **Eastern North America** | *C. edentula* | 55 | 26 | 2.03 |
| | *C. lanceolata* | 2 | 2 | 1 |
| **Europe and northern Africa** | *C. maritima* subsp. *integrifolia* and *baltica* | 12 | 12 | 1 |
| | *C. maritima* subsp. *maritima* | 12 | 12 | 1 |
| | *C. maritima* subsp. *islandica* | 1 | 1 | 1 |
| **Western North America** | *C. edentula* | 39 | 4 | 4 |
| | *C. maritima* | 79 | 10 | 5.9 |
| | Hybrids | 2 | 1 (in mixed) | / |

| | | | | |
|---|---|---|---|---|
| | Unknown | 1 | 0 (in *C. edentula*) | / |
| | Mixed populations | | 3 | 15.6 |
| | Total | 120 | 17 | 7.05 |
| **New Zealand** | Unknown | 1 | 1 | 1 |
| **Australia** | *C. edentula* | 43 | 3 | 7.33 |
| | *C. maritima* | 110 | 11 | 8 |
| | Hybrids | 14 | 5 (in mixed) | / |
| | Mixed population | | 7 | 8.4 |
| | Total | 167 | 21 | 7.95 |

822

823

824 Table 2 Results of the Spearman's rank correlation test in the introduced ranges examining the association between species ancestry for *C.*
825 *edentula*, *C. maritima* and hybrids or hybrids and the rank order of sampling locations based on the distance along the coastline from the
826 first recorded case of *C. maritima* in western North America (San Francisco) or south-east mainland of Australia (Adelaide). Spearman's
827 Rank Correlation Coefficient ρ and p values are presented for correlation between Q-value of the *supervised run* of the *C. edentula* cluster

828 for each population in western North America and Australia and correlation between ancestry index (S) (Figure 4) and rank order of
829 sampling locations.

830

| | | | Q | | | S | |
|---|---|---|---|---|---|---|---|
| **Range** | Species | # populations (# individuals) | ρ | p | # populations (# individuals) | ρ | p |
| **south-east Australia** | *C. edentula*, *C. maritima*, hybrids | 10 (65) | 0.815 | **0.004** | 10 (65) | 0.815 | **0.004** |
| | Hybrids | 7 (30) | 0.893 | **0.012** | 8 (38) | 0.905 | **0.005** |
| **western North America** | *C. edentula*, *C. maritima*, hybrids | 10 (68) | 0.511 | 0.132 | 10 (68) | 0.576 | 0.088 |
| **all sampling locations** | Hybrids | 5 (11) | 0.300 | 0.683 | 10 (50) | 0.467 | 0.213 |
| **western North America** | *C. edentula*, *C. maritima*, hybrids | 8 (47) | 0.719 | **0.045** | 8 (47) | 0.810 | **0.022** |
| **north of San Francisco** | Hybrids | 5 (11) | 0.300 | 0.683 | 7 (30) | 0.679 | 0.110 |

