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Sequencing of historic and modern specimens reveals cryptic diversity in *Nothogenia* (Scinaiaceae, Rhodophyta)

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Abstract: *Nothogenia fastigiata* has been reported to exhibit great morphological variability and has been considered to be widely distributed in the Southern Hemisphere. To test its current circumscription, sequences from type material of *N. fastigiata* and other species currently synonymized with it were compared to those from recent collections of this and other species in the genus. Eight distinct species previously subsumed under the name *N. fastigiata* were identified. Multiple specimens from southern Chile and a single specimen from Campbell Island (subantarctic New Zealand) were conspecific with type material of *N. fastigiata* from the Falkland Islands. For other species, molecular analyses of recent collections using the nuclear ITS1-5.8S-ITS2 region of the ribosomal cistron, the chloroplast *rbc*L and *psb*A genes and the mitochondrial *COI* gene indicated a strong geographic pattern to species relationships. Other specimens identified as *N. fastigiata* from Chile represented up to five species, including *N. chilensis* and *N. fragilis*, based on sequences of type material; these Chilean species occurred on a monophyletic branch. We also recognized *N. lingula comb. nov.* from Tasmania, which is closely related to *N. fastigiata*, based on sequences of type material. Specimens from mainland New Zealand identified as *N. fastigiata* fell into a distinct clade with New Zealand *N. pulvinata* and represented a previously undescribed species, described here as *N. neilliae sp. nov.* Another New Zealand species, *N. pseudosaccata*, was distantly related to *N. variolosa* from Auckland Island and other subantarctic islands south of New Zealand. The New Zealand species were more closely related to South African *N. erinacea* and *N. ovalis* than to species of *Nothogenia* from Chile, including *N. fastigiata*, although bootstrap support for this interpretation was weak. These genetic data demonstrate that matching DNA sequences from archival *Nothogenia* material to modern specimens can be used to identify and define new and old cryptic species.

Key Words: Biogeography, *COI*, ITS, *Nothogenia*, Phylogeny, *psb*A, *rbc*L, Sequencing type material, Species, Taxonomy

INTRODUCTION

Nothogenia Montagne, a genus of the Scinaiaceae (Order Nemaliales), currently contains six species that occur intertidally in the Southern Hemisphere (AlgaeBase, as of 13 March 2014): South African *N. erinacea* (Turner) P.G. Parkinson and *N. ovalis* (Suhr) P.G. Parkinson, New Zealand *N. pseudosaccata* (Levring) P.G. Parkinson and *N. pulvinata* (Levring) P.G. Parkinson, Peruvian *N. fragilis* Montagne and the widely distributed *N. fastigiata* (Bory) P.G. Parkinson. Most of these species previously had been assigned to *Chaetangium* Kützing, but Parkinson (1983) considered that genus to be a synonym of *Suhria* J. Agardh ex Endlicher. A summary of Parkinson's taxonomic conclusions can be found in Silva *et al.* (1996, p. 104.)

The genus *Nothogenia* was created by Montagne (1843, p. 302) to accommodate *N. variolosa* (Montagne) Montagne, a repeatedly dichotomous and linear cartilaginous species with thin medullary filaments, a cortex of submoniliform cells and cystocarps encircled by a dense pericarp. Montagne's material was collected on the Auckland Islands by Dumont D'Urville aboard the *Astrolabe* (Montagne 1842, 1845). His species, however, is considered a later heterotypic synonym of Bory de Saint-Vincent's *Halymenia fastigiata* (= *N. fastigiata*), originally collected on the Falkland Islands (Parkinson 1983). In addition to *N. variolosa*, two other species are considered synonyms of *N. fastigiata*: *N. chilensis* (J. Agardh) Montagne (basionym: *Chaetangium chilense*) and *Chaetangium lingula* Harvey with type localities of Valparaíso, Chile, and Brown's River, Tasmania, Australia, respectively.

As noted by Huisman & Womersley (1994, p. 110), 'The type species of *Nothogenia*, *N. variolosa* from the Auckland Is., as illustrated by Montagne (1845, fig. 3) is a much branched plant with narrow branches. Chapman (1969, p. 70) comments on the extreme variability of the New Zealand taxon, and Ricker (1987, p. 168) also doubts whether this material (and the type of *N. variolosa*) is the same as *N. fastigiatum* [*sic*] from the Falkland Is. and other subantarctic islands; clearly further study is needed'. Additionally, *N. fastigiata* was described as morphologically highly plastic along the Chilean coast (Hoffman & Santelices 1997).

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Differences between two distinct morphotypes in central Chile were attributed to adaptive responses to abiotic factors, although genetic differentiation was also suggested by Ramírez (1988).

The present study was undertaken to authenticate the identity of true *Nothogenia fastigiata*, to test the taxonomic positions of its synonyms using DNA from modern and type material and to determine species relationships.

MATERIAL AND METHODS

DNA was extracted from silica gel desiccated material (Table S1) using the CTAB mini-extraction protocol as described in Lindstrom & Fredericq (2003). Total extracted DNA was amplified for the nuclear ribosomal ITS regions, the chloroplast *rbc*L and *psb*A genes and the 5′ end of the mitochondrial *COI* gene using the primers listed in Table S2. For the ITS regions, primers ITS1 and JO6 were used initially. If these reactions were unsuccessful in producing a visible band, 1 µl of reaction product was used as the template in a subsequent reaction using ITS1Pa as the forward primer and ITS2Pa or JO6 as the reverse primer. If the initial reaction produced multiple bands, 1 µl of crude DNA was used as the template in a subsequent reaction using ITS1No and ITS4No as primers. The *rbc*L gene was amplified as a single fragment using the F57 and RrbcSst primers or in two fragments using F57-R1150K and F753No-RrbcSst as the primer pairs, and the *psb*A gene was amplified using the primers psbAF1 and psbAR2. The 5′ end of the *COI* gene was amplified using GazF1 and GazR1 as primers, or, if initially unsuccessful, CO1S1 and GazR1 were used. All reactions contained 5 or 6 µl 10× NEB Thermopol buffer (New England BioLabs, Whitby, Ontario), 0.5 or 1.0 µl 10 mM dNTP mix, 0.4–1.0 µl each forward and reverse primers (at a concentration of *c*. 32 pmol μl^{-1}), 0.35 µl NEB DNA Taq polymerase, *c*. 1 µl crude DNA (or PCR product) and distilled deionized water to a final volume of 50 µl. The ITS regions were amplified using the protocol of Hughey *et al*. (2001). If a PCR product was reamplified, we used the protocol of Broom *et al*. (1999). For *rbc*L and *psb*A, we used the PCR protocol of Lindstrom & Fredericq (2003). For *COI*, we used the PCR protocol of Saunders (2005). PCR products were sequenced using the ABI Applied Biosystems (Foster City, California USA) Big Dye Terminator v3.1 cycle sequencing kit by the Nucleic Acid Protein Service Unit (University of British Columbia, Vancouver, British Columbia, Canada) with the same primers used for amplification (but at reduced concentrations).

