The *Bostrychia tenella* species complex: morphospecies and genetic cryptic species with resurrection of *B. binderi*

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ABSTRACT: The question of whether morphological differences observed in specimens is due to multiple species or one variable species has always caused problems for taxonomists. The most recent taxonomic treatment of the '*Bostrychia tenella* species complex' suggested that much of the morphological variation represented a single highly variable entity. We used molecular data from all three genomes to clarify the phylogeny, species status and phylogeography of samples collected world-wide, and also in sympatry, of this complex. Our data strongly supports five genetic species in this complex, but only three morphological entities were recognized. The first, divided into two genetic species, fits characters associated with *B. binderi*, occasionally possessing short monosiphonous determinate laterals but lacking them most of the time. We therefore resurrect *B. binderi*, even though we could not assign a name to either of the two genetic species as we are missing molecular evidence from the type specimen. One genetic species was morphologically recognized as *B. montagnei*. Another lineage consisted of the two genetic species that fall into a new circumscription of *B. tenella*, with long monosiphonous determinate laterals. Again we were unable to assign either of these two lineages to a type, nor could we find morphological differences between the two lineages. Many of the genetic species have world-wide distributions, except for *B. montagnei*, which appears to be restricted to the Americas. Our molecular assisted taxonomy has helped clarify some of the morphological variation within the *Bostrychia tenella* species complex into three named species, but two cryptic species were still recognized that remain morphologically cryptic.

Keywords: *Bostrychia tenella*, *cox*2-3 spacer, large-subunit ribosomal DNA, phylogeny, *rbc*L, Rhodophyta, Rhodomelaceae, RuBisCo spacer, species delimitation.

INTRODUCTION

The question of whether morphological differences observed in specimens is due to multiple species or one variable species has always caused problems for taxonomists. Environmental conditions are known to change the morphology of genetically similar entities (phenotypic plasticity) and genetically distinct entities have been found that fulfil most of the criteria used in species delimitation (i.e. genetically distinct, reproductively isolated), but can not be distinguished morphologically (cryptic species)(e.g. Zuccarello & West 2003; Payo *et al.* 2012). These problems are even greater in organisms that have relatively simple morphologies to begin with and few morphological characters for species distinction. With molecular data we now have a method to easily designate putative species based on DNA sequencing (molecular-assisted alpha taxonomy) (Cianciola *et al.* 2010; Leliaert *et al.* 2014).

The taxonomy of *Bostrychia* has received a great deal of attention using molecular methods, since the last monographic account of King & Puttock (1989; reviewed in Zuccarello & West 2011). These studies have shown that the species diversity is greater than previously assumed (Zuccarello & West 2003, Muangmai et al. 2014) and have lead to a rearrangement of the generic circumscriptions within the Bostrychieae (Zuccarello & West 2006). For example, the genus *Stictosiphonia* which was distinguished from *Bostrychia* based on the number of tier cells per axial cell (>3 versus 2, respectively)(King & Puttock 1989) was merged with *Bostrychia* as tier cell number has evolved multiple times and is even variable within species (*B. montagnei*, Zuccarello *et al.* 2012).

Molecular studies have so far mostly focussed on the ecorticate species. The species diversity and taxonomy of corticated species have been largely ignored.

Corticated species of *Bostrychia* restricted to the southern hemisphere are *Bostrychia arbuscula* J.D. Hooker & Harvey and *B. gracilis* (R.J. King & Puttock) Zuccarello & J.A. West, both with three or more tier cells per axial cell (Muangmai *et al.* 2014).

The most common tropical, and warm temperate, corticated marine *Bostrychia* with (mostly) two tier cells per axial cell is a group of species variously described as *Bostrychia tenella* (Lamouroux) J. Agardh, *B. binderi* Harvey, *B. montagnei* Harvey and *B. flagellifera* E. Post. These species make up the *B. tenella* species complex. These names, except for *B. montagnei*, were synonymized into one species (King *et al.* 1988; King & Puttock 1989). The morphological characters used to distinguish these species for the most part overlapped (King *et al.* 1988), except for samples identified as *B. flagellifera* which were morphologically significantly different and assigned to a separate subspecies, *B. tenella* subspecies *flagellifera* (E. Post) R.J. King & Puttock. Later it was shown that *B. tenella* ssp. *flagellifera* was distantly related to other samples of the *Bostrychia tenella* species complex and it was returned to species status (Zuccarello & West 2006). Within the remaining species, except for *B. montagnei*, only the proportion of monosiphonous ultimate laterals appears to have any taxonomic value but even this was believed to be continuous and be part of a single highly variable species (King *et al.* 1988). Taxonomic questions still remain as to the relationships of the remaining species.

Molecular-assisted taxonomy (Cianciola *et al.* 2010) is a powerful tool to aid in untangling morphological variation (intra-species plasticity) from species status (species diagnostic characters) and allows for more natural taxonomies to be produced. By using molecular data from many worldwide samples are we able to better understand the taxonomy of the *B. tenella* species complex? Genetic species have been discovered

often with a modification of the 'barcode gap' (i.e. clear difference between inter- versus intra-species variation), a procedure that has been automated (ABGD- Puillandre *et al.* 2012). There are also other methods that take into account particular population and evolutionary parameters (general mixed Yule-coalescent, GMYC; Pons *et al.* 2006). These methodologies have been summarized from an algal perspective (Leliaert *et al.* 2014). The genetic clusters produced have been used to form tentative groups that then can be assessed using more traditional methods (i.e. search for diagnostic morphological characters, reproductive isolation) (Tronholm *et al.* 2010; Vieira *et al.* 2014).

We investigated the phylogeny and species level relationships of the *B. tenella* species complex to determine if we can produce a more natural taxonomy. As with many well sampled widely distributed tropical species we expect multiple cryptic species and it is possible that genetic species delimitation will facilitate the discovery of morphological characters that can be used in the field to identify these lineages.

MATERIALS AND METHODS

Most samples were field collected and dried in silica-gel soon after collection. Some samples were maintained in unialgal culture for up to 33 years following the procedures in West & Zuccarello (1999) and West (2005).

DNA extraction followed a Chelex extraction procedure (Zuccarello *et al.* 1999). Amplification of the plastid-encoded large subunit of the ribulose bisphosphate carboxylase/oxygenase gene (*rbc*L) used primers presented by Nam *et al.* (2000) and Freshwater & Rueness (1994). RuBisCo spacer amplification protocols followed Zuccarello *et al.* (1999a). Amplification of an approximately 900-1000 bp region (Y-fragment) of the nuclear encoded large subunit ribosomal DNA gene (LSU), followed

116 Harper & Saunders (2001). Amplification of the *cox*2–*cox*3 spacer followed Zuccarello
117 et al. (1999b). All successfully amplified products were cleaned using an ExoSAP-IT
118 (USB, Cleveland, Ohio) before being sequenced commercially (Macrogen Inc., Korea)
119 Sequences were edited, assembled and aligned using the Geneious software
120 package version 7 (Biomatters, available from http://www.geneious.com/). Alignment
121 was straight forward as no gaps were found in the *rbc*L data set. Alignment for the
122 RuBisCo spacer used MAFFT (Katoh *et al.* 2002) as implemented in Geneious and
123 visually refined. LSU alignment used the online version of MAFFT v7 and the Q-INS-I
124 algorithm that takes into account RNA secondary structure (Katoh & Standley 2013)
125 and visually refined. *Cox*2-3 spacer sequences were difficult to align, and thus we
126 aligned using *a posteriori* lineages (block alignment). The 5-prime end of *cox*2 was
127 alignable between all samples, and produced a phylogeny consistent with the other
128 genes (not shown), the spacer was then aligned as blocks between samples from the
129 different lineages. For the *rbc*L and LSU analyses the outgroup used was *Bostrychia*
130 *kelanensis* a species with cladohaptera, in a group that is shown to be distinct from
131 species with peripherohaptera in the tribe Bostrychieae (Zuccarello & West 2006).
132 The program Modeltest version 3.7 (Posada & Crandall 1998) was used to find
133 the model of sequence evolution that best fit the data set by a Akaike Information
134 Criterion (AIC) (Posada & Crandall 2001). Maximum likelihood was performed with
135 RAxML 7.2.8 (Stamatakis 2006). RAxML was performed, with all three codons
136 partitioned (where appropriate) and the GTR+gamma model and 500 non-parametric
137 bootstrap replicates (Felsenstein 1985). Bayesian inference was performed with
138 MrBayes v3.2 (Ronquist *et al.* 2012). Analyses consisted of two independent
139 simultaneous runs of one cold and three incrementally heated chains, and 3 x 10^6

generations with sampling every 1000 generations. The log files of the runs were checked with Tracer v1.5 (Rambaut & Drummond 2009) and a burn-in sample of 500 trees was removed from each run before calculating the majority rule consensus tree. TCS 1.21 (Clement *et al.* 2000) was used to construct a haplotype network based on RuBisCo spacer sequence data.