831

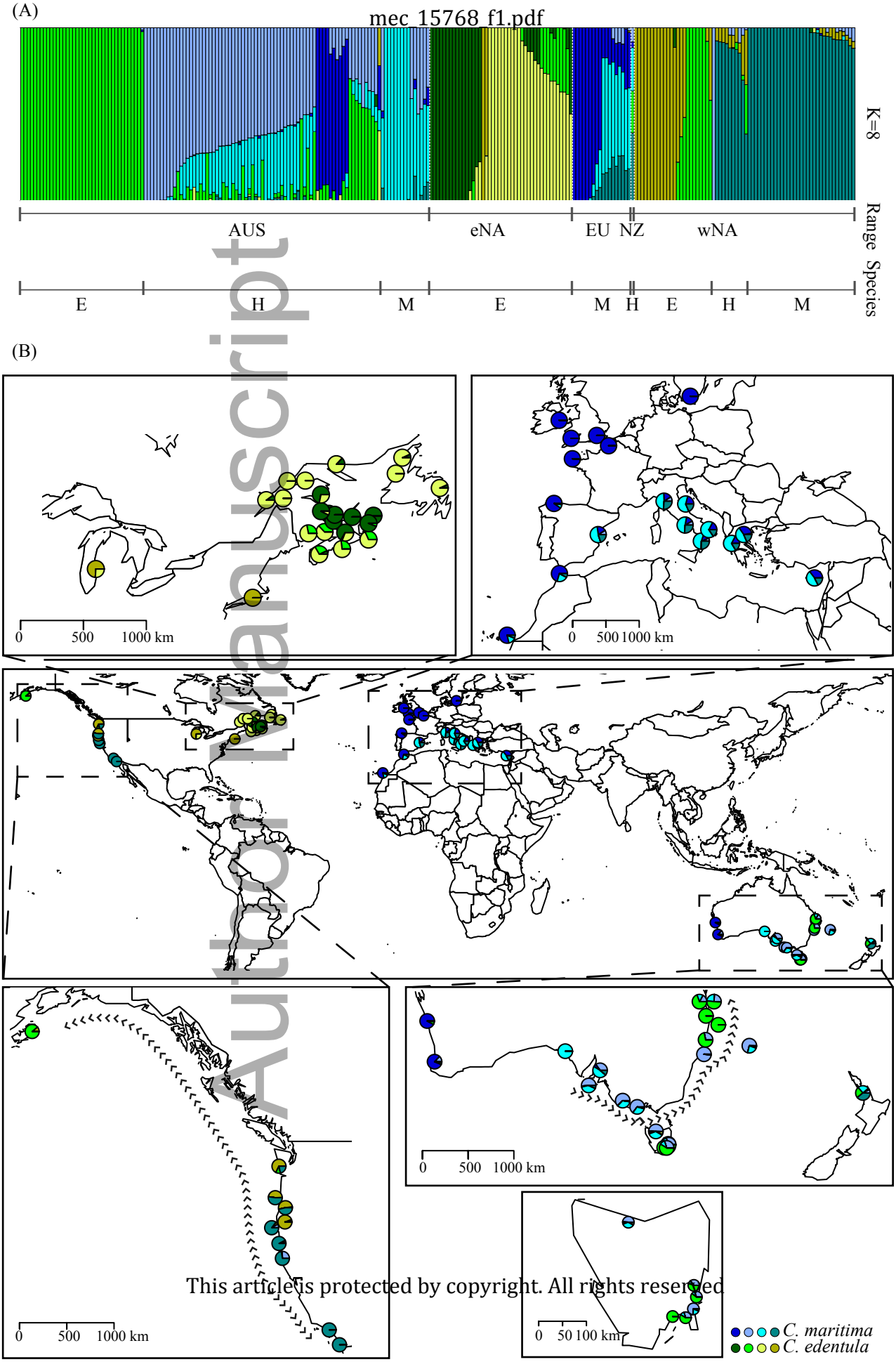
832 Table 3 Results of the $f_3$ statistic using TreeMix. Tests of admixture in the invasive range of Australia and western North America were done
833 separately and both were based on three groups (hybrids, *C. edentula, C. maritima*). Hybrid classification was done according to the
834 *supervised run* of Admixture. The $f_3$ statistic, the standard error of $f_3$ and the Z-score are reported.