Fully alignable *rbc*L, *psb*A and *COI* sequences from the specimens listed in Table S1 were concatenated and subjected to maximum likelihood (ML) analyses using PAUP* 4.0b10 (Swofford 2002) and RAxML 7.2.6 [as implemented on the T-REX website (http://www.trex.uqam.ca/index.php?action=raxml; Stamatakis 2006; Buc *et al*. 2012)]. The appropriate model of evolution for the PAUP ML analysis using the Akaike information criterion was determined from Modeltest 3.7 (Posada & Crandall 1998; Table S3), and data were partitioned by gene and codon position for the RAxML analysis. Separate analyses were carried out for these genes individually, as well as for the ITS region of the nuclear ribosomal cistron. Bootstrap proportions were determined based on 100 replicates for PAUP ML and 1000 replicates for RAxML. Bayesian phylogenetic analyses were performed on the Bio-Linux7 platform (Field *et al*. 2006) with MrBayes 3.2.1 (Huelsenbeck *et al*. 2001; Ronquist & Huelsenbeck 2003). Markov chain Monte Carlo runs were all executed with the GTR+I+G model. This substitution model was used because it corresponds most closely to the Tamura-Nei and Transitional models indicated in Table S3 (Zakharov *et al*. 2009; Skillings *et al*. 2011). The number of generations performed varied for each data set. As an indicator of convergence, we followed the MrBayes 3.2 manual, which recommends continuing analyses by increasing the number of generations until the average standard deviation of split frequencies drops below 0.01. All runs were performed using a sample frequency of 10 with two independent analyses. To calculate the Potential Scale Reduction Factor and posterior probabilities, the burn-in values were set to discard 25% of the samples.

Out-groups were selected based on blastn searches of GenBank. For *rbc*L, these included DQ787562 (*Nemalion* sp.; Yang & Boo, unpublished), KC134333 [*Scinaia confusa* (Setchell) Huisman; Scott *et al*. 2013] and AB258450 [*S. okamurae* (Setchell) Huisman, Huisman & Kurihara, unpublished]. For *psb*A, this included DQ787638 (*Nemalion* sp.; Yang & Boo, unpublished). For *COI*, they included HM916493 [*Nemalion multifidum* (Lyngbye) Chauvin; Le Gall & Saunders, unpublished], HM916595 (*S. confusa*; Le Gall & Saunders, unpublished) and HQ544543 [*S. interrupta* (A.P. de Candolle] M.J. Wynne; Le Gall & Saunders, unpublished). We also used unpublished sequences of Palmariaceae (Lindstrom, unpublished) since GenBank also indicated high similarity of our *Nothogenia* sequences to species in this family.

DNA from potential type material and other historical specimens (Table S4) was extracted, amplified and sequenced following the protocol described in Lindstrom *et al*. (2011) except for using 3× the primer concentration used previously. To amplify part of the *rbc*L gene, primers F753 and R900 were used (Table S2). Sequences were aligned with contemporary collections in BioEdit (Hall 1999).

RESULTS

The phylogenetic analysis of the concatenated data set of *rbc*L, *psb*A and *COI* sequences (Fig. 1) indicates that *Nothogenia* is a monophyletic genus. In general, divergences within species were universally small (mostly less than 1% and usually much less than 1%), and divergences between species were usually very large (often 6–13%). The two South African species, *N. ovalis* and *N. erinacea*, occurred in a strongly supported clade that was sister to a clade of New Zealand species, but this relationship had weak bootstrap support in the RAxML analysis. Among the New Zealand species, only the sibling relationship between *N. pulvinata* and *N. neilliae* was strongly supported. A sibling relationship between *N. pseudosaccata* and *N. variolosa* was only moderately supported by the PAUP and RAxML boot-

