Algal Research 90 (2025) 104182

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Insights into the molecular and morphological diversity of *Asparagopsis taxiformis* in north-eastern Australia

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ARTICLE INFO

Keywords:
Asparagopsis
Great Barrier Reef
Genetic diversity
Phylogeography
Macroalgae
Molecular marker

ABSTRACT

The red seaweed, *Asparagopsis taxiformis*, consists of multiple genetic lineages that have spread globally through natural and human-mediated dispersal. Endemic to Australia and the Indo-Pacific, *A. taxiformis* has become an invasive pest in regions such as the Mediterranean Sea. Despite the commercial and ecological importance of this species, the genetic and morphological diversity of Australian populations remains understudied. Understanding this diversity, particularly within biodiversity hotspots like the Great Barrier Reef (GBR), is essential for advancing ecological knowledge and improving conservation and commercial management. This study investigated the genetic and morphological diversity of *A. taxiformis* within north-eastern Australia, by collecting tetrasporophyte specimens from 13 near and offshore locations in Queensland, including the GBR Marine Park. DNA was extracted from specimens and the chloroplast RuBisCO spacer and mitochondrial *cox2-3* intergenic spacer (*cox2-3)* were sequenced to assess genetic diversity. Nine morphological characteristics of the tetrasporophyte were also measured. While little genetic variation was found using the RuBisCO spacer, *cox2-3* analysis revealed that lineage 5 (L5) was present at two locations, lineage 6 (L6) dominated southern Queensland and lineage 4 (L4) was prevalent in the northern GBR, marking its first record in Australian coastal waters. Morphometric analyses identified significant differences in axial cell width and filament width among L4 and L5, with these traits modelled to be most useful for differentiating between lineages. The findings highlight substantial genetic and morphological diversity within *A. taxiformis* populations of Australia and the need for further systematic sampling in underrepresented locations.

1. Introduction

The tropical red seaweed *Asparagopsis taxiformis* (Delile) Trevisan, is recognised as an invasive species in many parts of the world, although it has also recently gained commercial importance in aquaculture, particularly in Australia, where it is considered endemic [1]. This species is a significant source of bioactive compounds, most notably bromoform, which is effective in reducing methane emission in ruminants when used as a feed additive [1,2]. The genus of *Asparagopsis* consists of two described species that are characterised by a triphasic heteromorphic lifecycle, alternating between a tetrasporophyte (2n), gametophyte (n) and carposporophyte (2n). Taxonomic identification of the tetrasporophyte phase of the lifecycle is considered challenging using morphological characteristics, therefore, the use of molecular techniques has been widely employed to assess species diversity and "invasive risk" [3,4]. While research has largely focused on the invasion risk of *A. taxiformis* in non-endemic regions [5,6], there is limited understanding of the genetic and morphological diversity that exists within Australian *A. taxiformis* populations.

The concept of a 'lineage' or 'operational taxonomic unit' (OTU) in evolutionary biology refers to a group of species or populations that share a common ancestor, with genetic distinctions arising from actions of evolutionary forces [7,8]. In the context of *A. taxiformis*, genetic lineages are defined by well-supported, monophyletic branches of phylogenetic trees, most commonly constructed from the mitochondrial cox2-cox3 intergenic spacer (*cox2-3*) and the chloroplast RuBisCO large subunit gene (*rbcL*) [5,9]. Lineages can be further divided into 'haplogroups', which are combinations of unique sequences or indels within

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https://doi.org/10.1016/j.algal.2025.104182
Received 27 March 2025; Received in revised form 30 June 2025; Accepted 1 July 2025
Available online 2 July 2025

each region of the genome. Lineages can signify distinct evolutionary histories or ecological isolation, with unique haplogroups often reflecting adaptation to specific environments [5].

Molecular markers, such as the *cox2-3* and the *rbcL*, have be used in conjunction with species delimitation models to help assess whether lineages meet thresholds for species differentiation [10,11]. Morphological differences, particularly in the gametophyte phase, alongside evidence of reproductive isolation, are also important indicators of speciation and ecological adaptations [5]. Additionally, environmental niche modelling and biogeographic surveys may further clarify whether lineages have adapted to distinct ecological conditions or exhibit geographic isolation, supporting evolutionary divergence [8]. Chemical analysis of secondary metabolites may also provide further insights into functional differentiation between lineages, with potential applications in guiding seedstock collection for aquaculture production [12].

Previous genetic studies of *A. taxiformis* have revealed six mitochondrial lineages, each with varying geographical distributions and ecological adaptations, primarily distinguished by differences in the rapidly evolving *cox2-3* spacer [10]. Divergence of *A. taxiformis* into two primary clades was first identified by Ni Chualain et al. [4] and further developed by Dijoux et al. [11], who grouped Lineage 1 (L1), Lineage 2 (L2), and Lineage 5 (L5) into one clade and Lineage 3 (L3) and Lineage 4 (L4) into another. Andreakis et al. [10] later expanded the first clade to include Lineage 6 (L6). L1 is found in the Hawaiian Islands and Panama, adapted to tropical reef environments at depths up to 15 m [13]. L2 in both Hawaii and Japan are reported to be native, based on diversity of haplotypes present at each location [13,14]. Both populations exhibit structuring of haplotypes across environmental gradients, aligning with a broad North Pacific temperate distribution of this lineage. L2 is also the dominant invasive lineage in the Mediterranean Sea, where the invasive success has been attributed to high morphological plasticity and year-round vegetative and sexual reproduction [15]. In the Mediterranean, L2 has replaced temperate *A. armata* populations due to climate-driven warming, with models indicating that as waters continue to warm, the distribution and abundance of *A. taxiformis* will increase to dominate these regions [8].

The origins of L3 are suggested to be within the Western Atlantic, and presence in the eastern Mediterranean is considered a recent invasion [4,5]. Observations of L3 within the Mediterranean were limited to tetrasporophyte samples, leaving its sexual reproduction potential and invasive risk uncertain [8]. L4 spans tropical Indo-Pacific regions, including Thailand, Micronesia, Papua New Guinea, the Hawaiian Islands, and the Cocos-Keeling Islands, indicating tolerance for high temperatures and intense light [10,14]. Despite these observations, there is a significant gap in sampling through northern Australia, leaving the presence of L4 in this region unclear. L5 is distributed across the South Pacific, including the Keppel Islands and Lord Howe Island, while L6 is considered endemic to both the east and west coasts of mainland Australia [10].

In addition to molecular diversity, morphological differences between lineages provide valuable insights into evolutionary adaptation and offer practical applications in aquaculture, particularly for identifying strains suited to cultivation [15,16]. While tetrasporophytes of *A. taxiformis* were historically considered morphologically indistinguishable, traits such as filament width, apical cell width and thickness of the cell wall have been identified as distinguishing characteristics between lineages [15]. Furthermore, variations in the reproductive structures of the gametophyte phase suggest that lineages have evolved and adapted to specific environmental pressures [17]. These traits, along with evidence of phenotypic variation, both within and between lineages [4,12], highlight the utility of morphology as a complementary tool for lineage differentiation and may support the selection of regionally adapted strains for commercial production.

This study provides a baseline assessment of the genetic and morphological diversity of *A. taxiformis* within Queensland, with a particular focus on populations in and around the Great Barrier Reef (GBR) Marine Park. This research addresses key knowledge gaps in sampling through north-eastern Australia, to determine whether distinct genetic lineages are present and whether these represent native diversity or recent introductions. Characterising the population structure and morphological variation of this species is particularly important for informing conservation and management strategies in ecologically significant regions such as the GBR. In addition, improved understanding of genetic population structure may support the development of *A. taxiformis* for commercial aquaculture in north-eastern Australia by guiding seedstock collection and informing the risks associated with translocating genetic material within the region.