DNA-based species delimitation were conducted using the RuBisCo spacer data set (most complete sampling) by two different methods: GMYC (Pons *et al*., 2006) and ABGD (Puillandre *et al*. 2012). For the GMYC delimitation method, an ultrametric tree was constructed in BEAST v2.0.2 (Drummond *et al*. 2012), relying on the uncorrelated lognormal relaxed clock, the GTR + I + R and a coalescent tree prior. Bayesian Markov chain Monte Carlo (MCMC) was run for 20 million generations, and trees and parameters sampled every 1000 generations. Log files were visualized in Tracers v1.5 (Rambaut & Drummond 2009) for assessing the stationary state of parameters based on the value of estimate effective sample size. After removing 25% of trees as burn-in, the remaining trees were used to generate a single summarized tree in TreeAnnotator v2.0.2 (BEAST v2.0.2 package) as an input file for GMYC. The GMYC analyses with a single- and multiple- threshold model were performed in R (R Core Team, 2013) under "splits" package using the 'GMYC' function (R – Forge, http://r-forge.r-project.org/projects/splits/). The ABGD method was tested via a web interface (ABGD web, http://wwwabi.snv.jussieu.fr/public/abgd/ abgdweb.html).

RESULTS

The *rbc*L data set consisted of 1163 basepairs (bp) and 58 specimens. The sister relationships to the *B. tenella* species complex are not well resolved, nor is the *B.*

tenella species complex strongly supported with this data set (73% ML bootstrap support, 1.0 Bayesian posterior probabilities; Fig. 1). Within the *B. tenella* species complex three major clades are evident. One clade is sister to the other two, designated lineage A, consisting of samples from around the world (e.g. Brazil, Indonesia, Australia; Fig. 1., Supplementary Table 1). Within lineage A, a single sample is genetically distinct, sublineage A1. Samples identified as *B. montagnei* also form a well supported clade and are exclusively from tropical America. The clade sister to *B. montagnei* is composed of two sublineages with high support. These sublineages are designated B and C. This *rbc*L phylogeny is fully compatible with the LSU phylogeny (Supplementary Fig. 1), although LSU has only moderate support for sublineage C.

The RuBisCo spacer was sequenced for nearly all the samples (n=137, aligned data set 303bp) and produced 27 unique haplotypes (Fig. 2). Again the phylogeny produced three main clades (Fig. 2). Lineage A consisted of a sister group (sublineage A1) with six samples all of the same RuBisCo haplotype (1A1; Fig. 2, Supplementary Table 1) from the Pacific and southern Indian Ocean (South Africa, Guam, Thailand, Papua New Guinea, Philippines). Samples of the remaining samples, sublineage A, had 11 haplotypes, all derived from common haplotype, A1 (n=34; Fig. 2). Haplotype A1 is found in the western Pacific, all other haplotypes are found throughout the Indo-Pacific, except haplotype A5 found in central Atlantic (Puerto Rico) and A9 found exclusively in Brazil (Supplementary Table 1). *Bostrychia montagnei* makes a supported clade with the RuBisCo spacer data with two haplotypes. Haplotype M1 is exclusive to the American Atlantic (USA, Brazil) and M2 is found in both the Atlantic and Pacific (e.g. Belize, El Salvador; Fig. 2, Supplementary Table 1). The third clade is again divided into two sublineages, B and C, that are well supported. Sublineage B has seven

188 haplotypes with the putative ancestral one found throughout the world (B1; n=39, e.g.
189 Cuba, Panama, South Africa, Australia, Papua New Guinea, Korea; Fig. 2). The other
190 six haplotypes are found only in the western Pacific (e.g. the Philippines, Palau).
191 Sublineage C consists of 6 haplotypes with the putative ancestral haplotype, C1 (n=17),
192 again found throughout the world (e.g. Brazil, Madagascar, Papua New Guinea).
193 Haplotype C3 is from Madagascar, C4 from the eastern Indian Ocean (Western
194 Australia), while the remainder are from the western Pacific. The *cox*2-3 spacer data
195 (n=101) produced a phylogeny congruent with all other phylogenies (Supplementary
196 Fig. 2) although not all lineages received strong support.
197 The GMYC model produced grouping significantly different from the null
198 model (one population) and estimated five entities (putative species), these correspond
199 to sublineages A1, A, B, C and *B. montagnei*. The ABGD also proposed five species
200 groups corresponding to the same entities as the GYMC model (Table 1).
201 Morphological investigation based on samples from culture and the field from
202 around the world revealed that samples in clade A in the phylogenetic analyses had very
203 short or few monosiphonous laterals and were heavily corticated in culture. Samples
204 from culture either lacked secondary laterals on the determinate branches (Figs 3, 4) or
205 had short 'spine-like' polysiphonous secondary laterals (Figs 5, 6). Field samples from
206 Western Australia were consistent with culture material. Both possessed spine-like
207 secondary laterals and the main axis was heavily corticated (Fig. 6). Samples in lineages
208 B and C had varying degrees of monosiphonous secondary laterals (Figs 7-10) and
209 cortication, at least at the apex of primary shoots (Fig. 7). Monosiphonous sections on
210 secondary laterals were mostly over 10 cells long, and were abundant, especially in
211 culture (Figs 7, 8). Field samples from the Atlantic and Indian Oceans also had long

monosiphonous lateral branches and light cortication on the secondary laterals (Figs 9, 10). We found no consistent pattern in these characters to distinguish between lineages B and C using samples from the Atlantic, Indian and Pacific Oceans.

The morphological difference (degree of monosiphonous filaments on secondary branches) between samples in clade A and clade B-C investigated in samples collected on the same date from Dyual Island, New Ireland, Papua New Guinea showed that samples in sublineage A were heavily corticated and only had short monosiphonous filaments (5-10 cells long) at the tips of unbranched secondary laterals (Figs 11, 13). Monosiponous laterals were often lacking on secondary laterals (Fig. 12). Samples in sublineage C had abundant and long monosiphonous branches on secondary laterals (Figs 14, 15). This abundance of monosiphonous laterals in isolates genetically identified as belonging to sublineage B or C was seen in all isolates investigated.