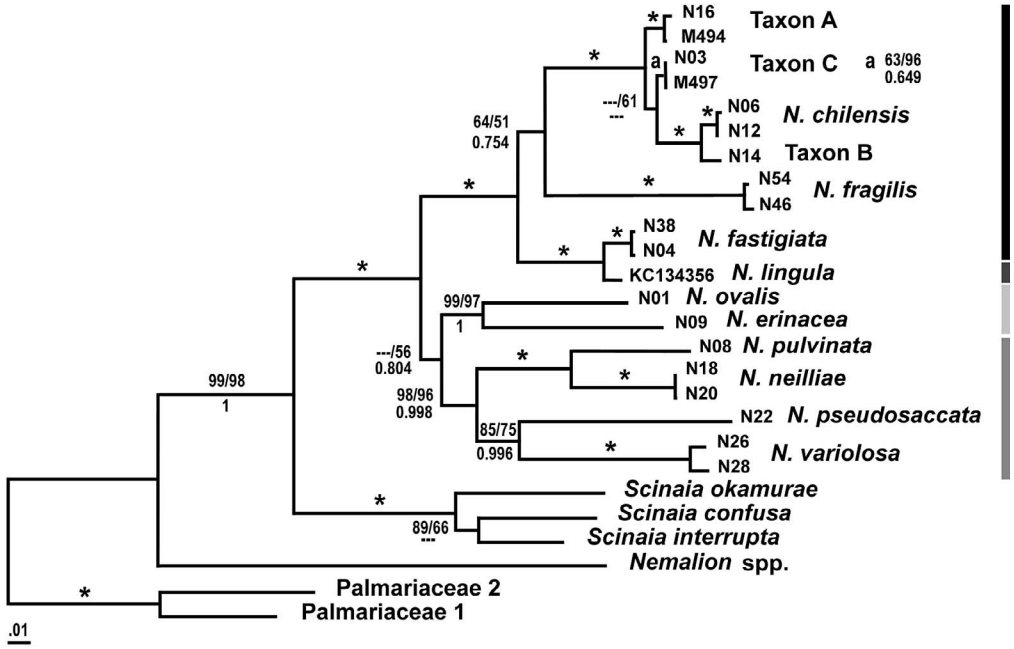

Fig. 1. Maximum likelihood analysis of concatentated *rbc*L, *psb*A and *COI* sequence data for species of *Nothogenia*. Bootstrap values represent left-to-right PAUP (nreps = 100) and RAxML (nreps = 1000); Bayesian posterior probabilities appear below these values. An asterisk indicates bootstrap values of 100 for the ML analyses and posterior probability of 1.000 for MrBayes. Regional provenances of samples represented by vertical line on right: South America (100% opacity), Australia (74% opacity), New Zealand including Subantarctic islands (48% opacity) and South Africa (24% opacity).

straps. The third geographic clade, which was strongly supported in all analyses, included species primarily from Chile. This clade included *N. fastigiata*, which is also known from the Falkland Islands, its type locality, and the closely related *N. lingula* from Tasmania (this taxon was represented by a GenBank *rbc*L sequence, KC134356, since we did not have contemporary material). Of the remaining Chilean species, only *N. chilensis* and *N. fragilis* have been described. *Nothogenia fragilis*, the most northerly of the South American species, occurred on a very long branch. The remaining Chilean species occurred in a terminal cluster. Although branches were shorter than those of other species of *Nothogenia*, most branches were strongly supported. The exception was Taxon C, which occurred along a branch from which other species diverged, suggesting that it has experienced little genetic differentiation.

We also examined phylogenetic relationships among species using individual genes to assess the relative contributions of these genes to the overall pattern as well as to include additional individuals. These figures are presented as Fig. S1 (*rbc*L gene), Fig. S2 (*psb*A gene) and Fig. S3 (*COI* gene). In general, patterns for the individual genes mirrored those of the concatenated data set. However, some of the individual branches of the deeply diverging species showed no clear relationships with other taxa in the *psb*A analysis compared to the *rbc*L or concatenated analyses. These analyses also included a single Chilean specimen (N57) that did not cluster with any of the other taxa and may represent an additional undescribed species. The positions of the terminal Chilean clades (*N. chilensis* and Taxa A–C) varied based on the gene analysed, but all species were moderately to strongly supported in all analyses. We also observed unusual *COI* genotypes for some of the Chilean species, which occurred on a very long branch sister to all of the other species of *Nothogenia*, and were also highly divergent from each other (data not shown). These anomalies were observed independently by E. Macaya and may represent numts (nuclear mitochondrial DNA) or pseudogenes.

The ITS data set was characterized by a large number of large indels (there were more than eight greater than 10 base pairs [bp] in length, the longest being > 100 bp). Because of this, we analysed the data using MP with gaps treated as a fifth base, with indels reduced to the number of steps that might have been required to achieve the alignment of species in our data set (data not shown but available upon request; since other types of analyses treat gaps as missing data, they were not performed). The resulting phylogenetic tree was similar to those produced by other analyses but with less resolution. Among Chilean species, *N. fragilis* was strongly supported, but *N. chilensis* and Taxon B were intermixed on a strongly supported branch, and most Taxa A and C occurred on unsupported to weakly supported branches. *Nothogenia fastigiata* also occurred on its own weakly supported branch but with some strongly supported internal branches. The subantarctic *N. variolosa* was also strongly supported (and also had moderately to strongly supported internal branches), but New Zealand *N. neilliae* and *N. pulvinata* were intermixed. There was weak support for the South African species occurring on the branch from which the New Zealand and subantarctic species diverged.

Below we provide details of the species examined in this study, including the results of sequencing type material. The distribution of these species in the Southern Hemisphere is shown in Fig. 2.