2. Methods

2.1. Collections

Tetrasporophytes ($n = 107$) of *A. taxiformis* were collected from 13 locations along the Queensland coast, between August 2022 and August 2024 (Table 1). Collections were authorised under permit from the Great Barrier Reef Marine Park Authority (G24/50049.1) and Department of Agriculture and Fisheries accepted development notification (2405–168,788 ADR-MP). A single tetrasporophyte was collected from nine of these locations, with larger collections from Lizard Island ($n = 24$), Cairns (n = 24), Magnetic Island ($n = 30$) and Kissing Point ($n = 20$). At sites where multiple samples were collected, individuals were collected by hand, at a distance ≥20 m from the previous sample in order to reduce the chance of sampling clonal specimens. Samples were transported live in individual tubes to a laboratory at James Cook University, Douglas, Queensland, manually cleaned of epiphytes, and apical cells isolated to establish clonal cultures of each tetrasporophyte. Cultures were propagated and maintained under controlled laboratory conditions for at least 2 months before harvest, ensuring sufficient mass of clonal cultures free from visually obvious contaminants. A total of 63

Table 1
Collection date and number of *Asparagopsis taxiformis* samples collected and sequences generated (for lineage assignment) from sites in north-eastern, Australia. Thirteen sampling 'locations' are further grouped by geographic region. The mitochondrial *cox2-cox3* intergenic spacer (*cox2-3*) and the chloroplast RuBisCO spacer (*rbcL-rbcS*) were sequenced for each sample. Where not specified, the number of sequences was the same for both markers.

Region	Location	Collection date	Samples collected (n)	Sequences retained for lineage assignment
Lizard Island	Lizard Island	July 2024	24	14 (*cox2-3*), 13 (*rbcL-rbcS*)
Cairns	Cairns	October 2023	24	9 (*cox2-3*), 10 (*rbcL-rbcS*)
Townsville	Magnetic Island	June 2023	30	16
	Kissing Point	May 2023	20	13
	Arthur Bay	August 2024	1	1
	Townsville Reef	June 2023	1	1
Bowen	Bowen	August 2022	1	1
	Bowen South	September 2022	1	1
Rockhampton	Rockhampton	January 2023	1	1
Bundaberg	Bundaberg	January 2023	1	1
Caloundra	Caloundra	January 2023	1	1
	Caloundra Beach	January 2023	1	1
	Mooloolaba	January 2023	1	1

surviving specimens were harvested, dried of excess water and snap-frozen in liquid nitrogen. Samples were stored at −80 °C prior to DNA extraction.

2.2. DNA extraction and amplification

Approximately 25 mg of snap-frozen tissue was weighed and placed into a tube containing 700 μL of preheated cetyl-trimethylammonium bromide (CTAB) buffer with 2 % PVP-40. The tissue was then ground within the solution using a microfuge pestle, followed by the addition of 10 μL of proteinase K (20 mg/mL) and 17 μL of β-mercaptoethanol (2.5 % *v*/v). After incubation at 60 °C for one hour, 10 μL of Qiagen RNase A (100 mg/mL) was added to the solution and allowed to incubate at room temperature for a further 10 min. DNA was extracted using 700 μL of chloroform-isoamyl alcohol (24:1), followed by precipitation with isopropanol and incubation at −20 °C for 16 h to enhance DNA recovery. The DNA pellet was washed sequentially with 100 % and 70 % ethanol to remove impurities. The pellet was air dried for 20 min, then rehydrated with 50 μL of Qiagen EB buffer. DNA concentration and quality were assessed using a Nanodrop spectrophotometer. The integrity of the DNA was further verified on a 1 % agarose gel.

From the extracted DNA, all 63 samples were amplified individually for *cox2-3* [18] and RuBisCO spacer [17]. Polymerase chain reactions (PCR) for *cox2-3* were conducted in 50 μL volumes, using AccuPower Hot Start PCR pellets, with 1 μL template DNA, 2 μL bovine albumin serum (BSA), 1.5 μL of each forward and reverse primer (100 uM), 0.5 uL MgCl2 and 43.5 μL of ultrapure water. Amplification conditions for the *cox2-3* follow the method outlined in [18].

For the RuBisCO spacer, reactions and amplification conditions followed the protocol described in [17], with the exception of BSA, which was replaced by ultrapure water.

PCR products were visualised on a 2 % agarose gel after electrophoresis at 80 V for 60 min. PCR products were then sent to Macrogen Inc., Korea for purification and strand sequencing in both directions.

2.3. Molecular analysis

Forward and reverse sequences of each target gene were assembled into a consensus sequence for each individual sample using the default assembler in Geneious Prime 2024.0.7 and then aligned against consensus sequences from other individuals using the Geneious Clustal Omega 1.2.2 function with 10 refinement iterations. Sequences were trimmed manually from their ends to avoid the influence of missing data and BLASTn [19] was used to confirm that all sequences presented a similarity of >98 % to *A. taxiformis*.

Phylogenetic analysis was computed using IQ-TREE 2 v2.3.5 [20] with the best-fit model according to ModelFinder [21], 1000 ultrafast bootstraps [22] and Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT) [23]. Maximum likelihood (ML) trees were visualised using FigTree v1.4.4 [24] and haplotype networks were constructed using the median joining network method in PopART v1.7 [25].

For lineage assignment, 65 representative *cox2-3* sequences and eight representative RuBisCO spacer sequences of known lineage from GenBank [26] (see Supplementary Table 2) were aligned against sequences obtained in this study. The datasets generated for each marker were used to create final ML trees, using the same method described above. For the *cox2-3* analysis, *Asparagopsis armata* (AY589521 [3]) was added as an outgroup and used to root the tree. Tamura-Nei genetic distances were calculated using the ape package in R [27]. The dist.dna function was employed to compute the pairwise distances between lineages with standard error computed from 1000 bootstrap replicates.

To prevent the influence of missing data on population diversity analysis, the *cox2-3* data set from this study was further trimmed to 311 bp and seven individual samples were removed (see Sup. Table 1). Population diversity indices, including the number of sequences (n), number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π), average number of nucleotide differences (k), and the number of segregating sites (S), were calculated using DnaSP6 v12.03 [28]. Population genetic structure was visualised using Principal Component Analysis (PCA) and Netview analysis implemented in R v4.3.0 [29], utilising the packages adegenet v2.1.10 [30] and igraph v1.5.0 [31]. Haplotype accumulation curves were generated using the ape package [27], based on 200 permutations. Analysis of Molecular Variance (AMOVA) was performed in R using the poppr v2.9.4 package [32] to partition genetic variance within and among populations, with significance assessed using 999 permutations.

2.4. Morphological characteristic assessment and analysis

To determine if differences exist in morphological characteristics between lineages of *A. taxiformis* on the GBR, single tetrasporophytes from 10 individuals of known lineage were harvested from laboratory cultures. Apical tips of each tetrasporophyte were chopped and transferred to a 3 cm Petri dish with autoclaved filtered seawater and half strength F/2 media [33]. Filaments of each tetrasporophyte were grown under controlled laboratory conditions (26 °C, 10 h light:14 h dark) for 4 weeks, to ensure environmental conditions were consistent for all specimens.

A sample of filaments from each individual were stained for 10 min in 1 % aniline blue solution (1 g aniline blue in a 1:99 solution of glacial acetic acid: deionised water), rinsed in autoclaved sea water and mounted on a glass slide. To account for biological variability within individuals, 12 separate filaments from each individual were viewed and photographed by microscope (Olympus SZ61). For each filament, nine morphological parameters were measured using ImageJv1.54g [34] (Fig. 1): 1) width of apical cell (wac), 2) length of apical cell (lac), 3) thickness of cell wall (tcw), 4) width axial cell (waxc), 5) length axial cell (laxc), 6) filament width (fw), 7) vesical cell diameter (vcd), 8) length of 10th cell (l10), 9) pericentral cell distance (dpc). All non- apical cell parameters were measured at, or as close as practicable, to the 10th cell from the apical tip.