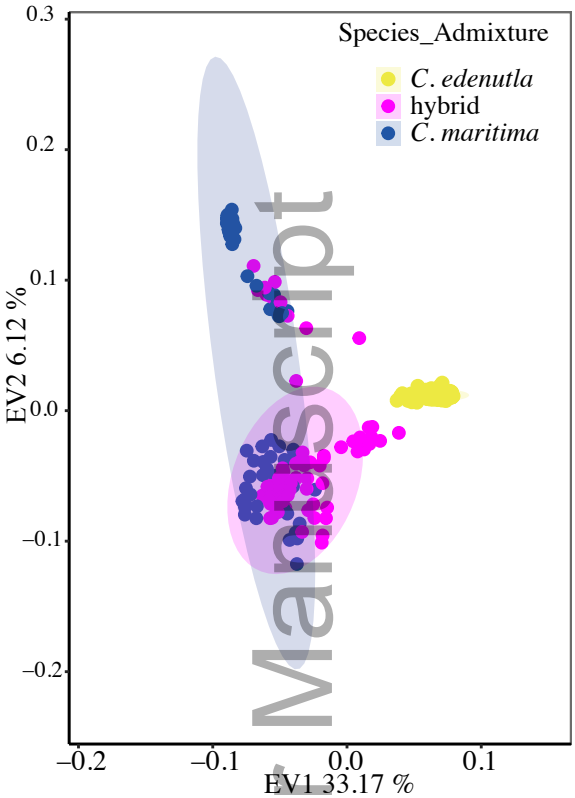
835

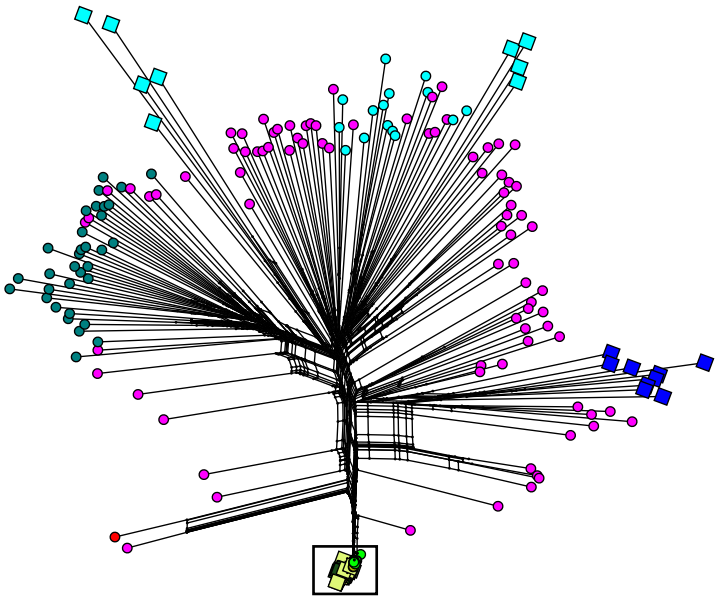
| **Range** | **Target** | **Source 1** | **Source 2** | $f_3$ | **Standard error** | **Z-score** |
|---|---|---|---|---|---|---|

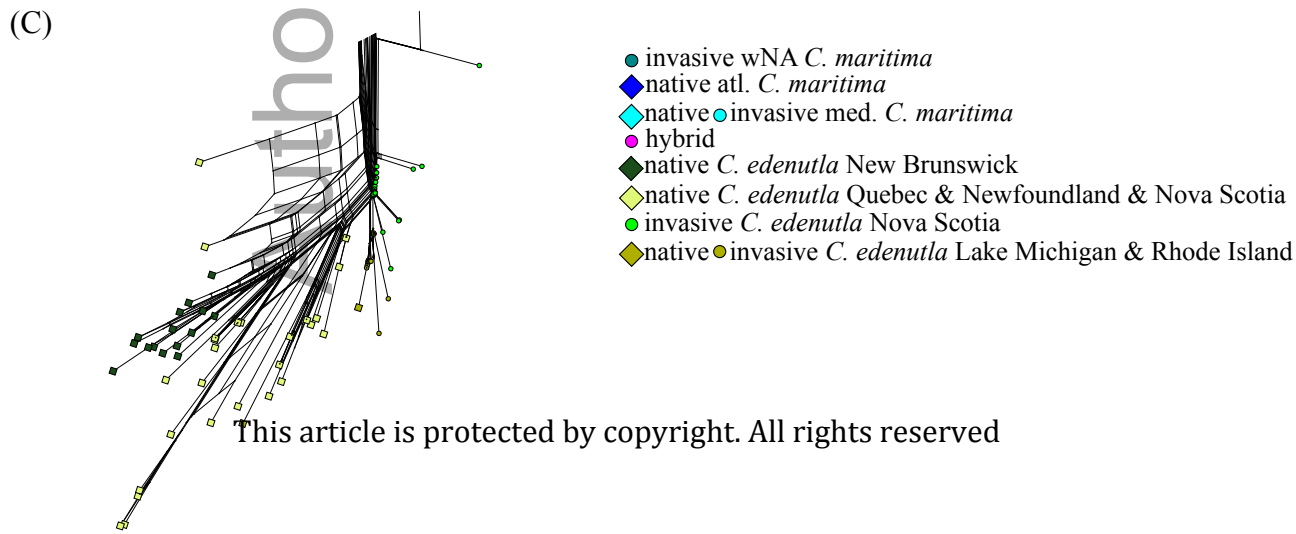
| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | **of $f_3$** | |
| **Australia** | Australian hybrids | Australian C. edentula | Australian C. maritima | **-0.0058** | 0.0002 | **-31.9723** |
| **w. North America** | w. North American hybrids | w. North America C. edentula | w. North American C. maritima | **-0.0049** | 0.0002 | **-23.2228** |