DISCUSSION

Our data revealed the phylogenetic relationships within the *Bostrychia tenella* species complex. We showed that this complex contained multiple lineages, some of which can be characterized based on their morphological variation and some can not. These data again highlight the utility of molecular assisted taxonomy in unravelling a morphologically variable and widely distributed species. Our results clearly showed that the *Bostrychia tenella* species complex consists of several 'putative genetic species' using two different species delimitation methods (Leliaert *et al.* 2014). As model assumptions were different for different techniques more than one method indicating the same species delimitations is recommended (Carsten *et al.* 2013). Both methods showed that there are five 'species' in the *Bostrychia tenella* species complex. Several of these

236 'genetic species' are morphologically distinguishable. *Bostrychia montagnei*, while
237 morphologically similar to *B. tenella,* is clearly distinct because of its circinate apices
238 (King & Puttock 1989). *Bostrychia montagnei* is restricted to the Americas (Atlantic
239 and Pacific Oceans) and at present has limited genetic variation with the markers used.
240 *Bostrychia montagnei*, therefore, meets two important criteria used to define species
241 (i.e. morphological and phylogenetic).
242 The remainder of the isolates fell into four genetic species (A, 1A, B, C). One
243 large clade consists of samples, in two groupings, which we have designated as A and
244 1A. Most of the samples are in sublineage A and found world-wide. A sister sublineage
245 (1A) is also recognized as a species using species delimitation criteria. This sublineage
246 consists of only one haplotype, is rarer, but also widely distributed, although not found
247 yet in the Americas. These entities (sublineages A and 1A) are also morphologically
248 distinct from other samples of the *Bostrychia tenella* species complex both in culture
249 and from sympatric field samples. *Bostrychia binderi* was synonymized with *B. tenella*
250 (King & Puttock 1989) based on a morphological study indicating that the distribution
251 and length of monosiphonous laterals did not clearly distinguish between isolates *a*
252 *priori* identified as *B. binderi* and *B. tenella* (King *et al.* 1988). This study indicated that
253 the only character that could be used to separate the species was the length of
254 monosiphonous cells in determinate lateral branches. Still King *et al.* (1988) proposed
255 that these samples were part of a morphological continuum within one morphologically
256 variable species and merged the two species (King & Puttock 1989). The question of
257 whether observed morphological variation in a character is due to inter- or intra-species
258 variation is one of the main issues in taxonomic studies. Quantitative characters (e.g.
259 degree of monosiphonous lateral branches) are known to vary based on environment

plus genetics, and untangling these factors in species descriptions is difficult. It is unclear from the King *et al.* (1988) paper how the species were designated for the *a priori* classification, except maybe voucher specimen designation. Now with molecular data we can assign samples to evolutionary lineages and we see a fairly consistent morphological pattern. Samples in sublineages A and 1A seem to be more robust (more heavily corticated) both in culture and the field, and have no, or sparse and short, monosiphonous laterals. Therefore we resurrect *Bostrychia binderi*.

Bostrychia binderi Harvey

W. H. Harvey. *Neries australis*. 68. *pl. XXVIII* (upper group of figures).

Resurrecting *B. binderi* clearly separates the *B. tenella* species complex into a taxonomy that more closely reflects the phylogeny of the group (*B. binderi*, *B. montagnei*, *B. tenella*). And yet important issues remain unresolved. First, our genetic species delimitation data indicate at least two 'genetic species' under this nomenclature for both *B. binderi* (lineage A and A1) and *B. tenella* (lineages B and C). While most of our samples of *B. binderi* are found in lineage A, the only sample from South Africa is in lineage A1 (JAW3176, Isipingo, Natal). Durban, Natal province is the type locality for *B. binderi* (King & Puttock 1989). We attempted to sequence DNA from the type specimen of *B. binderi* (MEL672330, Durban, Port Natal, *c.* 1839 Krauss or Ecklon) but were unsuccessful (designed RuBisCo spacer primers targeting a 175bp fragment, information available on request). This type sample and the illustrations (Harvey 1847) of the type match the above description of *B. binderi* as lacking monosiphonous lateral branches. As our sampling was poor in South Africa it is unclear if samples of both lineage A and 1A are found in the type locality, as they are in other more extensively

284 sampled sites (Thailand, Papua New Guinea- this study). It is increasingly evident that
285 increased sampling uncovers genetic diversity not seen with limited sampling
286 (Zuccarello *et al.* 2006; Dijoux *et al.* 2014). We reserve the assignment of the species
287 name to one or the other lineage and consider all samples in this clade, with the set of
288 morphological characters, as *B. binderi* with only DNA sequencing able to distinguish
289 the species within this cryptic species complex.
290 It is also clear that there are two 'genetic species' of *B. tenella* (B and C). The
291 type for *B. tenella* is from St. Croix, Caribbean Sea, and the only samples we collected
292 of *B. tenella* from that area (Puerto Rico) is in lineage B. Should this be the designated
293 *B. tenella* lineage? At present we can not distinguish morphologically between the two
294 lineages, nor do we have access to the type for potential genetic characterization.
295 Therefore we do not designate the species name to either lineage, at present, and
296 consider *B. tenella* another cryptic species complex with lineages only designated
297 molecularly. This conservative approach takes into account the idea that morphology
298 may never distinguish between these cryptic lineages. Sequencing of the type will help
299 pinpoint the lineage that is associated with the type specimen, and this has been done in
300 other algae (e.g. Lindstrom *et al.* 2011; Hughey & Gabrielson 2012; Hind *et al.* 2014).
301 While samples of *B. binderi* are morphologically distinct from *B. tenella* by
302 lacking monosiphonous determinate laterals, there are cases where samples of *B.*
303 *binderi* can be confused with *B. tenella*. Some samples identified morphologically as *B.*
304 *tenella*, due to the presence of short monosiphonous laterals, are genetically in the *B.*
305 *binderi* lineage. So while we have improved the taxonomy and determined characters
306 that best identify lineages, clear diagnostic characters are still elusive. Morphological
307 species boundaries are difficult to characterize in many cases as many environmental

characters can influence morphology (light levels, disturbance). While morphometrics and other methods are informative in morphological species delimitation in organisms with complex morphologies (e.g. *Halimeda*, Verbruggen *et al.* 2005) in simple filamentous algae these methods may not useful.

An important criterion in species recognition and evolution is reproductive isolation. Are these genetic species reproductively compatible? In other *Bostrychia* species cryptic lineages are reproductively isolated (Zuccarello & West 1997, 2003). We attempted to perform crosses between culture isolates of *B. tenella* and *B. binderi* but were unsuccessful. In culture, isolates became bisexual and self-fertile. Bisexuality is well known in *Bostrychia* species, and even mixed-phase reproduction occurs in *B. tenella* (West & Calumpong 1988) and *B. moritziana* (West & Zuccarello 1999). Results indicated that the successful crosses were self-crosses.

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463 Table 1. Species delimination techniques with all RuBisCo spacer haplotypes of the *B.*

464 *tenella* species complex.

465

GMYC analysis	Single threshold	Multiple threshold
Log likelihood of null model (one population)	154.82	154.82
Log likelihood of GMYC model	159.69	159.69
Result of likelihood ratio test	$p = 0.208$*	$p = 0.208$*
Number of GMYC entities (confidence intervals)	5 (5-14)	5 (4-11)
ABGD analysis		
Partitions (groups)	5	

466

467

468

Figure Legends

Fig. 1. Maximum-likelihood tree of *rbc*L sequence data of *Bostrychia* species, highlighting the position and multiple lineages within the *B. tenella* species complex. Thickened branches = ≥95% ML bootstrap values and ≥0.95 Bayesian posterior probabilities. Other values associated with branches = RaxML bootstrap percentage/Bayesian posterior probabilities.

Fig. 2. Left: Maximum-likelihood tree of the 27 RuBisCo spacer haplotype of the *B. tenella* species complex showing the 6 lineages inferred as genetic species by species delimitation algorithms (*B. binderi* = A, A1; *B. tenella* = B, C; *B. montagnei* = M). **Right:** Haplotype network of the 27 RuBisCo spacer haplotypes for five putative genetic species (A1 only one haplotype, left out). Line= one mutational step, small circle= missing haplotype. n= number of samples.

Figs 3-10. Cultured and field specimens of samples *B. binderi* and *B. tenella*. Samples live or from dried specimens. 4-digit numbers = JAW culture numbers (more details in Supplementary Table 1).