For the morphological characteristics, coefficient of variation (CV) and standard error (SE) were calculated to assess natural variability and pseudo-replication within replicate measurements for each individual. Tests for multivariate normality were conducted using the MVN v5.9 package [35] to classify characteristics into normal and non-normal groups. For normally distributed characteristics, a mixed-effects model with a Gaussian identity-link was fitted using lme4 v1.1–34 [36], while non-normal characteristics were analysed with a mixed-effects model incorporating a gamma log-link. Post hoc pairwise comparisons were performed using the emmeans v1.8.8 package [37]. A random forest model was developed using randomForest v4.7–1.1 [38] to classify individuals to populations, and variable importance was assessed via the MeanDecreaseGini metric.

3. Results

3.1. Molecular analyses

Following successful PCR amplification for each region, 61 consensus sequences were generated for the *cox2-3* and 62 for the RuBisCO spacer (Table 1). Post alignment, *cox2-3* sequences were trimmed to 336 bp (ungapped alignment) and RuBisCO spacer sequences trimmed to an average of 278 bp. The alignment of the RuBisCO spacer sequences contained an indel gap of 5 bp in some samples, indicating a possible insertion or deletion event. Within the 61 *cox2-3* sequences retained, 272 (79.5 %) sites were identical. Less sequence variation was observed within the RuBisCO spacer, with 275 (97.5 %) identical sites. All sequences generated from this study were confirmed to match *A. taxiformis* through the Blastn database search. A subset of unique sequences from this study were deposited in GenBank under accession numbers provided in the supplementary material (see Sup.

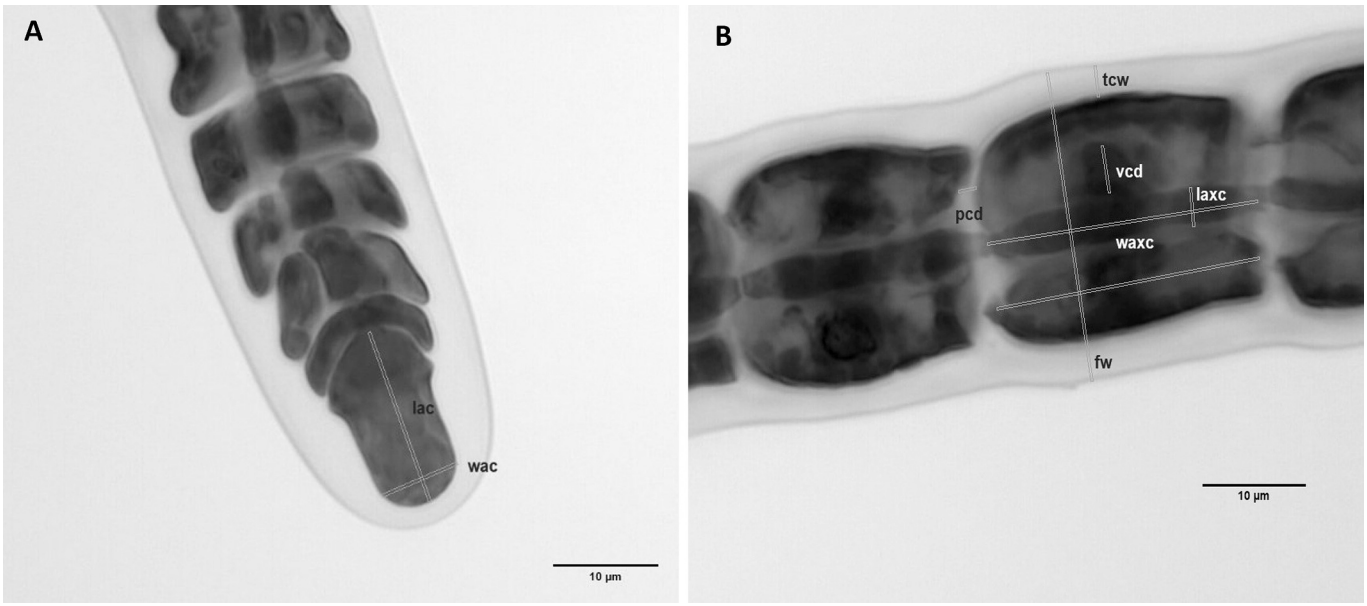

Fig. 1. Morphological characteristics of *Asparagopsis taxiformis* tetrasporophyte measured at the (A); apices of the filament, wac – width of apical cell and (B); lac – length of apical cell at the tenth cell, tcw – cell wall thickness, waxc – width of axial cell, laxc – length of axial cell, vcd – vesicular cell diameter, fw – filament width, pcd – pericentral cell distance.

Table 1).

Phylogenetic analysis and ML tree construction of the *cox2-3* sequences generated in this study clearly resolved three of the six previously proposed lineages for *A. taxiformis* (Fig. 2b) [10]. Of the 61 *cox2-3* sequences, 65.6 % (40 samples) clustered within L6, 4.9 % (3 samples) clustered within L5 and 29.5 % (18 samples) clustered within L4. In contrast, ML analysis of the RuBisCO spacer sequences, which exhibited lower sequence variability than the *cox2-3*, resolved only two clades. Samples assigned to lineages L5 and L6 based on *cox2-3* grouped into a single clade, distinct from L4 (SH-aLRT = 91.7 %, ultrafast bootstrap = 82 %) (see Sup. Fig. 1).

On a population level, L6 was present across all sample sites, with L4 only present at Lizard Island, Cairns, and one location in Townsville. L5 was found to be present only in the two northern sample sites of Cairns and Lizard Island. The Lizard Island population was composed of 50 % L4, 14.3 % L5 and 35.7 % L6 (Fig. 3). It is worth noting that within this location, L5 were only found on the exposed edge of the northern-most part of the island and were not present in the south-western lagoon. The Cairns population had the highest proportion of L4 at 55.6 %, 11.1 % L5 and 33.3 % L6. The Townsville population (inclusive of four sample locations; Table 1) had the highest proportion of L6 at 80.6 %, with the remaining 19.4 % of the population being L4. Within this population, one site was entirely composed of L6, with another composed of a mix of L4 and L6. All samples collected from locations south of Townsville were L6 (Fig. 3). Within the three locations (Lizard Island, Cairns and Magnetic Island) that were composed of both L4 and L6, there did not appear to be any obvious visual pattern of structuring, with individuals of each lineage dispersed across the transect in sympatry.

Following phylogenetic analysis and lineage assignment, the reduced 311 bp dataset of 54 *cox2-3* sequences was used to examine haplotype variation among lineages and to calculate population diversity indices. The remaining 14 L4 samples could be separated into two haplotypes, sharing 99.7 % of sites, with one base difference between them. The 37 L6 samples shared 96.8 % of sites with five separate haplotypes, three of which were only represented by one sample and therefore cannot be ruled out as anomalous. The three L5 samples were identical, sharing 100 % of sites.

When compared with other *cox2-3* sequences of *A. taxiformis* available in GenBank (Fig. 2a & b), the most common L6 haplotype from this study was identical to the L6 'GBR strain' identified in [9]. The main cluster of L6 samples from this study varied by 7 nucleotides (2.25 %) to the samples identified as L5, and by a minimum of 22 nucleotides (7.07 %) to the samples identified as L4 in this study. The L5 samples share a 100 % similarity to haplotype G [11] samples previously collected from the Keppel Islands in the GBR, Lord Howe Island and New Caledonia, with a single nucleotide difference between haplotype F from Norfolk Island, French Polynesia and Kermadec Island [10]. The majority of L4 samples from this study differed from the L5 samples by a minimum of 15 nucleotides (4.82 %) and were identical to historical sequences of haplotype D, from Cocos-Keeling Islands, Hawaii and Naos Island, Panama [10,11,14]. The second haplotype of the L4 samples, shared by two individuals from this study, were identical to haplotype C, from Guam and Papua New Guinea (Fig. 2b).