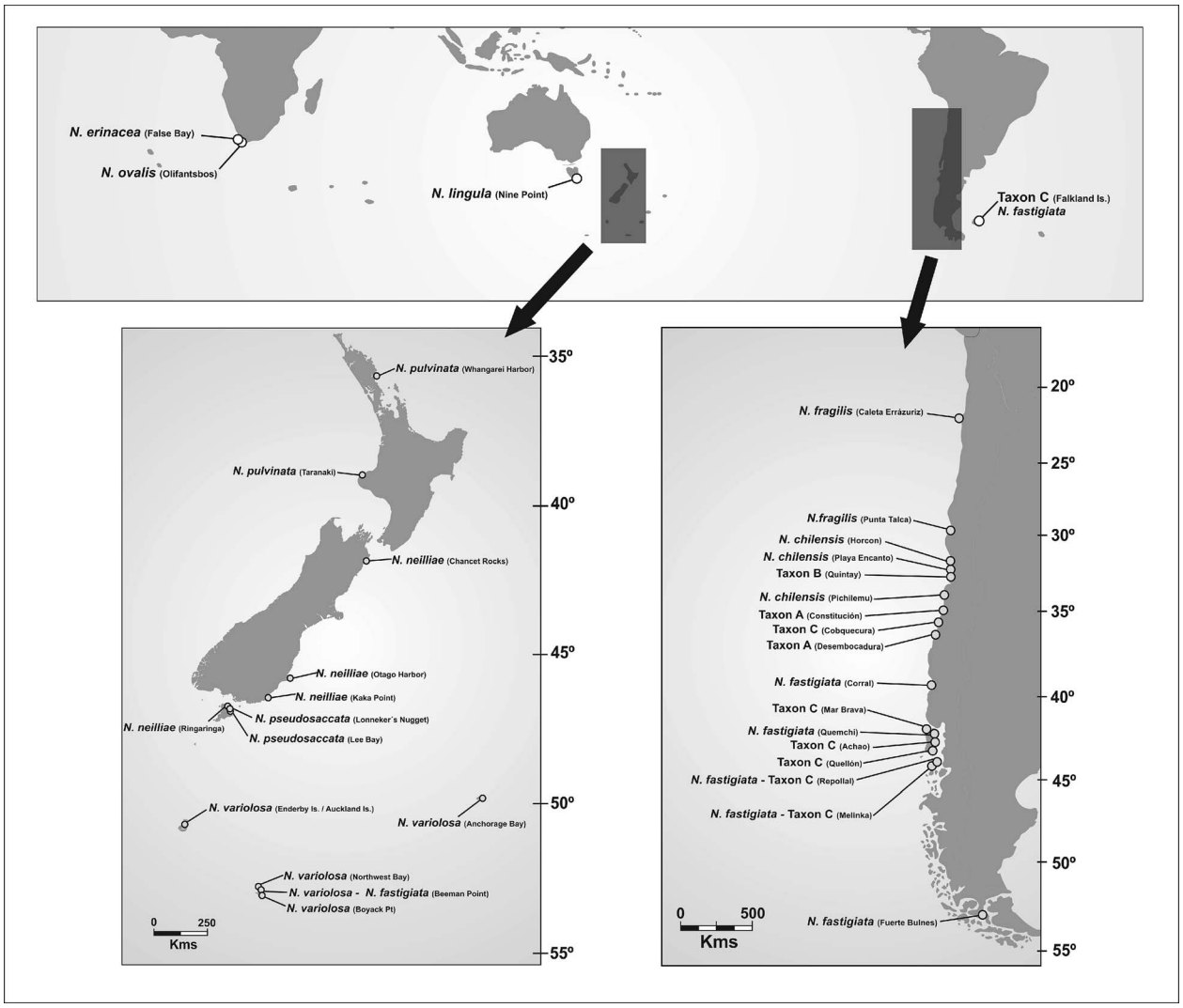

Fig. 2. Distribution of species of *Nothogenia* in the Southern Hemisphere.

South African species

Nothogenia ovalis (Suhr) P.G. Parkinson 1983, p. 609

BASIONYM: *Dumontia ovalis* Suhr 1840, p. 274.

TYPE LOCALITY: Cape of Good Hope (see Silva *et al*. 1996: 112).

KNOWN DISTRIBUTION: South Africa, Namibia, Tristan da Cunha (Guiry & Guiry 2014).

We did not sequence type material of this species, but the morphology, genotypes and provenance of the sequenced material suggest it is a distinctive species.

Nothogenia erinacea (Turner) P.G. Parkinson 1983, p. 609

BASIONYM: *Fucus erinaceus* Turner 1808, p. 55, pl. 26.

TYPE LOCALITY: Cape of Good Hope (Silva *et al*. 1996, p. 112).

KNOWN DISTRIBUTION: South Africa, Namibia (Guiry & Guiry 2014).

We did not sequence type material of this species. As with *N. ovalis*, the morphology, genotypes and provenance of the sequenced material suggest it is a distinctive species. Anderson & Stegenga (1985) observed a *Cruoriopsis*-like crustose tetrasporophyte in the life cycle of both South African species. Collantes *et al*. (1981) had earlier illustrated tetrasporangia in crusts from Chilean *N.* '*fastigiata*', and Delépine *et al*. (1979) illustrated crusts from natural populations from the Kerguelen Islands.

South American species

Nothogenia chilensis (J. Agardh) Montagne 1854, p. 326

BASIONYM: *Chaetangium chilense* J. Agardh 1847, p. 10.

TYPE LOCALITY: Valparaíso, Chile.

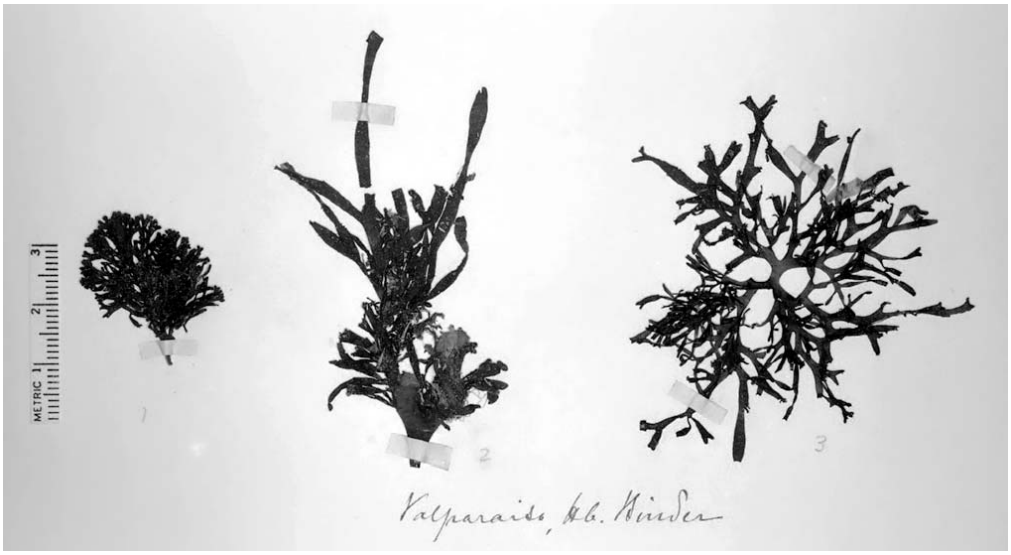

Fig. 3. Lectotype of *Nothogenia chilensis* (LD 32578).

KNOWN DISTRIBUTION: Near Valparaíso, Chile (this study).

Three fragments from the three specimens comprising Herb. Ag. 32578 in LD (6N, 9N, 17N; Table S4), identified as 'Chaetangium chiloense', had *rbc*L sequences identical to N06 (from Horcón, north of Valparaíso) and N12 (from Playa El Encanto, near Valparaíso). The Herb. Ag. material is in complete agreement with the protologue (Agardh 1847), including the type locality of Valparaíso and the material being from the Binder herbarium (Fig. 3). Therefore, we herein designate LD 32578 as the lectotype. Agardh (1847) briefly noted that his species was nearly identical with Montagne's *Nothogenia variolosa*. *Nothogenia chilensis* can be applied to N06, N12 and N56 among our collections. The relationship of these specimens to N13 and N14 is discussed below under Taxon B.

Nothogenia fastigiata (Bory) P.G. Parkinson 1983, p. 609

BASIONYM: *Halymenia fastigiata* Bory 1825, p. [22] 594.

TYPE LOCALITY: Iles Malouines [Falkland Islands].