Fig. 3. *B. binderi*, 2514, Puerto Rico, lineage A. Culture specimen with polysiphonous primary laterals, secondary laterals absent. Scale bar = 100 µm.

Fig. 4. *B. binderi*, 2641, Brazil, lineage A. Culture specimen with polysiphonous primary laterals, secondary laterals absent. Peripherohaptera (arrowheads) at intervals along the main shoot. Scale bar = 50 µm.

Fig. 5. *B. binderi*, 2851, Queensland, Australia, lineage A. Culture specimen with only spine-like polysiphonous laterals. Scale bar = 100 µm.

Fig. 6. *B. binderi*, 3743, Western Australia, lineage A. Field specimen. Primary polysiphonous laterals with spine-like polysiphonous secondary laterals. Scale bar = 100 µm.

Fig. 7. *B. tenella,* 2515, Puerto Rico, lineage B. Culture specimen with monosiphonous primary laterals and lightly corticated main axis. Peripherohapteron (arrowhead) opposite indeterminate shoot. Scale bar = 100 µm.

Fig. 8. *B. tenella,* 2815, Queensland, Australia, lineage B. Shoot apex of cultured specimen with polysiphonous primary laterals and many monosiphonous laterals. Scale bar = 50 µm.

Fig. 9. *B. tenella,* 3655, Brazil, lineage C. Field specimen with polysiphonous laterals and many monosiphonous laterals. Scale bar = 100 µm.

Fig. 10. *B. tenella,* 3807, Western Australia, lineage C. Field specimen with primary polysiphonous laterals and many monosiphonous laterals. Scale bar = 100 µm.

Figs 11-15. Specimens from Dyual Island, New Ireland, Papua New Guinea.
Figs 11-13, *B. binderi* G311, lineage A. Figs 14-15, *B. tenella* G396, lineage C.

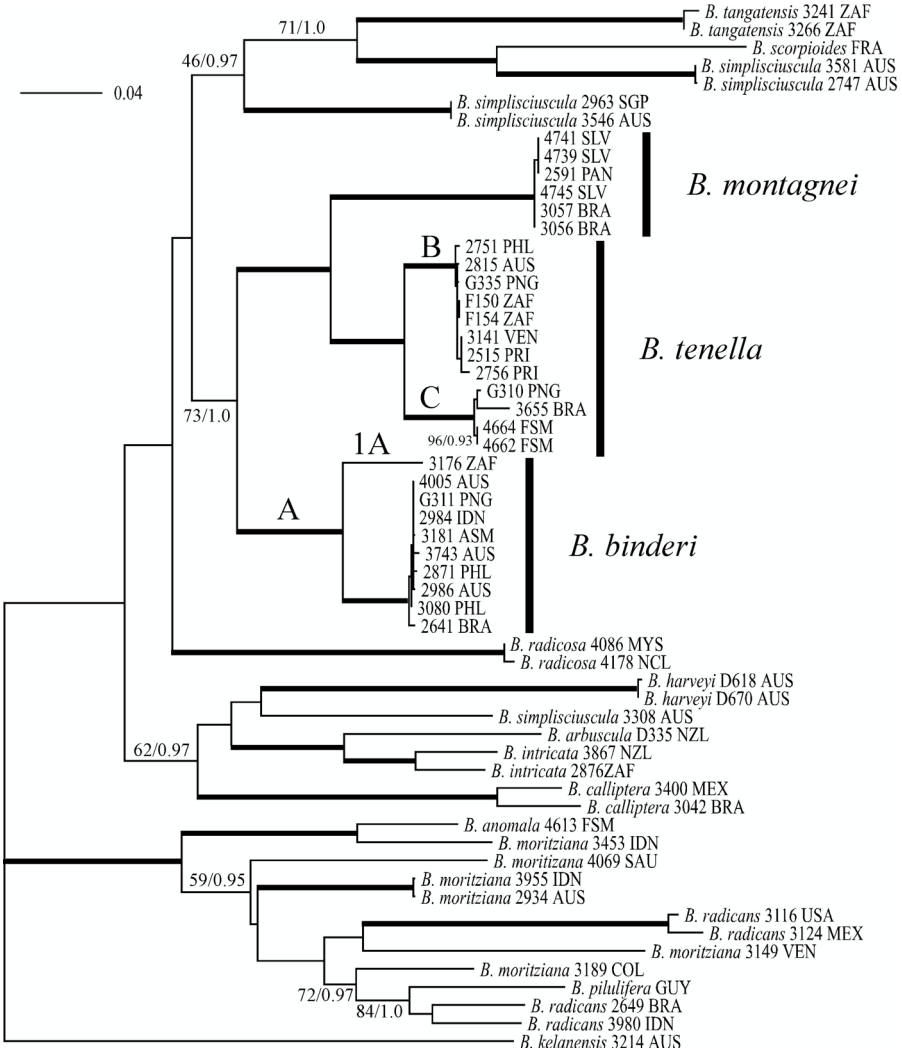
Fig. 11. Heavily corticated main axis with peripherohaptera (arrowheads, P). Heavily corticated primary laterals , secondary laterals, spine-like, with polysiphonous base and occasionally short monosiphonous tips. Scale bar = 100 μm.

Fig. 12. Heavily corticated primary laterals, short polysiphonous secondary laterals. Scale bar = 50 μm.

Fig. 13. Older lower axis with primary laterals corticated at bases and monosiphonous upper sectors (to 10-15 cells long), bearing secondary laterals with very short (2-3 axial cells long) polysiphonous bases and upper monosiphonous sectors up to 20 cells long. Scale bar = 100 μm.

Fig. 14. Upper main shoot and primary laterals not corticated, secondary laterals entirely monosiphonous. Scale bar = 100 μm.

Fig. 15. Mid-shoot axis lightly corticated, primary laterals polysiphonous but not corticated, secondary laterals entirely monosiphonous. Scale bar = 150 μm.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B. simplisciuscula 2747 AUS
B. simplisciuscula 2963 SGP
B. simplisciuscula 3546 AUS
4741 SLV
4739 SLV
2591 PAN
4745 SLV
3057 BRA
3056 BRA
B. montagnei
B
2751 PHL
2815 AUS
G335 PNG
F150 ZAF
F154 ZAF
3141 VEN
2515 PRI
2756 PRI
B. tenella
C
G310 PNG
3655 BRA
73/1.0
96/0.93
4664 FSM
4662 FSM
1A
3176 ZAF
4005 AUS
G311 PNG
2984 IDN
3181 ASM
3743 AUS
2871 PHL
2986 AUS
3080 PHL
2641 BRA
A
B. binderi
B. radicosa 4086 MYS
B. radicosa 4178 NCL
B. harveyi D618 AUS
B. harveyi D670 AUS
B. simplisciuscula 3308 AUS
B. arbuscula D335 NZL
62/0.97
B. intricata 3867 NZL
B. intricata 2876ZAF
B. calliptera 3400 MEX
B. calliptera 3042 BRA
B. anomala 4613 FSM
B. moritziana 3453 IDN
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B. moritziana 3955 IDN
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B. radicans 3124 MEX
B. moritziana 3149 VEN
B. moritziana 3189 COL
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84/1.0
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B. radicans 3980 IDN
B. kelanensis 3214 AUS