The RuBisCO spacer sequences of *A. taxiformis* obtained from Genbank supported the separation of sequences obtained in this study in to two genetic clades, with L6 and L5 samples clustered together and L4 samples separated by only two nucleotides. Notably, invasive L4 samples from Israel do not cluster with L4 samples from this study, although only a single base different. The Israeli samples appear to be more closely related to sequences from samples previously described for the Canary Islands and Puerto Rico, which were assigned to L3 [9] (Sup. Fig. 1).

The Tamura-Nei (TN) corrected distances between all six lineages in the combined dataset (Genbank and this study) revealed substantial genetic divergence (Fig. 4). The highest mean divergence occurred between L3 and L6 (0.063 ± 0.002) and L4 and L6 (0.062 ± 0.002), while L1 and L2 and L5 and L6 had the lowest divergence (0.021 ± 0.002 and 0.023 ± 0.002, respectively). These findings indicate that L3 and L6, followed by L4 and L6 are the most genetically distinct, whereas L1 and L2, along with L5 and L6, are more closely related.

3.2. Diversity indices

Cox2-3 sequences from this study, (Queensland population; Table 2), revealed a haplotype diversity of 0.604 ± 0.064 and nucleotide diversity of 0.028 ± 0.003, with 8 haplotypes identified among 54 sequences. The number of variable sites (31) showed evidence for a moderate level of genetic differentiation, while Tajima's D (0.943, $P > 0.01$) indicated

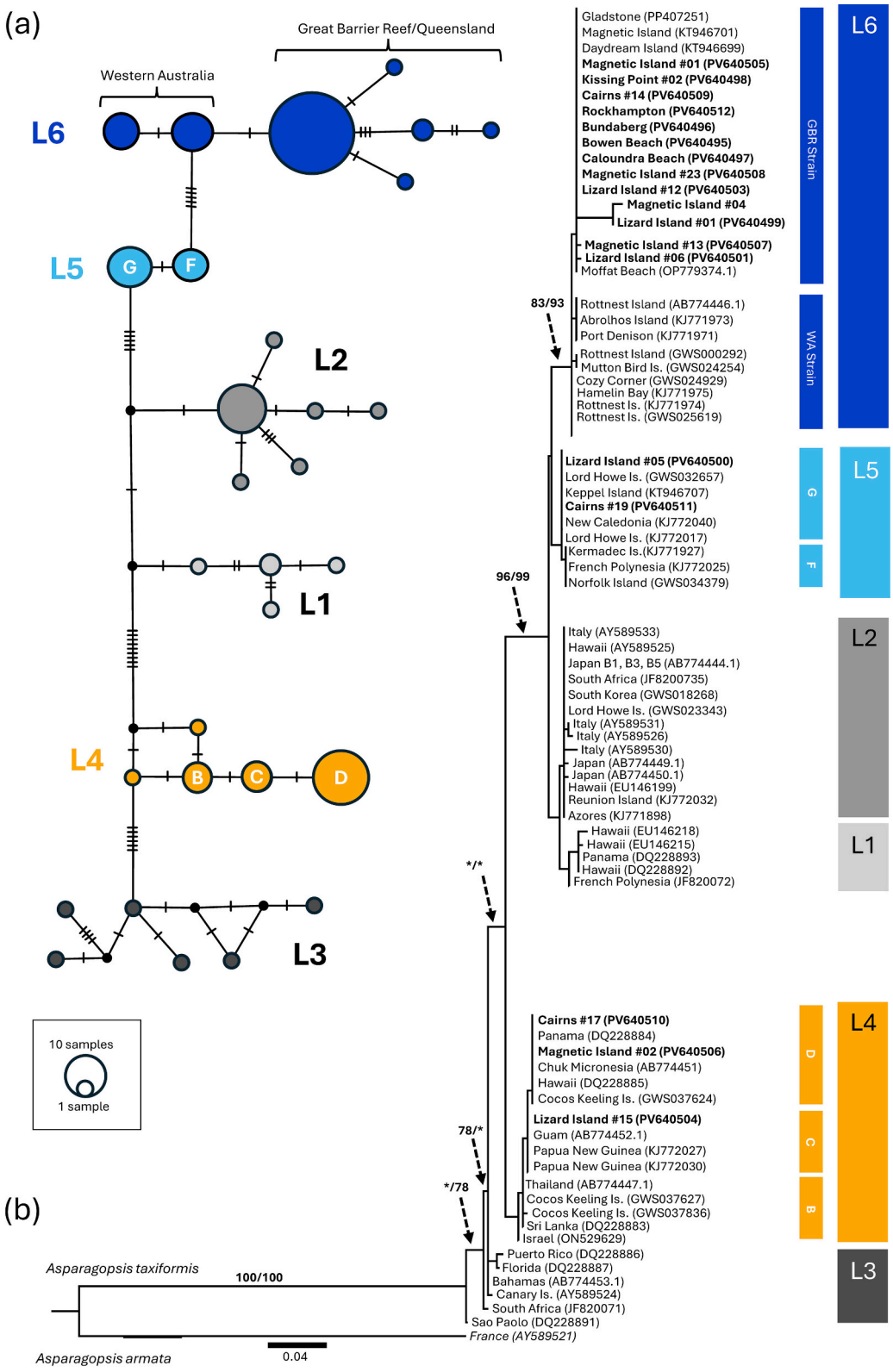

Fig. 2. Phylogenetic relationships of *Asparagopsis taxiformis* lineages 1–6, based on 65 historical *cox2-3* intergenic spacer (*cox2-3*) sequences and 54 novel sequences generated in the present study. Haplotypes B—D and G-F (Dijoux et al., 2014) denote the most frequently observed haplotypes of Lineage 4 and 5 respectively. The Great Barrier Reef (GBR) and Western Australia (WA) strains are annotated according to the classifications of Andreakis et al. (2016). **(a)** Median-joining haplotype network. Circles represent individual haplotypes, with circle size proportional to haplotype frequency. Black 'dots' indicate a median vector (unsampled or extinct taxa) between the lineages. Hatch marks along the lines represent number of mutational steps between haplotypes. **(b)** Maximum likelihood phylogenetic tree (log likelihood = −1011.06), rooted with *Asparagopsis armata* as the outgroup. Branch support values are shown as Shimodaira-Hasegawa approximate likelihood ratio test (%) and ultrafast bootstrap support (%). Support values below 70 % are represented by an asterisk (*). Samples highlighted in bold represent unique sequences identified in the present study.