KNOWN DISTRIBUTION: Falkland Islands; southern Chile, from Corral near Valdivia south through Isla Chiloé to at least Magellan Strait; Campbell Island (south of New Zealand; this study).

Despite having a widely applied name, this species appears to be more restricted geographically than previously understood. It has a distinct morphology of narrow, fastigiate branches. Other taxa that have been misidentified as this species but have distinct genetic signatures are usually broader with fewer, sparser branches. *Nothogenia fastigiata* occurs on its own well-supported branch in all analyses for all loci (*rbc*L, *psb*A, *COI* and ITS). It is closely related to *N. lingula* from Tasmania; these species occur on a strongly supported branch sister to all other Chilean species.

The 102-bp fragment of type material (Herb. Ag. 32591 in LD—11N; Table S4), 'Dumontia fastigiata Bory herb. Halymenia #23 fl. Mal.', was identical to five of our specimens (M498, N04, N37, N38 and N44) from the coast of Chile; we therefore confirmed them as *N. fastigiata*. Specimens 2N (Herb. Ag. 32576 from Ancud, Isla Chiloé), 13N (Herb. Ag. 32577 from Sandy Point, Fort Magellan, Chile) and 16N (an unlocalized fragment from Chile in the PC herbarium) also had identical 102-bp sequences to Herb. Ag. 32591. The sequence from a specimen (N31) from Campbell Island (south of New Zealand) indicates that this species may indeed be widespread. The Campbell Island specimen diverged from the Western Hemisphere specimens by 0.2% in the *rbc*L gene. The morphology of the Campbell Island specimen differed slightly from other Campbell Island specimens identified as *N. variolosa* by having shorter and broader branches.

Nothogenia fragilis Montagne 1852, p. 318

TYPE LOCALITY: Cobija, Peru.

KNOWN DISTRIBUTION: Cobija, Caleta Errázuriz, Punta Talca, and Valparaíso, Chile.

The 118-bp fragment of type material (Table S4) was identical to four contemporary specimens except for one nucleotide in the area overlapping the reverse primer used to amplify the type sequence (here the type sequence had the same nucleotide as the primer rather than the nucleotide found in the longer sequences of the contemporary material). Three of the contemporary specimens were from Caleta Errázuriz, near Antofagasta, the fourth from Punta Talca, south of Coquimbo; these differed by 0.3%. All of these sequences matched an *rbc*L sequence from an historical specimen (12N) said to be from Valparaíso, Chile (Table S4). These specimens occurred on a well-supported branch sister

to *N. chilensis* and Taxa A–C. Specimens are usually 5–6 cm high, dichotomously branched at first, then slightly irregular; segments are of variable length and 1–3 mm diameter; texture is firm; colour is brown-red. This species is easy to differentiate from the other Chilean *Nothogenia* because thalli are cylindrical to slightly compressed. A dark red tetrasporophytic crust with irregular margins was described for this species (Ramírez 1988).

Taxon A

KNOWN DISTRIBUTION: Near Constitución and Concepción, central Chile (this study).

Three specimens (M494, N05 and N16) were identified as Taxon A based on similar or identical *rbc*L and *psb*A sequences, and all were collected near Concepción. These specimens occurred on their own strongly supported branch in the *rbc*L and *psb*A. Only a single specimen was sequenced for *COI*.

In addition to the contemporary specimens we sequenced, we matched two historic specimens (7N and 10N; Table S4) to this species. The contemporary specimens were from near Concepción; the two historic specimens lacked information on provenance, but the identity of their sequences with the more recent collections suggests they may also have been from near Concepción.

Taxon B

KNOWN DISTRIBUTION: Near Valparaíso (Quintay), Chile (this study).

This taxon is closely related to *N. chilensis* in all analyses, but the distinction of the two taxa is supported by strong bootstrap values in analyses of *rbc*L and *psb*A sequences. The two specimens (N13 and N14) identified as this species had identical *rbc*L and *psb*A sequences and were both collected at the same site at the same time. Despite this, they were morphologically distinct: N13 was up to 3 cm tall with open, spreading branches ending in acute tips; whereas N14 was up to 2 cm tall, densely branched and with truncate tips. No historical specimens had sequences that allied them with this taxon.

Taxon C

KNOWN DISTRIBUTION: Falkland Islands; Cobquecura, Isla Chiloé, Melinka and Repollal, Chile (this study).

Specimens identified as Taxon C shared identical or nearly identical *rbc*L, *psb*A and *COI* sequences and occurred together on moderately to strongly supported branches (*rbc*L and *COI* analyses) or along the branch that gave rise to Taxon B and *N. chilensis* (*psb*A). No historical specimens had sequences that allied them with this taxon.

Naming of Taxa A to C will be done after further research on these by E. Macaya. A single specimen (N57) not clearly related to any of the other Chilean specimens occurred among these taxa in *rbc*L, *psb*A and *COI* analyses. This specimen remains unascribed (Figs S1–S3). Further collections are required before it can be described.

New Zealand species

Nothogenia pseudosaccata (Levring) P.G. Parkinson 1983, p. 609

BASIONYM: *Chaetangium pseudosaccatum* Levring 1955, p. 423.

TYPE LOCALITY: Blind Broad Bay, Stewart Island, New Zealand.

TYPE SPECIMEN: GB, Lindauer No. 7732; 21 November 1945 (Andersson & Athanasiadis 1992; Nelson & Phillips 2001).

KNOWN DISTRIBUTION: Southeast coast of South Island, Stewart Island and Snares Islands (Adams 1994); Macquarie Island?

This species shows a moderate to weak relationship to *N. variolosa* and is only distantly related (Figs 1, S1, S3). We did not sequence type material of this morphologically distinct species, which is inflated, club-shaped, simple or bi- or trifurcate, with a short stalk (Levring 1955; Adams 1994; Nelson 2013). We did sequence two specimens identified as this species from Stewart Island, New Zealand: N21 from Lonneker's Nugget and N22 from Lee Bay. Ricker (1987) reported inflated specimens from Macquarie Island that he considered intermediate in form between *N. pseudosaccata* and *N. fastigiata*. Recent photographic quadrats of intertidal communities at Macquarie Island reveal specimens with a morphology very similar to that of Stewart Island *N. pseudosaccata*. Further work is required to compare material from Macquarie Island with that from Stewart Island.