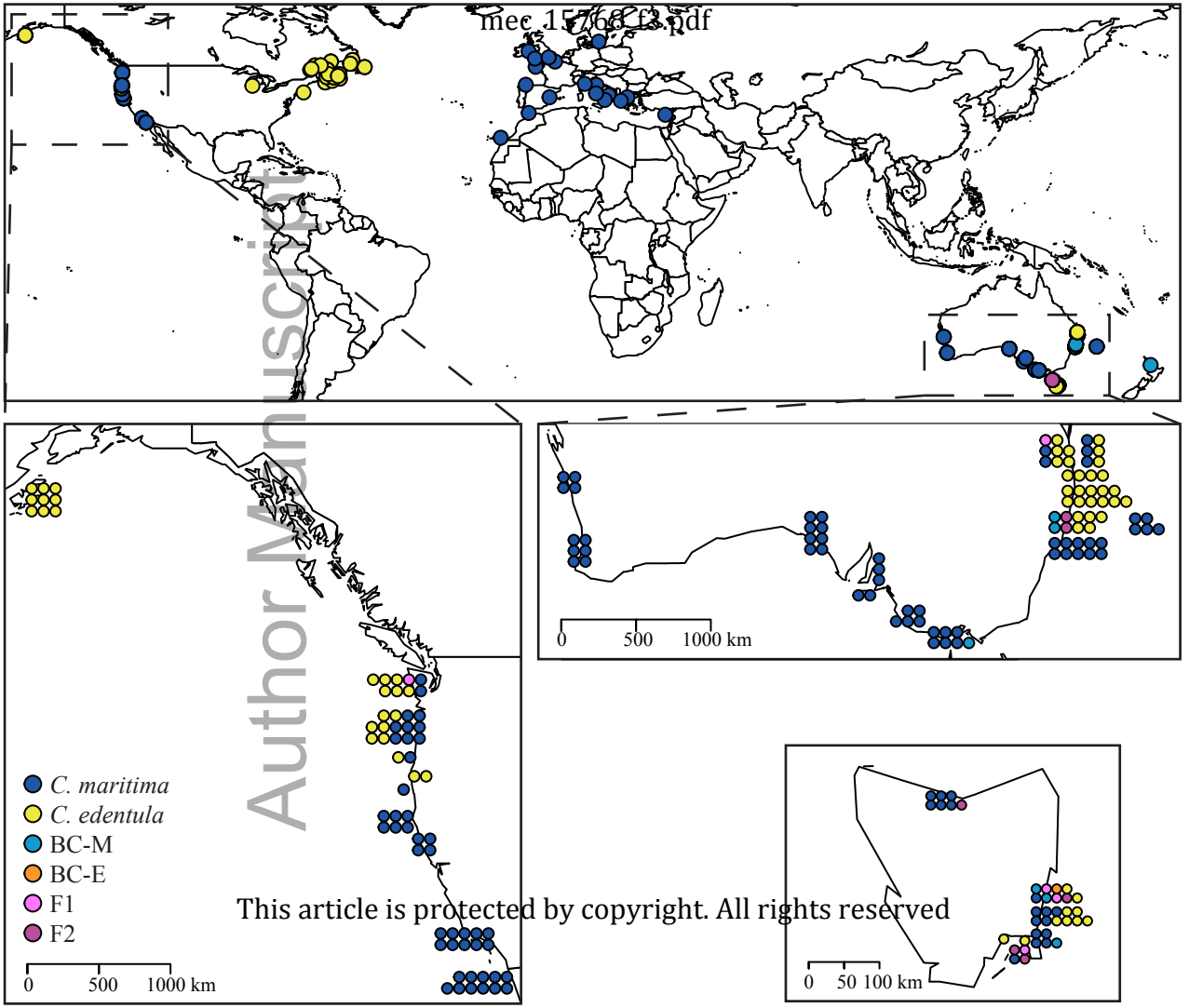
836