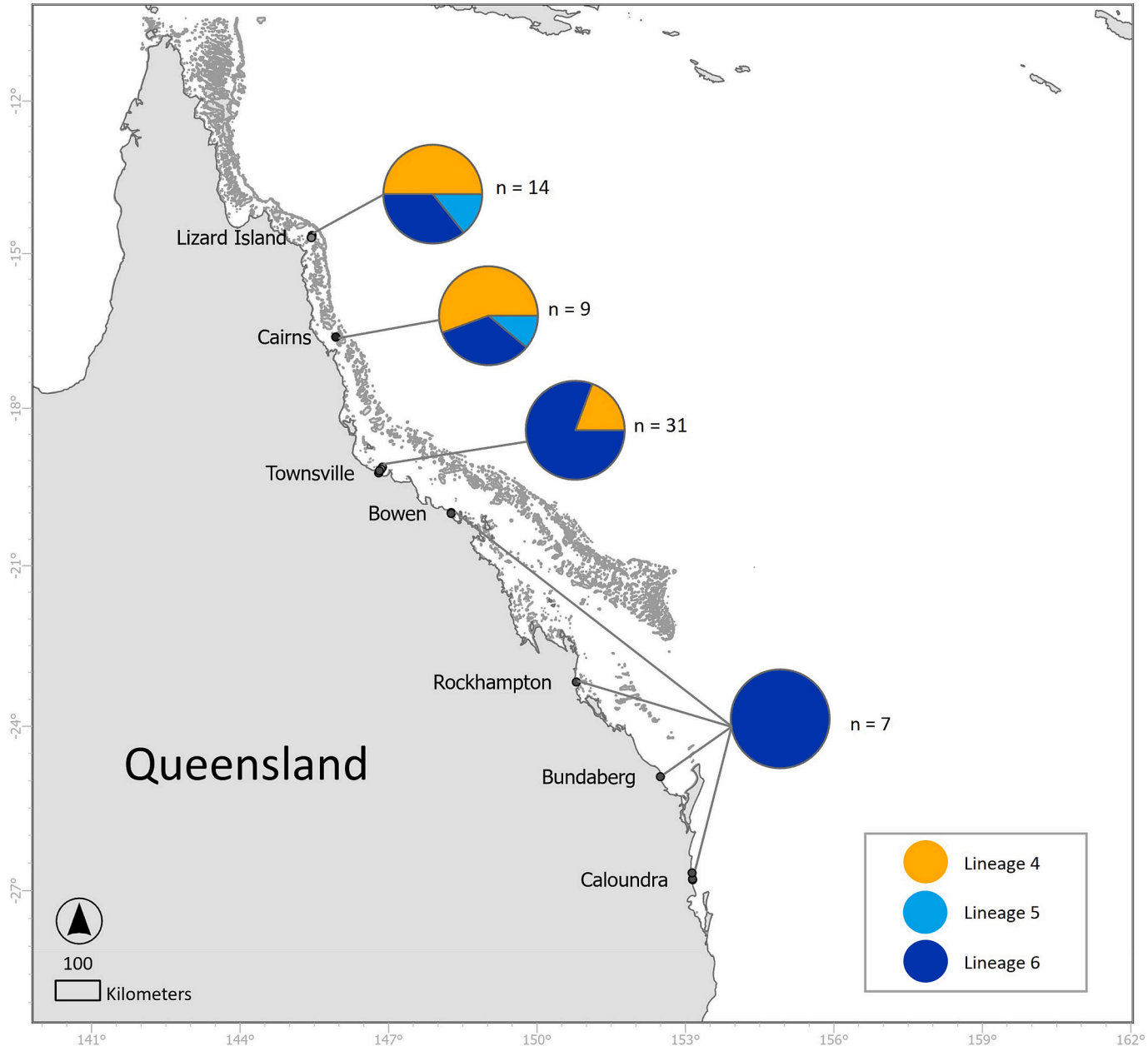

Fig. 3. Mitochondrial lineage composition of *Asparagopsis taxiformis* populations in north-eastern Australia. Individual pie charts grouped by collection 'Region' in Table 1. n = number of sequences included in population. Map created using ArcGIS Pro [39].

neutrality, with no strong evidence of recent selection or demographic shifts. Site-specific patterns revealed that Lizard Island exhibited the highest diversity (Hd = 0.889 ± 0.075, π = 0.037 ± 0.006), with 6 haplotypes among 10 sequences. Cairns showed moderate diversity (Hd = 0.639 ± 0.126), however, the Tajima's D (1.912) suggested potential balancing selection or population structure. The diversity of Magnetic Island was similar to the overall Queensland population (Hd = 0.608 ± 0.086), suggesting it may act as a genetic intermediary or reservoir. Kissing Point displayed the lowest diversity (Hd = 0.200 ± 0.154, π = 0.005 ± 0.003) with two haplotypes among 10 sequences, and negative Tajima's D (−1.839). The haplotype accumulation curves (Fig. 5) further support these findings, indicating variation in haplotype richness between populations, with higher richness and potentially undiscovered haplotypes in northern populations. Although most population curves did not reach an asymptote, the curve for Queensland suggests that sampling was sufficient to detect the majority of haplotypes across the broader study area.

The AMOVA conducted on all *cox2-3* sequences (n = 54), revealed significant genetic differentiation among the populations sampled. The total genetic variance was partitioned into 16.28 % among populations and 83.72 % within populations (Sup. Table 3). A permutation test (999 replicates) confirmed that the among-population variance component was statistically significant (Φ_{ST} = 0.163, p = 0.014), indicating moderate genetic structure. The within-population variance component, treated as residual variation, was not independently tested for significance.

The first two principal components (PC1 and PC2) of the PCA explained a significant portion of the genetic variance among individuals, accounting for 58.78 % and 23.45 % of the total variation, respectively (Fig. 6). The PCA plot reveals that individuals cluster primarily according to mitochondrial lineage rather than geographic population. The NetView analysis complements these results by revealing two distinct genetic clusters among the samples (Sup. Fig. 2). Each cluster contained a mix of populations, indicating that the clustering is

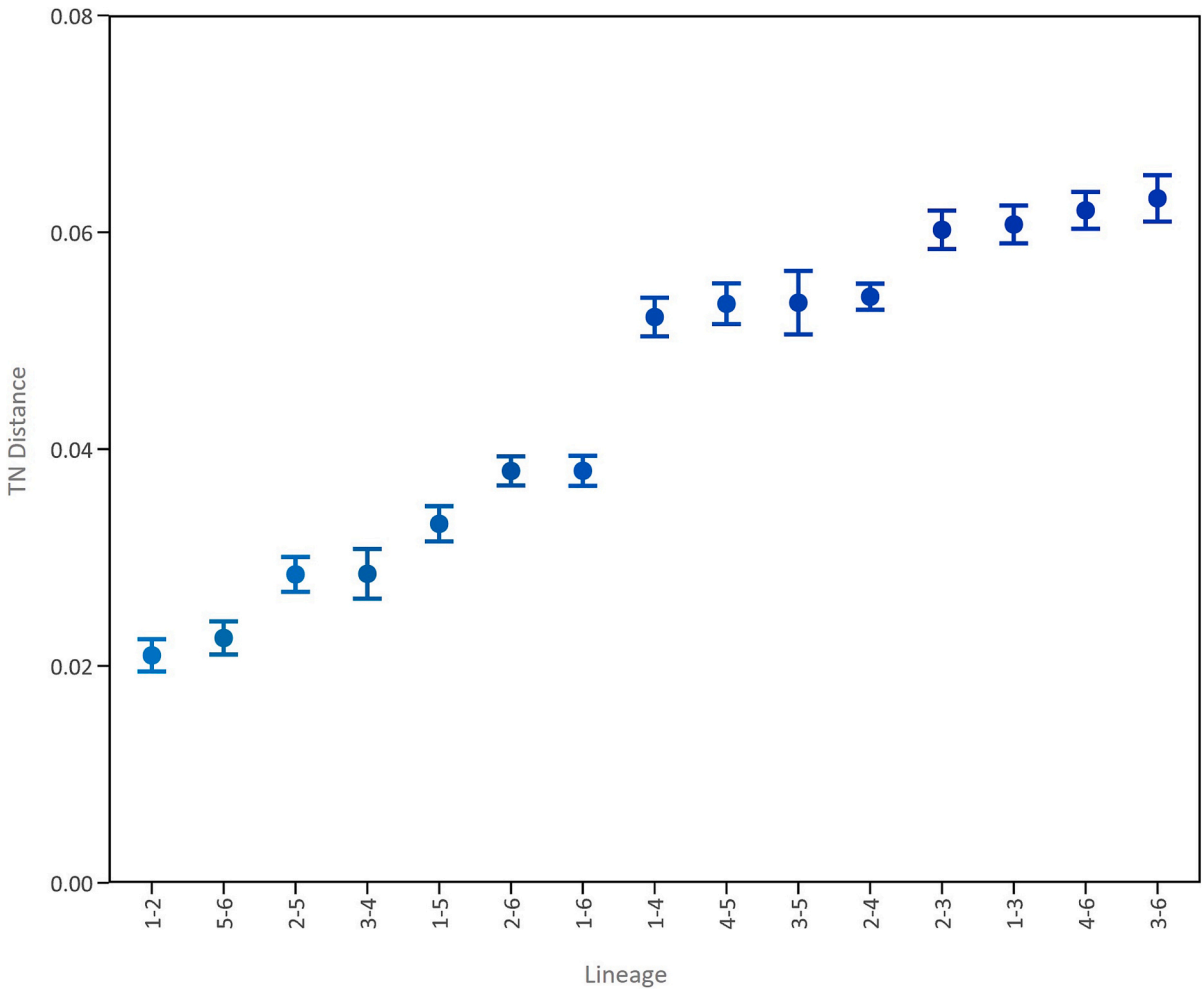

Fig. 4. Tamura-Nei genetic distances (± SE) between lineages (1–6), calculated on 1000 bootstrap replicates of 119 *Asparagopsis taxiformis* mitochondrial *cox2-cox3* intergenic spacer (*cox2-3*) sequences.