Nothogenia pulvinata (Levring) P.G. Parkinson 1983, p. 609

BASIONYM: *Chaetangium pulvinatum* Levring 1955, p. 422.

TYPE LOCALITY: Temple Bar, Russell, Bay of Islands, North Island, New Zealand.

TYPE SPECIMEN: GB; Levring No. 88-5, 24 March 1948, *Levring* (Nelson & Phillips 2001).

KNOWN DISTRIBUTION: North Island: mainly on the east coast (Adams 1994).

This species shows a strong relationship to *N. neilliae* (Figs 1, S1–S3). We sequenced two specimens of this species: N08 from Reotahi, Whangarei Harbour, North Island, and N17 from Bayly's Rd., Taranaki, North Island (a new southern distribution record for this species). This distinctive species, forming domed, densely branched tufts of narrow cylindrical branches with pointed tips (Levring 1955; Adams 1994; Nelson 2013), is known only from the North Island of New Zealand.

Nothogenia neilliae W.A. Nelson *sp. nov.*, Fig. 4

TYPE LOCALITY: 46°22.93′S, 169°46.98′E; intertidal on rock; Kaka Point, southeast Otago, South Island, New Zealand.

TYPE SPECIMEN: WELT A032881, 26 November 2011, Leg. W. Nelson, K. Neill, J. Dalen.

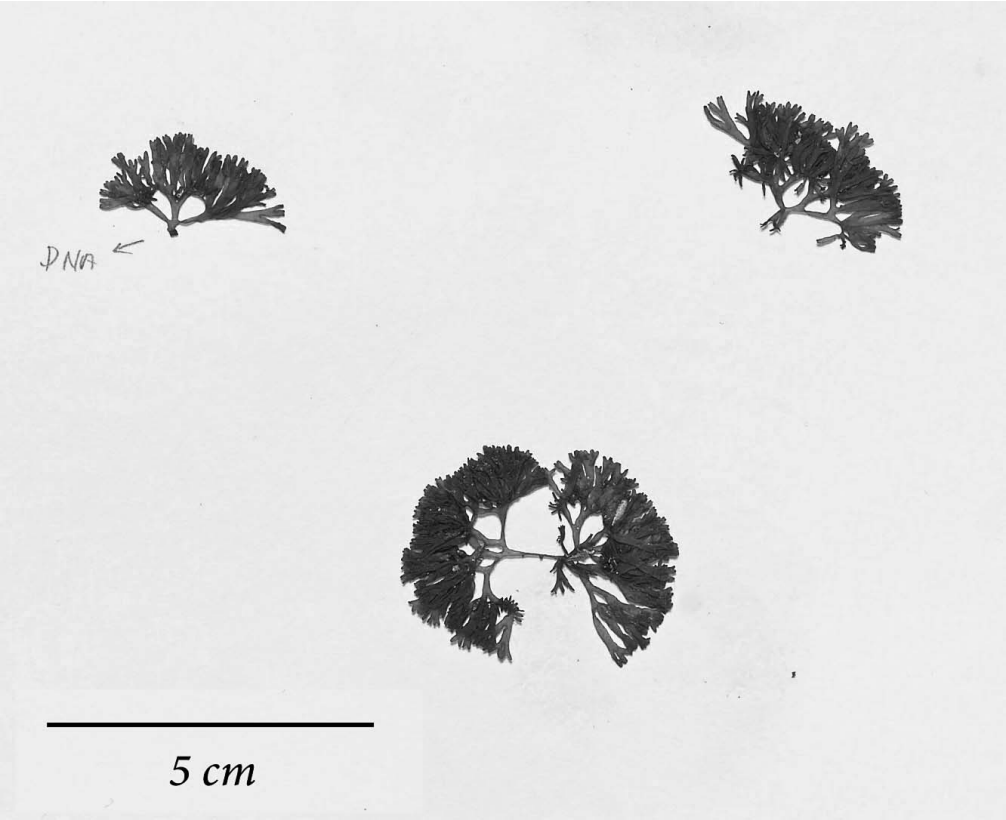

Fig. 4. Holotype of *Nothogenia neilliae* (WELT A032881).

KNOWN DISTRIBUTION: Southern North Island (Cook Strait), South Island, Stewart Island (this study).

DESCRIPTION: Thalli usually 3–5 cm high, tufted, bushy, repeatedly dichotomously or irregularly branched; axes terete to compressed below, becoming flattened distally. Growing from a crust-like pad with multiple axes arising from it. Dome-shaped cystocarps with conspicuous pore on upper branches. Texture firm, colour brown-red to purple, bleaching ginger-red in summer.

ETYMOLOGY: In recognition of contributions made by Kate Neill to New Zealand phycology.

This species had previously been identified as *N. fastigiata* in New Zealand (Adams 1994; Nelson 2013).

Nothogenia variolosa (Montagne) Montagne 1843, p. 303

BASIONYM: *Chondrus variolosus* Montagne 1842, p. 6.

TYPE LOCALITY: Auckland Island.

KNOWN DISTRIBUTION: Auckland, Antipodes, and Campbell Islands (this study).

Three specimens (3N, Herb. Ag. 32575 in LD; 14N, PC—Gen; 15N, L 941 51 22; Table S4) represent potential type material of *Chondrus variolosus*. All are from Auckland Island. 3N and 14N had identical *rbc*L sequences to our specimens from Antipodes Island (N11) and Campbell Island (N28 and N29), and they varied at one bp position from the contemporary specimen from Auckland Island (N26), which had a sequence identical to 15N. We recognize 14N from PC—Gen as the lectotype specimen since PC is where Montagne worked on these collections. We consider the L and LD specimens to be isolectotypes.

Montagne (1845) illustrated *N. variolosa* with a narrow and much-branched thallus. Further work is warranted to clarify morphological variation in this species and *N. fastigiata* within the New Zealand subantarctic region.

Australian species

Nothogenia lingula (Harvey) S.C. Lindstrom & Hughey *comb. nov.*

BASIONYM: *Chaetangium lingula* Harvey 1860, p. 316.

TYPE LOCALITY: Brown's River, Tasmania.

KNOWN DISTRIBUTION: Tasmania.