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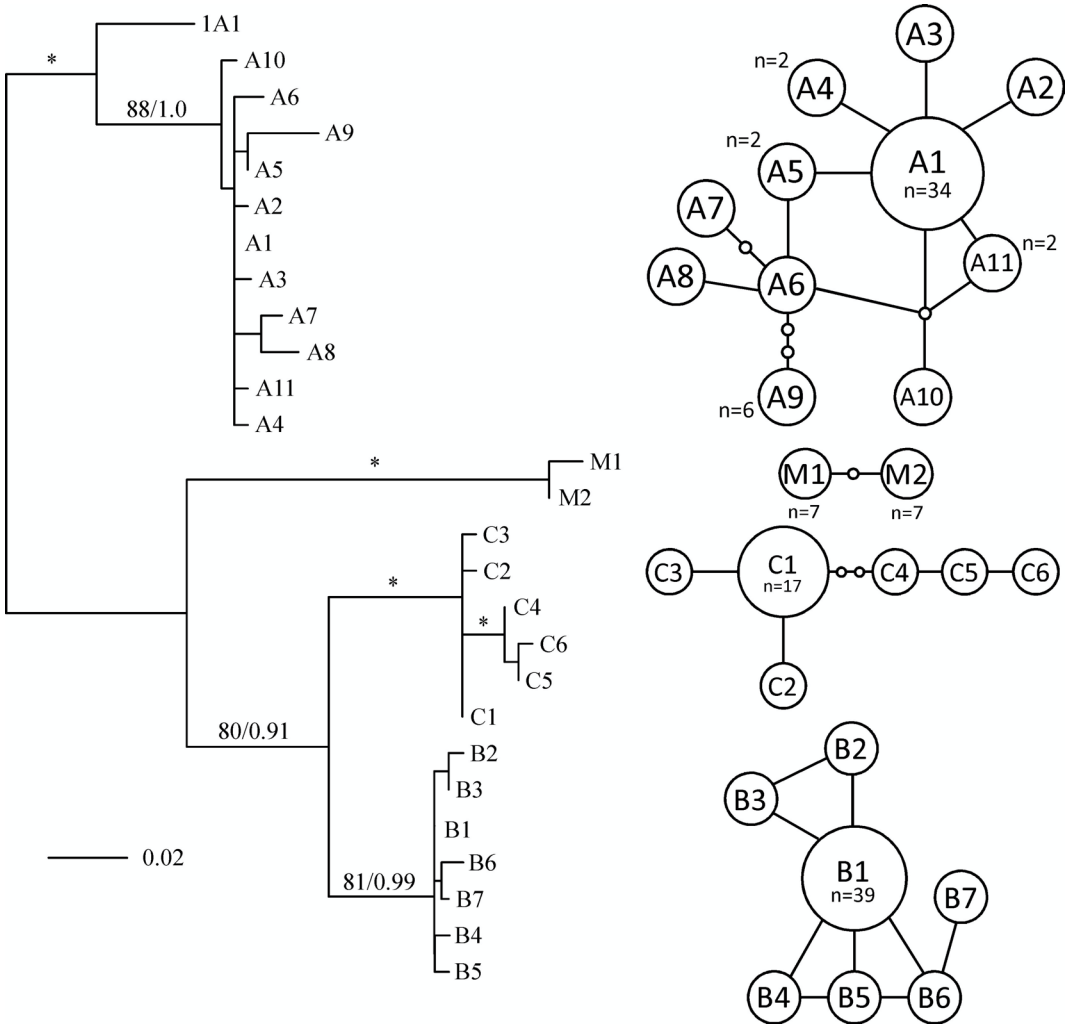
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M2
C3
C2
C4
C6
C5
C1
80/0.91
B2
B3
B1
B6
B7
B4
B5
81/0.99
0.02
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A4
A2
A1
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A10
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B5
B6

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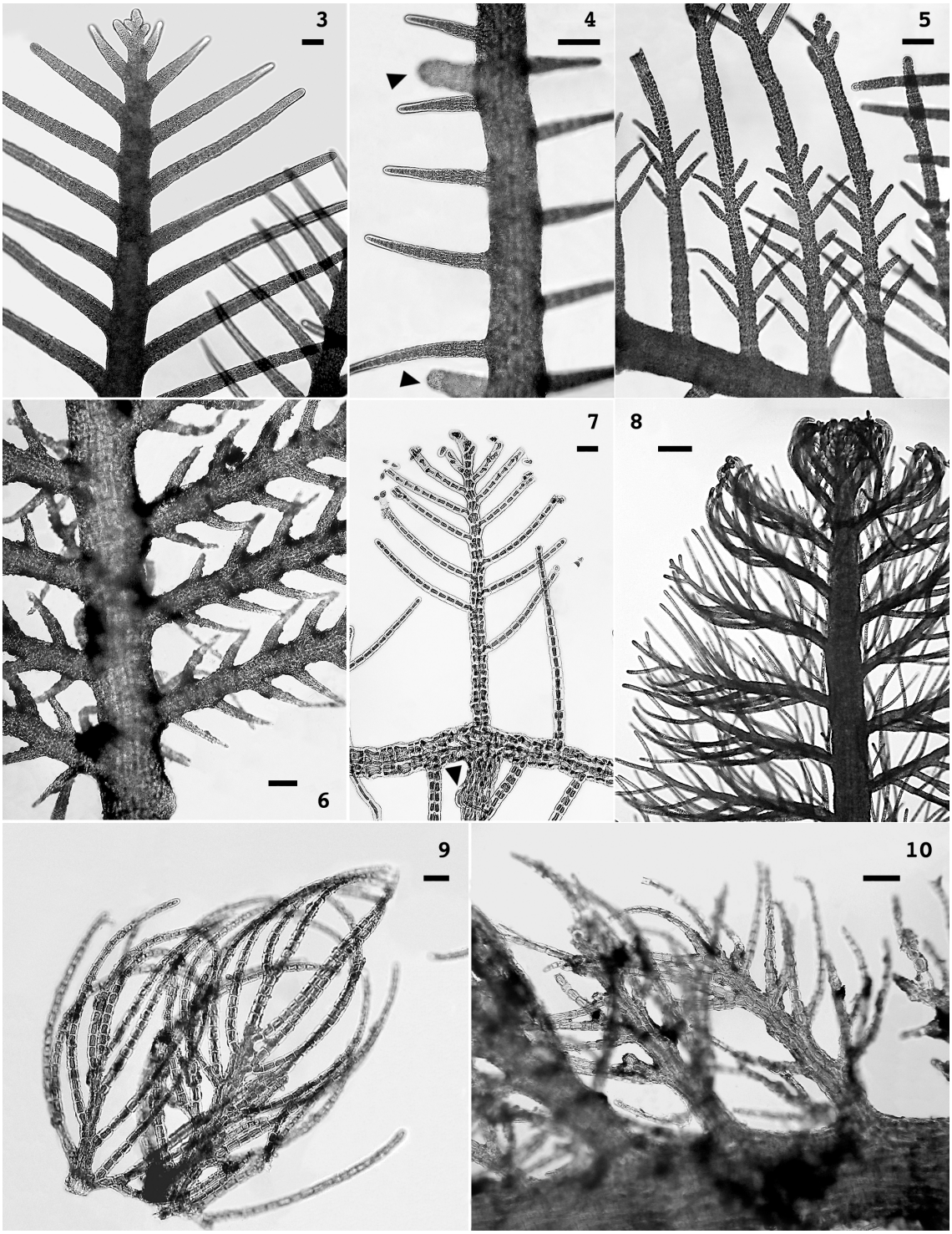