Table 2
Genetic diversity indices of *Asparagopsis taxiformis* based on the mitochondrial *cox2-cox3* intergenic spacer (*cox2-3*). 'Queensland' represents all 54 sequences from this study, with the remaining 'populations' grouped by geographical sampling locations.

Population	Locations	n	Total n	H	Hd	π	k	S	Tajima's D
Queensland	All	54	54	8	0.604 ± 0.064	0.028 ± 0.003	8.746	31	0.943
Lizard Island	Lizard Island	10	10	6	0.889 ± 0.075	0.037 ± 0.006	11.356	29	0.517
Cairns	Cairns	9	9	3	0.639 ± 0.126	0.035 ± 0.005	10.722	21	1.912
	Magnetic Island	16							
Magnetic Island	Arthur Bay	1	18	4	0.608 ± 0.086	0.032 ± 0.005	10.050	29	0.771
	Townsville Reef	1							
Kissing Point	Kissing Point	10	10	2	0.200 ± 0.154	0.005 ± 0.003	1.400	7	−1.839
	Bowen	1							
	Bowen South	1							
	Rockhampton	1							
Other Queensland	Bundaberg	1	7	0	n/a	n/a	n/a	n/a	n/a
	Caloundra	1							
	Caloundra Beach	1							
	Mooloolaba	1							

n – number of sequences included in analysis; H – number of haplotypes; Hd – haplotype diversity and standard deviation (sd); π – nucleotide diversity and sd; k – average number of nucleotide differences; S – segregating sites. Tajima's D was not significant for any of the populations.

based on genetic lineage assignment rather than geographic location. This suggests that the genetic variation within the dataset is structured by lineage, with individuals from different populations grouping together based on their genetic similarities.

3.3. Morphological analysis

Due to the small and uneven number of individuals within each lineage, all replicate measurements for individuals were analysed. To account for pseudo-replication, the variance of replicate measurements for an individual was assessed using the coefficient of variation (CV) and

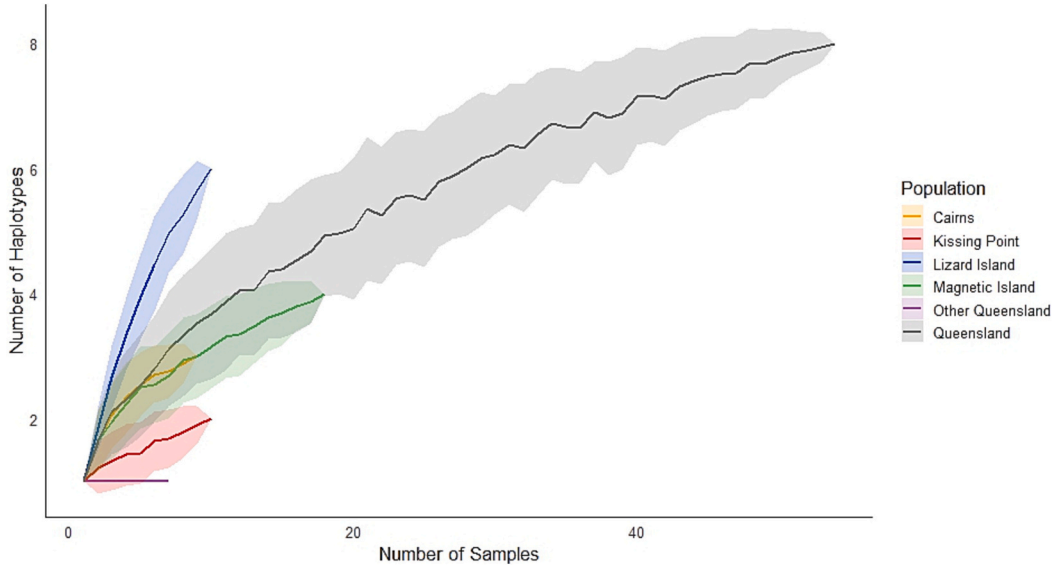

Fig. 5. Haplotype accumulation curves for *Asparagopsis taxiformis* populations defined in Table 2. Curves represent the mean number of unique mitochondrial *cox2-3* intergenic spacer (*cox2-3*) haplotypes detected in each population with increasing sample size, based on 200 random permutations of sample order. 'Queensland' represents the pooled sequences of all populations. Shaded areas indicate ±1 standard deviation.

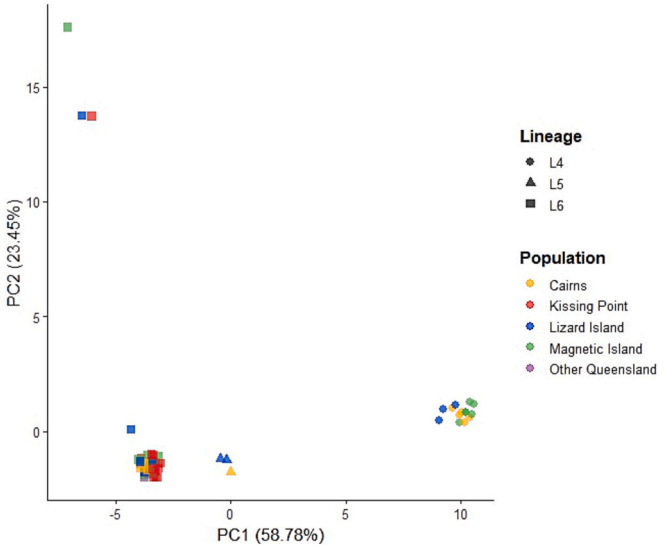

Fig. 6. Principal Coordinate Analysis (PCA) of *Asparagopsis taxiformis* individuals, based on pairwise Euclidian distances of the mitochondrial *cox2-3* intergenic spacer (*cox2-3*). Populations (defined in Table 2) are represented by colour, with mitochondrial lineage represented by shape. The PC1 and PC2 explain 58.78 % and 23.4 % of the total genetic variation, respectively. A jitter filter was applied along both axes to reduce overlap among data points.

standard error (SE). Variation was observed within all individuals for each characteristic. However, the mean CV across individuals for each characteristic was consistent, with a small SE. Cell wall thickness and pericentral cell distance were the most variable characteristics (CV > 20 %) within individuals, while filament width exhibited the lowest variability (CV = 8.75 % ± 0.97).