We sequenced the type specimen housed in TCD. The 102-bp fragment matched exactly GenBank KC134356 from Bicheno, Tasmania, which in turn was similar to a specimen from Ninepin Point, Tasmania, collected in April 1958 by W.M. Curtis and housed in HO (72638); this latter specimen differed at two bp positions (2% divergence) from the type specimen and at three bp (0.9% divergence) from KC134356 over a longer alignment. Sequence differences among these

samples suggest possible cryptic diversity in this distinctive species—the specific epithet referring to the flat, lanceolate branches that distinguish it morphologically from other species in the genus. We also examined but did not sequence specimens in NSW (392817, 392818); these specimens were formerly housed in AD (as A56468 and A57076) and formed the basis of Huisman & Womersley's (1992) description of postfertilization development in *N. fastigiata*. All of these specimens share the same morphology as the type and HO specimens: thalli up to 4.6 cm tall, mostly 2–4 (4.5 maximum) mm wide, flattened, usually once or twice bifurcate (up to a maximum of four times), with relatively long branches tapering to narrowly rounded branch tips.

DISCUSSION

This study confirms the distinctiveness of currently recognized species of *Nothogenia* (*N. erinacea*, *N. fastigiata*, *N. fragilis*, *N. ovalis*, *N. pseudosaccata* and *N. pulvinata*). Also, we were able to show that several genetic lineages, some of them formerly subsumed under the name *N. fastigiata*, can be linked to previous names based on sequencing of type material. These include the herein resurrected *N. chilensis*, *N. lingula* and *N. variolosa*. A new species name, *N. neilliae*, was created for the New Zealand species formerly identified as *N. fastigiata*. There are still several lineages that as yet are unnamed, especially along the central Chilean coast.

The amount of genetic diversity uncovered in this study among species previously subsumed in *N. fastigiata* is high even considering that they occur in the upper intertidal, a habitat previously identified by Kelly & Palumbi (2010) as rife with genetic subdivision among invertebrates. The ITS region of the nuclear ribosomal cistron is particularly divergent between cryptic species, but significant divergences were revealed by all gene regions sequenced, as evidenced in the phylogenetic trees.

We observed the most consistent results in the *rbc*L and *psb*A gene sequences. Janouškovec *et al*. (2013), who studied the architecture of four red algal plastid genomes, found plastid DNA to be useful for resolving relationships; they noted that the *rbc*L gene was particularly good at resolving both evolutionarily deep as well as species/subspecies-level relationships. Our results also confirm previous observations (e.g. Kim *et al*. 2006) that the *rbc*L gene is a more sensitive marker compared to the *psb*A gene: the latter gene did not resolve relationships nearly as clearly as the former. The intermixing of genotypes for the nuclear ITS region in our study, especially among closely related species, suggests the possibility of incomplete lineage sorting, hybridization, introgression or thallus coalescence among these species. Problems using the ITS region, including slow genetic coalescence and intragenomic variation, have also been identified as reasons to use caution in employing ITS sequence data in phylogenetic and taxonomic studies (e.g. Álverez & Wendel 2003; Lane *et al*. 2007; Leliaert *et al*. 2014). Finally, we note the highly divergent *COI* sequences for some Chilean specimens. Whereas the majority of *COI* sequences produced a phylogenetic pattern similar to that observed for the plastid genes, a few diverged by 20–26% from sequences of other specimens of the same species. All of these divergent specimens occurred together in a clade sister to the remaining species of *Nothogenia* but still within the Sciniaceae, and they were all highly divergent from each other. Such a pattern of extreme divergence has not been previously reported for this frequently used 'bar-coding' gene; these sequences may represent numts (nuclear mitochondrial DNA) or pseudogenes.

Another contributing factor to the large genetic divergences observed among species is their geographic isolation in the Southern Hemisphere, where there are large distances between landmasses. An exception is the genetic diversity observed along the Chilean coast, where species differentiation appears to occur among proximate collections. In a study of the kelp *Durvillaea antarctica* (Chamisso) Hariot, Fraser *et al*. (2010) found low genetic connectivity across central Chilean sampling localities, suggesting that this may be attributable in part to habitat discontinuity. In a different study, Montecinos *et al*. (2012) observed genetic discontinuity in *Mazzaella laminarioides* (Bory) Fredericq, an intertidal red algal species endemic to Chile and the subantarctic islands, at 32°37′S–34°05′S and at 37°38′S–39°40′S. The genetic differentiation between these populations for both the *rbc*L and *COI* genes indicates that they represented different species. In the present study, we observed breaks in populations of species previously identified as *N. fastigiata* at 32°57′S–33°11′S and at 33°11′S–36°49′S. The first break point for both *Mazzaella* and *Nothogenia* occurred in the same area near Valparaíso, indicating that the region between 30°S and 33°S is an important phylogeographic break point on this coast; this has been also reported for other marine organisms in several phylogeographic studies (Tellier *et al*. 2009; Macaya & Zuccarello 2010; Sanchez *et al*. 2011; Brante *et al*. 2012; Haye *et al*. 2014). The ancient origin of this break might increase the genetic differentiation in poorly dispersing species (Haye *et al*. 2014), including red algae such as *Mazzaella* and *Nothogenia* (Alveal 2001; Montecinos *et al*. 2012). However, our data also indicate the possibility of mixed genotypes among most of these putative species of *Nothogenia* from mainland Chile, where specimens could potentially hybridize. More samples and sites need to be analysed to provide a better understanding of the phylogeographic structure of species of *Nothogenia* along this coast.

Previously, *N. fastigiata* was recognized as a highly plastic morphological species along the Chilean coast (Ramírez 1988; Hoffman & Santelices 1997); however, our results demonstrate the presence of different species. Ramírez (1988), studying two populations of *N. fastigiata* growing in different ecological habitats in central Chile, found morphological variation, with individuals from a protected environment being flattened and samples from an exposed site having cylindrical thalli. DNA sequence data including sequences from type material indicate that our samples collected from Caleta Errázuriz (23°S) and Punta Talca (30°S) correspond to *N. fragilis* and have a similar morphology to those from the exposed site in Ramírez (1988, figs 8–13); therefore, the range of this species might extend from Peru to central Chile. However, no contemporary specimens from Peru have been analysed, and the type