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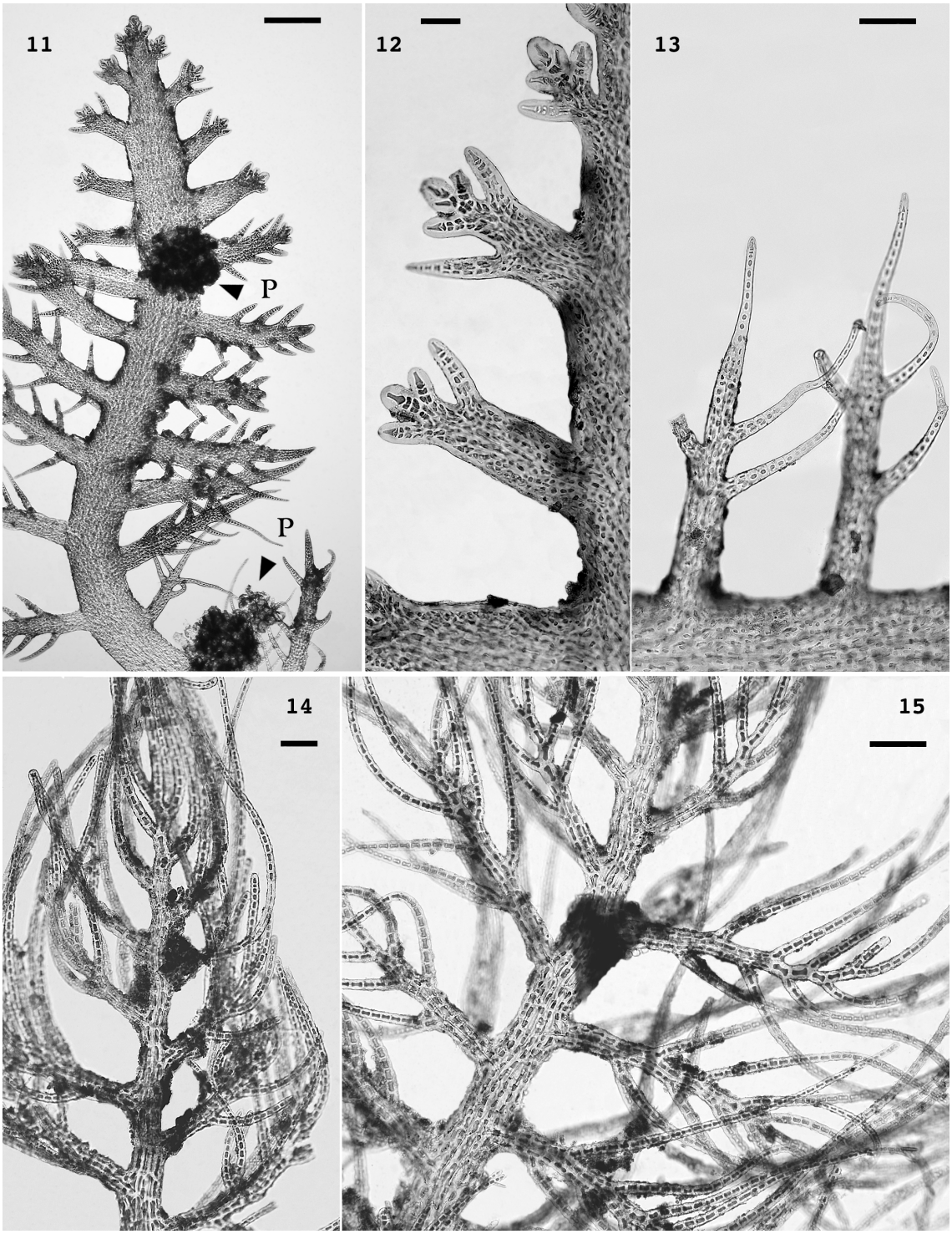

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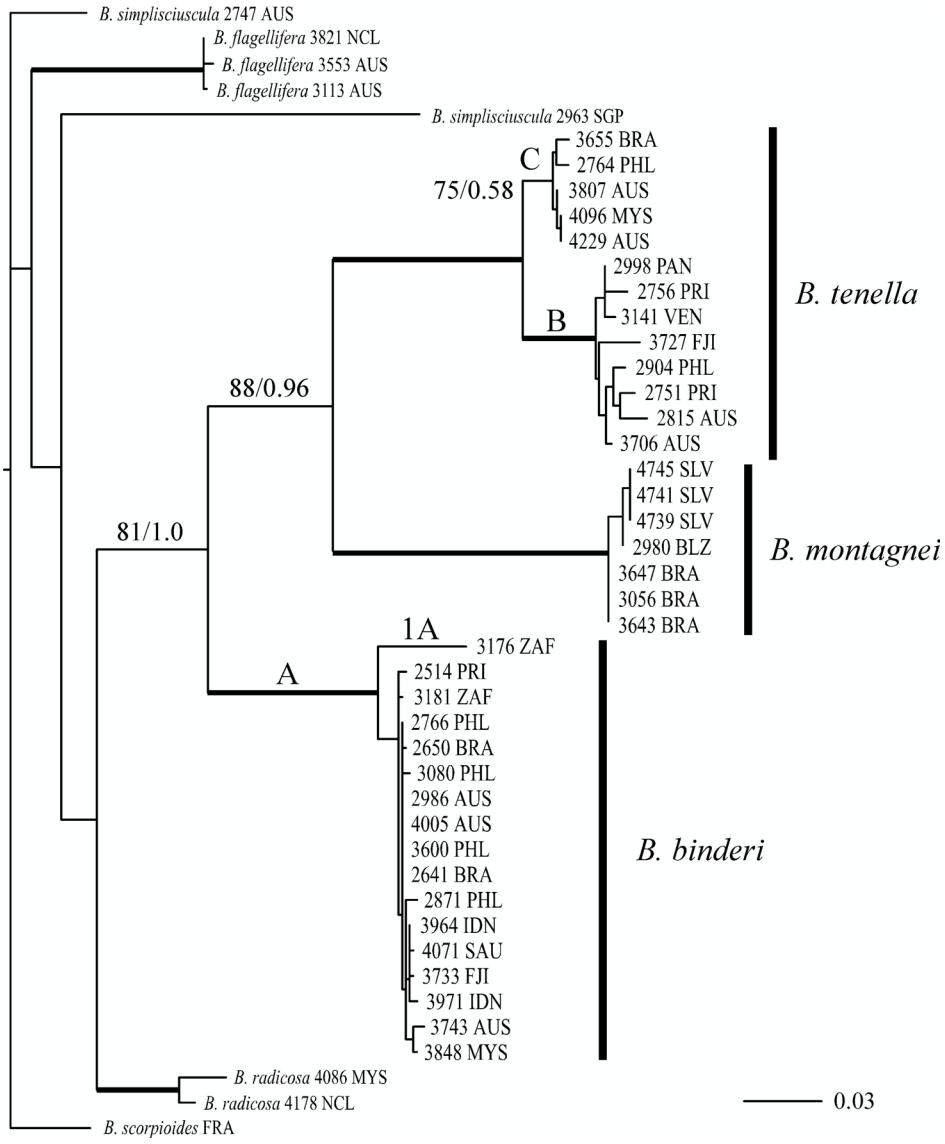
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B. flagellifera 3113 AUS
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C
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3655 BRA
2764 PHL
3807 AUS
4096 MYS
4229 AUS
2998 PAN
2756 PRI
3141 VEN
B
3727 FJI
2904 PHL
2751 PRI
2815 AUS
3706 AUS
B. tenella
88/0.96
4745 SLV
4741 SLV
4739 SLV
2980 BLZ
3647 BRA
3056 BRA
3643 BRA
B. montagnei
81/1.0
1A
3176 ZAF
A
2514 PRI
3181 ZAF
2766 PHL
2650 BRA
3080 PHL
2986 AUS
4005 AUS
3600 PHL
2641 BRA
2871 PHL
3964 IDN
4071 SAU
3733 FJI
3971 IDN
3743 AUS
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B. binderi
B. radicosa 4086 MYS
B. radicosa 4178 NCL
0.03
B. scorpioides FRA