Tests for multivariate normality revealed that not all morphological characteristics were normally distributed, justifying the separation of the data into normal and non-normal groups for statistical analysis. For normally distributed characteristics, a mixed-effects model with a Gaussian identity-link was employed, however, no significant differences among lineages were detected. For non-normal characteristics, a mixed-effects model with a gamma log-link was used, treating individuals as a random effect. Significant population-level differences were identified for axial cell width ($p = 0.048$) and filament width ($p = 0.018$). Post hoc analysis revealed that individuals from L5 had a significantly larger axial cell width (4.85 ± 0.17 μm) compared to L4 (3.74 ± 0.09 μm; $z = -2.322$, $p = 0.049$), though not significantly different from L6 (4.44 ± 0.18 μm; $z = 0.927$, $p = 0.623$). Similarly, filament width in L5 (40.87 ± 1.15 μm) was significantly larger than in L4 (30.11 ± 0.39 μm; $z = -2.738$, $p = 0.017$), with no significant difference observed between L5 and L6 (35.96 ± 1.15 μm; $z = 1.25$, $p = 0.424$). Lineage trends indicated that L5 consistently exhibited the largest mean measurements for traits including; apical cell width (8.77 ± 0.279 μm), apical cell length (17.40 ± 0.723 μm), width of axial cell and filament width, although the former two did not reach statistical significance (Table 3). In contrast, L4 generally displayed the smallest mean measurements across multiple characteristics, while L6 often presented intermediate values.

A random forest model incorporating all characteristics achieved an accuracy of 66.67 % in predicting membership of individuals to each lineage. The importance of each characteristic was quantified using the MeanDecreaseGini metric, which reflects the contribution of each characteristic to reducing uncertainty in the model. Filament width had the highest score (9.64), followed by axial cell width (8.02). These findings suggest that filament width is the most informative characteristic for distinguishing between lineages, whereas characteristics with high natural variability, such as pericentral cell distance, may be less reliable for this purpose.

4. Discussion

Each investigation into the biodiversity of *A. taxiformis* continues to uncover new insights, particularly in relation to cryptic lineages and native or invasive geographical ranges. Prior to this study, the mitochondrial genetic diversity of *A. taxiformis* within Australia was reported to be dominated by a single haplotype of L6 in eastern Australia and two haplotypes of L6 in Western Australia. Additional records indicate the presence of L5 within coastal habitats south of Rockhampton [10], as well as three separate records of L2 within Australian waters. It should be noted that the first report of L2 is a misclassification of an L6 'WA strain' from Rottnest Island, Western Australia (DQ228894) [5], which occurred prior to the identification of the sixth lineage. The second

Table 3
Morphological measurements of *A. taxiformis* tetrasporophyte, presented as mean ± standard error of each lineage. All measurements expressed as μm. Lowercase letters signify significant differences among lineages ($P < 0.05$). Coefficient of variation (CV) is expressed as a percentage, indicating the relative variability of each measurement within and across lineages.

	Individuals (n)	L4	L5	L6	CV (%)
		4	2	4	
1	Width apical (wac)	7.68 ± 0.20	8.77 ± 0.28	8.32 ± 0.18	13.74 ± 1.56
2	Length apical (lac)	16.70 ± 0.32	17.40 ± 0.72	15.90 ± 0.42	14.49 ± 1.07
3	Thickness cell wall (tcw)	3.95 ± 0.11	3.58 ± 0.25	3.65 ± 0.11	21.30 ± 2.06
4	Width axial cell (waxc)	3.74 ± 0.09[a]	4.85 ± 0.17[b]	4.44 ± 0.18[ab]	16.23 ± 1.32
5	Length axial cell (laxc)	26.91 ± 0.48	27.68 ± 1.07	29.31 ± 0.87	14.03 ± 1.58
6	Filament width (fw)	30.11 ± 0.39[a]	40.87 ± 1.15[b]	35.96 ± 1.15[ab]	8.75 ± 0.98
7	Vesical cell diameter (vcd)	5.22 ± 0.14	5.49 ± 0.17	5.75 ± 0.29	14.74 ± 2.44
8	Length tenth cell (l10)	26.63 ± 0.72	27.21 ± 1.20	28.83 ± 0.88	17.30 ± 1.25
9	Pericentral cell distance (dpc)	3.01 ± 0.14	2.66 ± 0.16	2.45 ± 0.11	27.8 ± 1.56

record is a specimen collected at Lord Howe Island in 2010 (GWS023343) [10] and the third is a specimen described in Zanolla et al. (2015) reportedly collected at Kissing Point, Townsville, Queensland. The presence of L2 at Lord Howe Island matches the reported geographical and environmental range associated with this lineage [4,15], however, the collection of an L2 specimen within the warmer region surrounding Townsville could represent a misclassification [11], an example of human mediated transport, or a local extinction event. Prior to this study, the only example of L4 within Australian waters was collected at Cocos-Keeling Islands, a remote territory in the Indian Ocean [10].

In this study, we report significant morphological differences and molecular diversity within *A. taxiformis* populations in north-eastern Australia, with the first documented presence of L4 in Australian coastal waters. Notably, other studies [10,12] have collected samples within our study sites of Townsville and as far north as Cairns, only revealing the presence of L6. This study establishes that three populations in the north of Queensland, including Magnetic Island, Cairns, and Lizard Island, are composed of multiple mitochondrial lineages and unique haplotypes, indicating substantial genetic diversity. In these three locations, samples were collected from shallow coral reefs when the water temperature was above 25 °C and water clarity was high. Some specimens at these locations were epiphytically attached to the shaded, central branches of corals of the genus *Acropora*. In contrast, the Kissing Point population exhibited low genetic diversity, consisting exclusively of L6. This population was sampled from rocks on an exposed tidal flat, subject to higher turbidity and temperature fluctuations than at the other locations. The different habitat and abiotic conditions between Kissing Point and the other collection sites suggests environmental pressures influence genetic diversity in *A. taxiformis* populations [40]. Unfavourable conditions may limit sexual reproduction, reduce recruitment from diverse source populations, and favour persistence through vegetative propagation [41]. Field observations support this, as gametophytes were consistently absent at Kissing Point but commonly observed at other sites. It is therefore plausible that the Kissing Point population represents a clonal colony, potentially derived from a nearby donor population such as Magnetic Island, by direct onshore winds and current [17].

Population structure of *A. taxiformis* across the northern GBR is not evidently driven by geographic location, with three lineages co-distributed in this region. Inferences cannot be made for southern Queensland populations due to the small population sizes, however, it is generally accepted that tropical marine environments have an increased level of diversification in taxa [42]. The occurrence of tetrasporophytes of three *A. taxiformis* lineages in sympatry, contradicts previous assumptions that intermingled cases of lineages are rare [11]. Lineage overlap in transition zones, such as Cocos-Keeling and Christmas Islands, has been observed for other marine taxa, including fish species [43]. Our findings suggest that, at least for the tetrasporophyte, sympatry is more common than previously thought. One reported case of sympatry in the *A. taxiformis* gametophyte from the Hawaiian Islands suggested that distribution was likely partitioned by reef habitat and competitive exclusion [13]. Although we did not see this is the tetrasporophyte across the sampled locations, habitat partitioning may still occur for the gametophyte stage, warranting further investigation.

The lack of historical presence of L4 in the GBR raises the question of this lineage's origin: is it endemic? The dominance of L4 in the northern GBR populations as found from this study suggests a specific plasticity to tropical conditions. It therefore is possible that this lineage has existed undetected, primarily due to a lack of sampling effort in the remote northern GBR [10]. The historical distribution of L4 within the Coral Triangle and other equatorial localities supports the conclusion that L4 is well established in this region [11,14]. The characterisation of L4 as a lineage dependent on warmer temperatures for photosynthesis [40] may provide one explanation for why this lineage is not yet present, or just undetected in southern Queensland.

The earliest herbarium record of *A. taxiformis* in Queensland, is a specimen collected in 1934 at Moreton Bay (Queensland Herbarium; BRI AQ0708369). To date, there have been no systematic surveys of this species in Queensland, with sampling efforts defined by haphazard collections of single samples or drift cast specimens. Until the present study, the only previous samples in Australia, collected north of Cairns, are represented by two herbarium records: from Thursday Island (Queensland Herbarium; BRI AQ0708264) in 1960 and Cape York (Queensland Herbarium; BRI AQ0708347) collected in 1985. Without the support of molecular information, these specimens can only be inferred to belong to a lineage or clade suited to tropical environments, matching the environmental preferences of a lineage such as L4 and further supporting its historical existence in the north of Australia [1,15].