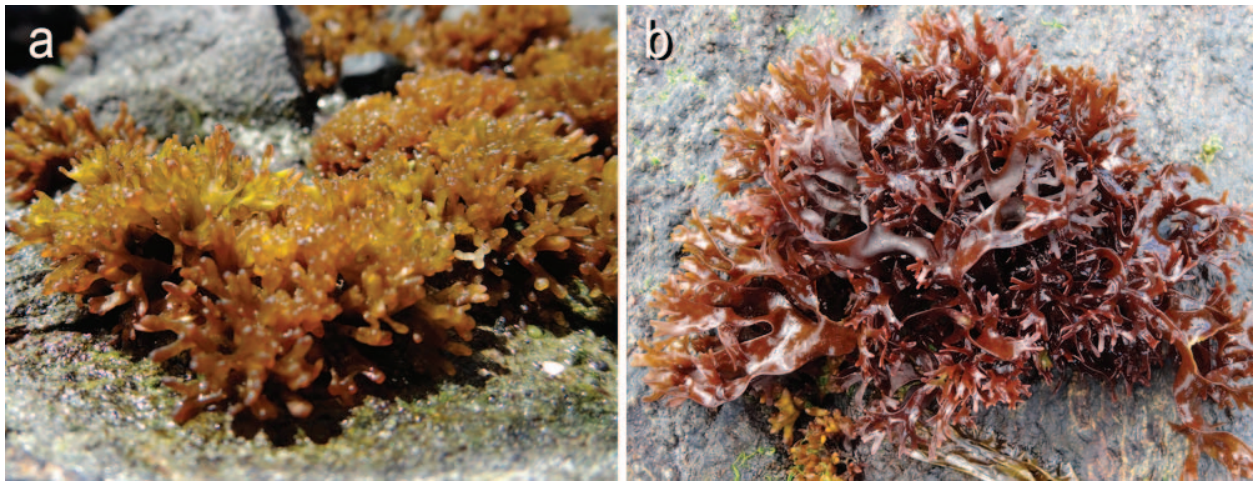

Fig. 5. Photos of the different morphologies of *Nothogenia fastigiata* (a) and Taxon C (b), the only species to co-occur in Chile.

collection, which was indicated to be from Peru, is from an area now part of Chile.

At most sites along the Chilean coast, only a single species was found, with the exception of Melinka and Repollal (43°S), where both *N. fastigiata* and Taxon C occurred in the mid-intertidal zone. These species are morphologically distinct (Fig. 5). Further work is needed to understand possible postglacial recolonization routes and/or possible glacial refuges of these species. For example, Taxon C might have recolonized from nonglaciated areas (e.g. Mar Brava, Cobquecura) after the Last Glacial Maximum (LGM).

On mainland New Zealand, the distribution of a northern species (*N. pulvinata*) and a southern species (*N. neilliae*) is consistent with well-documented patterns of geographic and genetic disjunctions among closely related species (e.g. *Glaphyrosiphon*—Hommersand *et al.* 2010; *Apophlaea*—Nelson 2013; *Melanthalia*—Nelson *et al.* 2013).

Within the subantarctic region of the Southern Hemisphere, the impacts of glaciation on the distribution of marine taxa have been investigated using molecular sequencing data to evaluate the connectivity of populations around the Southern Ocean and in southern South America (e.g. Fraser *et al.* 2009, 2012; Macaya & Zuccarello 2010; Reisser *et al.* 2011; González-Wevar *et al.* 2012). The impact of the LGM and the subsequent recolonization of habitats have been investigated for both macroalgal and invertebrate species. There is evidence that sea ice resulted in the removal of ice-sensitive shallow marine taxa, while ice-resistant taxa persisted through the LGM (e.g. Fraser *et al.* 2009, 2012; Reisser *et al.* 2011). Some biogeographic studies reveal the presence of circumpolar haplotypes and very low genetic diversity, indicating recent dispersal and population connectivity (e.g. Fraser *et al.* 2009; Macaya & Zuccarello 2010; Nikula *et al.* 2010). Other studies have documented species with very restricted distributions and high levels of genetic structuring (e.g. Reisser *et al.* 2011). The distribution of *N. variolosa*, restricted to the New Zealand subantarctic islands, suggests that this high intertidal species persisted through the LGM but has had limited capacity for dispersal. A more detailed investigation of population structure within and between islands would address questions about connectivity amongst the New Zealand subantarctic islands.

The record of *N. fastigiata* from Campbell Island, the southernmost island in the New Zealand subantarctic group, indicates that there has been recent connection around the Southern Ocean in this species. The most commonly invoked mechanism for genetic connectivity across vast ocean distances is rafting, and there is evidence of regular gene flow amongst populations of invertebrates associated with kelp rafts (e.g. Nikula *et al.* 2011a, b). It is less clear how a high to mid-intertidal macroalga such as *N. fastigiata* disperses, but in a recent article Fraser *et al.* (2013) reported evidence of transoceanic dispersal between New Zealand and South America of *Adenocystis utricularis* and *Bostrychia intricata*, two intertidal nonbuoyant algal species. They suggested attachment to buoyant macroalgae or floating wood, also a possible mechanism for dispersal of *N. fastigiata*. Moreover, inflated, buoyant specimens of *N. fastigiata* have been observed in the field (E. Macaya, personal observation). The single specimen of *N. fastigiata* found on Campbell Island and confirmed by sequencing was noted to be morphologically different from specimens growing on adjacent rocky substrates later confirmed to be *N. variolosa*. Further investigations of Campbell Island populations are warranted: *N. fastigiata* and *N. variolosa* appear to occupy the same niche within the mid- to upper intertidal shore.

These results add yet another family and order of red algae to a growing list in which sequenced type specimens allow us to unequivocally apply 19th- and early 20th-century names to modern collections (Hughey *et al.* 2001; Gabrielson 2008; Gabrielson *et al.* 2011; Lindstrom *et al.* 2011; Hind *et al.* 2014). Because of the cryptic diversity that is being uncovered with DNA sequencing within all orders of red algae, coupled with the morphological variability of some cryptic species, including Chilean species of *Nothogenia*, sequencing type specimens is necessary for the correct application of names. Moreover, Hughey *et al.* (2014) have demonstrated that entire plastid and mitochondrial genomes can be sequenced from tiny amounts of type material, a particularly appropriate application of NextGen sequencing,

which utilizes short sequences of DNA, exactly the kind of DNA present in type specimens 100 or more years old.

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SUPPLEMENTARY DATA

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