545

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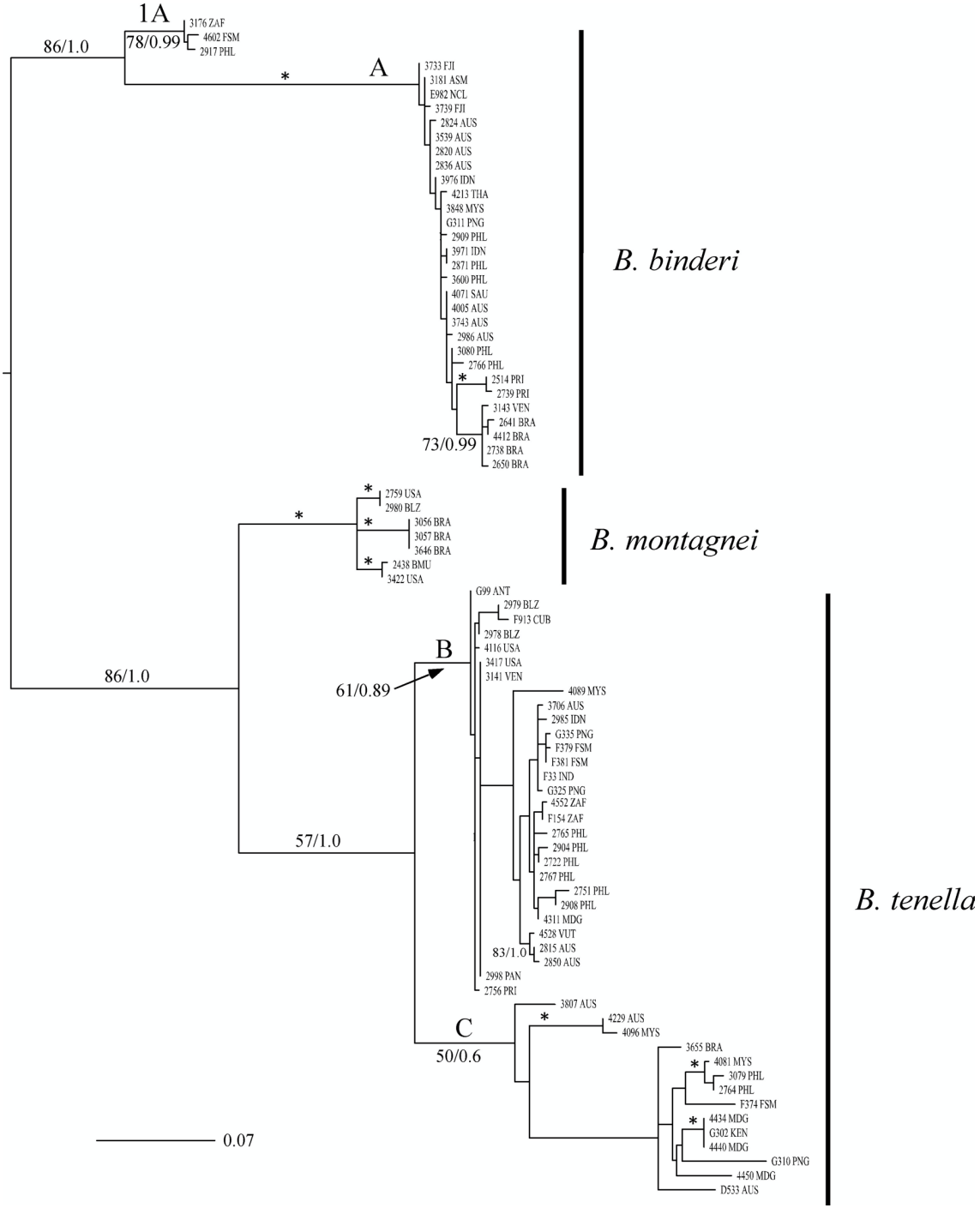
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A
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3539 AUS
2820 AUS
2836 AUS
3976 IDN
4213 THA
3848 MYS
G311 PNG
2909 PHL
3971 IDN
2871 PHL
3600 PHL
4071 SAU
4005 AUS
3743 AUS
2986 AUS
3080 PHL
2766 PHL
2514 PRI
2739 PRI
3143 VEN
2641 BRA
4412 BRA
2738 BRA
2650 BRA
73/0.99
B. binderi
2759 USA
2980 BLZ
3056 BRA
3057 BRA
3646 BRA
2438 BMU
3422 USA
B. montagnei
G99 ANT
2979 BLZ
F913 CUB
2978 BLZ
4116 USA
3417 USA
3141 VEN
B
61/0.89
86/1.0
4089 MYS
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2904 PHL
2722 PHL
2767 PHL
2751 PHL
2908 PHL
4311 MDG
4528 VUT
2815 AUS
2850 AUS
83/1.0
2998 PAN
2756 PRI
57/1.0
3807 AUS
4229 AUS
4096 MYS
C
50/0.6
3655 BRA
4081 MYS
3079 PHL
2764 PHL
F374 FSM
4434 MDG
G302 KEN
4440 MDG
G310 PNG
4450 MDG
D533 AUS
B. tenella
0.07

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Supplementary Table 1. *B. tenella* species complex samples used. Culture No.= JAW 4-digit culture number or field sample ID. Latitude and longitude where known. Genetic markers used: Plastid-encoded RuBisCo spacer, mitochondria-encoded *cox2-cox3* spacer, Plastid-encoded *rbc*L, nuclear encoded partial large subunit ribosomal rRNA. RuBisCo spacer haplotype designation or lineage designation in column.

Species	Culture No./ID	Lat.-Long.	Location	Coll. Date	RuBisCo haplotypes	*cox*2-3 spacer lineage	*rbc*L lineage	pLSU lineage
B. binderi	2917	5° 09’ N, 120° 04’ E	Tawi-tawi, Mindanao, PHI	29 v 1988	1A1	A1		
B. binderi	3176	30° 00’ S, 30º56’ E	Isipingo, Natal, ZAF	16 vii 1991	1A1	A1	A1	A1
B. binderi	4727	13° 29’ N, 144º52’E	Marbo Cave, GUM	15 x 2008	1A1			
B. binderi	G367	08º 47’ S, 115º 13’ E	Nusa Dua Beach, IDN	26 iv 2013	1A1			
B. binderi	G402	6° 38’ S, 99° 41’ E	Pulao Na, Satun, THA	8 iv 2008	1A1			
B. binderi	G410	--	Satun Province, THA	11 iii 2014	1A1			
B. binderi	2820	15º 59’ S, 145º 26’ E	Cowie Point, QLD, AUS	13 vi 1987	A1	A		A
B. binderi	2824	19º 08’ S, 146º 52’ E	Alma Bay, Magnetic I., QLD, AUS	4 vi 1987	A1	A		A
B. binderi	2836	19º 07’ S, 146º 52’ E	Florence Bay, Magnetic I., QLD, AUS	4 vi 1987	A1	A		A
B. binderi	2838	16º 09’ S, 145º 26’ E	Bouncing Stones Beach, QLD, AUS	13 vi 1987	A1	A		
B. binderi	2851	19º 07’ S, 146º 52’ E	Florence Bay, Magnetic I., QLD, AUS	4 vi 1987	A1	A		
B. binderi	2909	08º 30’ N, 124º18’ E	Libertad, Initao, Misamis Oriental, PHI	9 vi 1988	A1	A		A
B. binderi	2935	13° 29’ N, 144º52’ E	Marbo Cave, GUM	10 xii 1988	A1	A		
B. binderi	3181	14° 16’ S, 170º41’ W	Tutuila, ASM	26 vii 1991	A1	A	A	A
B. binderi	3539	15º 29’ S, 145º 16’ E	Quarantine Bay, QLD, AUS	4 x 1995	A1	A		
B. binderi	3733	18º 08’ S, 177º 24’ E	Yanuca I., Cuvu Bay, Viti Levu, FJI	6 vi 1997	A1	A		A
B. binderi	3739	17º 37’ S, 177º 25’ E	Saweni Beach, Viti Levu, FJI	5 vi 1997	A1	A		
B. binderi	3743	17º 57’ S, 122º 14’ E	Mangrove Trail, Broome, WA, AUS	18 vi 1997	A1	A	A	A