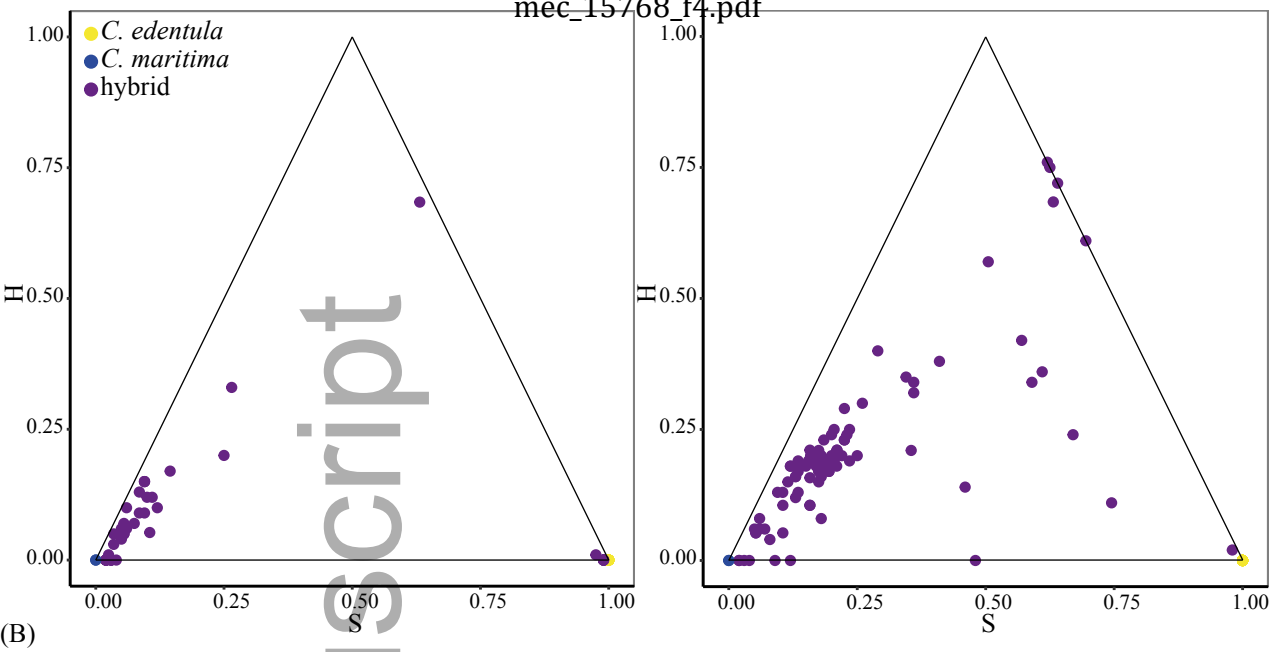




(A)




(B)