The distinct mitochondrial and plastid divergence between the specimens of L4 and L6 on the GBR is further evidence supporting the independent evolution of these two lineages. Divergence between the two primary clades, first defined in Ni Chualain et al. [4], is estimated to have occurred approximately 5.4 Ma, based on a 4.88 % sequence divergence, likely driven by adaptation to distinct niche environments [11]. In the present study, the divergence of L6 from L4 is 7.07 % for the *cox2-3* marker and 2.3 % for the RuBisCO spacer, with a TN corrected divergence of 0.062 ± 0.002, presenting a case for the separation of these lineages into subspecies. In contrast, the lesser divergence observed between L5 and L6 of 2.3 %, as indicated by mitochondrial markers, and the inability of the RuBisCO spacer to resolve these two lineages in this study, suggests either recent divergence or introgression between taxa that are not fully reproductively isolated [11]. Comparatively, *A. armata* comprises two clades that may be separate cryptic species, with divergences of 7.25 % for the *cox2-3* and 3.16 % for *rbcL* [11]. Interspecific divergence across the genus *Asparagopsis* is reported as 18.88 % for the *cox2-3* region and 9.14 % for the *rbcL*. Although divergence between the two recognised species is relatively high, similar molecular complexities to those reported in this study, have been

identified in other macroalgal genera including *Pyropia* [16] and *Gelidiales* [11]. One study observed interspecific divergence of 2.6 % - 17.1 % within the COI-5P + *cox2-3* spacer of seven species of the genus *Pyropia* in Korea [16]. The delineation of species within macroalgal genera is therefore typically achieved through an integrative approach combining both molecular and morphological analyses [44].

In addition to molecular differences, morphometric analysis conducted in this study revealed significant differences in axial cell width and filament width between L4 and L5 from the GBR. The tetrasporophyte of species within the genus *Asparagopsis* were historically assumed to be identical until Ni Chualain et al. (2004) noted distinct morphological differences between *A. armata* and *A. taxiformis*, attributing variations to both molecular and ecological factors. Similarly, Zanolla et al. (2014) reported that L3 tetrasporophyte specimens exhibited larger filament widths and axial cell dimensions compared to L1, L2, and L4. It should be noted that whilst some characteristics, such as filament width, may be useful for lineage separation or identification within tetrasporophytes, variability of some morphological characteristics within individuals is high and caution is advised. Differences in the morphometrics of the gametophyte between lineages is considered to be more pronounced, with the invasive L2 lineage in the Mediterranean exhibiting larger reproductive structures than native Hawaiian L2 populations [15]. The same study reported Hawaiian populations of L1 and L2 gametophytes to be monoecious, with L4 gametophytes absent of any reproductive structures. In contrast, the invasive L4 specimens of the Israeli Mediterranean Sea (IMS) were documented to be monoecious, as well as larger in many characteristics when compared with the L4 population in Hawaii [13,17]. Although difficult to accurately compare populations under different biotic conditions, the increased size and reproductive characteristics of some specimens may contribute to an ecological advantage, increasing likelihood of invasive success [17].

The identification of L4 samples within the northern GBR provides further support for the hypothesis that L4 has a natural distribution across the Indo-Pacific and particularly within the Coral Triangle. By extending the lineage's known range into northern Australia, we confirm that it is well-established in tropical regions of the Indo-Pacific Ocean [11,14]. Notably, the invasive L4 population reported in the IMS varies slightly to the two haplotypes identified on the GBR, being most similar to haplotypes from Thailand, Sri Lanka, and a second haplotype from the Cocos-Keeling Islands [17]. It is proposed that this invasive population of L4 was introduced to the Mediterranean via the Suez Canal, likely through human-mediated dispersal from Sri Lanka or South Africa [17]. Zanolla et al. [8] suggested that L4 may have been introduced to Hawaii, and its distribution may expand further with rising sea temperatures. Such environmental changes may enable L4 to colonise additional tropical regions, potentially outcompeting temperate-dominant lineages like L6 and L5 under warming ocean conditions.

The potential for L4 to expand into historically cooler regions of Australia raises important questions about the role of environmental conditions and barriers in shaping its future distribution. Zanolla et al. [8] modelled the range expansion of other *A. taxiformis* lineages, predicting significant demographic expansion of L1 in tropical regions by 2050 due to rising sea temperatures. Similarly, the invasion risk of L2 is inferred to be high. Temperature emerges as a key driver of range expansion for this species, particularly as temporal fluctuations differentially affect the success of different lifecycle stages to colonise new areas through clonal propagation or sexual reproduction [8].

An area warranting further investigation is the differential success of *A. taxiformis* lineages in completing their full lifecycle, particularly in populations where sympatry occurs. The abundance of certain lineages as tetrasporophytes may not necessarily reflect the same abundance of each lineage as gametophyte at the same location due to factors such as competitive exclusion or differential reproductive success [6,45]. For instance, if L4 on the GBR has reduced success in colonising habitats dominated by L6 in the gametophyte phase, it could explain why historical studies have primarily detected L6, as majority of studies have typically sampled gametophytes due to their ease of identification [10]. The present study highlights the biases inherent in sampling only one life stage of a macroalgae and emphasises that inferences of lineage origin or diversity based on single-phase sampling or small sample sizes should be approached with caution.

Systematic sampling and increased sample sizes have been shown to increase the resolution of genetic diversity, uncovering new haplotypes and cryptic lineages [13,14]. Whilst the systematic sampling design employed in this study was sufficient to capture genetic diversity across the broader Queensland region, additional haplotypes may remain undetected in northern populations such as Lizard Island (Fig. 5). Although increased sampling in these diverse northern areas could reveal further haplotypes, logistical challenges, time constraints, and cost often limit large-scale sampling efforts. As an alternative, smaller sample sizes collected systematically over broader spatial scales, coupled with multiple genetic markers [46,47], or advanced next-generation sequencing techniques, could enhance genetic resolution and diversity estimates [48]. Evidence from other macroalgae, such as *Nereia lophocladia*, demonstrates the utility of genome-wide SNP analyses in identifying location of origin, population diversity and signatures of selection for traits associated with climate-driven range shifts [49].

5. Conclusion

This study underscores the importance of combining molecular and morphometric analyses to enhance our understanding of the diversity of *A. taxiformis* in Australia. Future research should prioritise systematic sampling across broader spatial scales, including underrepresented regions like southern Australia, and incorporate multiple lifecycle stages to mitigate sampling bias. Addressing these gaps will also shed light on how climate change may influence the distribution of this species. With growing interest in the commercial production of *A. taxiformis* in Australia and worldwide, understanding the natural biodiversity of this species is essential not only for ensuring sustainable management and preserving the ecosystems that support these populations, but also for informing the selection of robust strains for cultivation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2025.104182.

Ethical consent

This study does not involve participation from humans or animals.

CRediT authorship contribution statement

Anna Wilson: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jessica E. Harris:** Writing – original draft, Investigation. **Rocky de Nys:** Writing – review & editing, Supervision, Funding acquisition. **Dean R. Jerry:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Anna Wilson reports financial support was provided by Sea Forest Ltd. Co-Author employed by Sea Forest Ltd. - R. D N If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was produced under the Australian Research Council Industry Research Transformation Hub for Supercharging Aquaculture

Through Genetic Solutions in partnership with Sea Forest Ltd. Funding support was provided by the Australian Research Council and Sea Forest Ltd. We thank Cassandra Karalis, Rebecca Tite and Hamish Tristram for their assistance with specimen collection, as well as Nikolaos Andreakis, Nga Vu and Kyall Zenger for ongoing research support.

Data availability

Unique sequences generated in this study and sequences used for lineage assignment are provided in the supplementary material. Additional data generated during the current study are available from the corresponding author on reasonable request.

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