B. binderi	3848	02º 31’ N, 101º 48’E	Port Dickson, Selangor, MYS	14 v 1998	A1	A		A
B. binderi	3964	08º 06’ S, 114º 30’E	Bali Barat National Park, IDN	8 iv 1999	A1	A		A
B. binderi	3976	08º 50’ S, 116º 24’E	Teluk Ekas, Lombok, IDN	27 iv 1999	A1	A		
B. binderi	4071	16º 56′ N, 42º 00′ E	Farasan Island, Red Sea, SAU	8 vii 2000	A1	A		A
B. binderi	4104	05 ° 18’ 48" S, 115 ° 23’ 14" E	Merumbok, Sabah, MYS	14 viii 2000	A1	A		
B. binderi	4176	21º 06’ 27” S, 164º 49’ 76” E	Plage de Foué Fishing Village NCL	02-vii-2001	A1	A		A
B. binderi	4543	168° 21’ E. 17° 48’ S	Eratap, Efate I., VUT	14 vi 2005	A1	A		
B. binderi	G315	2º 58’ S, 150º 53’ E	Dyaul Island, NI, PNG	8 vi 2013	A1			
B. binderi	G364	28º 23’ N, 129º 29’ E	Kasari, Kagoshima, JPN		A1			
B. binderi	G390	2º 58’ S, 150º 53’ E	Dyaul Island, NI, PNG	8 vi 2013	A1			
B. binderi	G393	2º 58’ S, 150º 53’ E	Dyaul Island, NI, PNG	8 vi 2013	A1			
B. binderi	G398	?	Kozban, Malang, IDN	6 ix 2012	A1			
B. binderi	G399	?	Kozban, Malang, IDN	6 ix 2012	A1			
B. binderi	G401	?	Satun Province, THA	7 iv 2012	A1			
B. binderi	G411	6° 50' N, 99° 41' E	Khao Yai, Satun Province, THA	11 iii 2014	A1			
B. binderi	G414	6° 50' N, 99° 41' E	Khao Yai, Satun Province, THA	11 iii 2014	A1			
B. binderi	G415	6° 53' N, 99° 43' E	La Ngu Canal, Satun, THA	12 iii 2014	A1			
B. binderi	G424	6° 53' N, 99° 43' E	La ngu Canal, Satun, THA	12 iii 2014	A1			
B. binderi	G526	6° 26’ N, 99° 51’ E	Langkawi, MYS	12 viii 2014	A1	A		
B. binderi	G527	6° 26’ N, 99° 51’ E	Langkawi, MYS	12 viii 2014	A1	A		
B. binderi	G528	6° 26’ N, 99° 51’ E	Langkawi, MYS	11 viii 2014	A1	A		
B. binderi	G529	6° 26’ N, 99° 51’ E	Langkawi , MYS	11 viii 2014	A1	A		
B. binderi	2766	20° 24’ N, 121° 56’ E	Mahatao, Batan I., Batanes, PHI	22 iv 1987	A10	A		A
B. binderi	2986	12º 21’ S, 130º 51’ E	Darwin, NT, AUS	4 vi 1989	A11	A	A	A
B. binderi	4005	12° 02’ 30" S, 134° 30’ 07" E	Maningrida, Arnhem Land, NT, AUS	22 viii 1999	A11	A	A	A

B. binderi	4213	8º 11’ 7" N, 98º 17’ 26" E	Sirinath National Park, Phuket I., THA	15 iii 2002	A2	A		A
B. binderi	3971	08º 41’ S, 115º 28 E	Nusa Lembongan, Bali, IDN	25 iv 1999	A3	A		A
B. binderi	3080	9° 34’ N, 123° 10’ E	Talabong, Negros Oriental, PHI	19 vii 1990	A4	A	A	A
B. binderi	3600	10° 24’ N, 123° 38’ E	Toledo, Cebu, PHI	1 v 1996	A4	A		A
B. binderi	2514	17° 58’ N, 67° 03’ W	La Parguera, PRI	20 iii 1981	A5	A		
B. binderi	2739	17º 58’ N, 62º 02’ W	La Parguera, PRI	14 viii 1986	A5	A		
B. binderi	2984	1° 04’ N, 103º 55’ E	Batam I., IDN	17 vi 1989	A6	A	A	
B. binderi	2871	5° 09’ N, 120° 04’ E	Tawi-Tawi, Mindanao, PHI	15 i 1988	A7	A	A	A
B. binderi	4225	10º 41’S, 142º 31’ E	Pajinka, Cape York, QLD AUS	19 vi 2002	A8	A		
B. binderi	2641	12° 45’ S, 38° 10’ W	Arembepe, Bahia, BRA	20 vii 1982	A9	A	A	A
B. binderi	2650	23° 48’ S, 42° 25’ W	São Sebastião, São Paulo, BRA	2 vii 1982	A9	A		A
B. binderi	2738	23° 59’ S, 46° 15’ W	Guaruja, São Paulo, BRA	18 viii 1986	A9	A		
B. binderi	4412	13° 22’ S, 38° 54’ W	Garapuá, Ilha de Tinharé, Bahia, BRA	1 i 2004	A9	A		A
B. binderi	E740	27º 08’ S, 48º 29’ W	Praia do Recanto, Santa Caterina, BRA		A9	A		
B. binderi	E741	27º 08’ S, 48º 29’ W	Praia do Ribeiro, Santa Caterina, BRA		A9			
B. binderi	3143	10º 18’ N, 64º 24’ W	I. Larga, Bahia Mochima, Edo Sucre, VEN	9 iv 1991		A		
B. binderi	4602	06º 48’ N, 158º 09’ E	Lehn Mesi River, Pohnpei, FSM	4 ii 2006		A1		
B. binderi	G311	2º 58’S, 150º 53’E	Dyaul Island, NI, PNG	8 vi 2013		A	A	A
B. binderi	E982	21º 10’ S, 165º 32’ E	Cape Bocage, NCL			A		A
B. montagnei	2759	24º 38’ N, 81º 15’ W	Bahia Honda, FL, USA	10 xii 1986	M1	X		
B. montagnei	3056	25º 07’ 28"S, 47º 54’ 15" W	Rio Sitio Grande, Ilha do Cardoso, S.P., BRA	5 iv 1990	M1	X	X	X

B. montagnei	3057	25º 07’ 28" S, 47º 54’ 15" W	Rio Sitio Grande, Ilha do Cardoso, S.P., BRA	5 iv 1990	M1	X	X	
B. montagnei	3428	24º 58’ N, 80º 32’ W	Plantation, Florida Keys, FL, USA	19 vi 1994	M1			
B. montagnei	3643	23º 03’ S, 43º 33’ W	Guaratiba, Rio de Janeiro, BRA	15 xi 1996	M1	X		X
B. montagnei	3646	2º 46’ S, 44º 18’ W	Parra Açu, Maranhão, BRA	17 xi 1996	M1	X		
B. montagnei	3647	2º 46’ S, 44º 18’ W	Parra Açu, Maranhão, BRA	20 xi 1996	M1	X		X
B. montagnei	2438	32º 21’ N, 64º 42’ W	concrete block on main island, BMU	16 ix 1980	M2	X		
B. montagnei	2591	9º 23’ N, 79º 52’ W	Galeta, PAN	10 xii 1981	M2	X	X	
B. montagnei	2980	16º 49’ N, 88º 06’ W	Twin Cays, BLZ	15 vii 1989	M2	X		X
B. montagnei	3422	27º 28’ N, 80º 19’ W	Intracoastal Waterway, Ft. Pierce, FL, USA	19 vi 1994	M2	X		
B. montagnei	4739	13º 15’ N 88º 39’ W	3 km from Isla Mendez, SAL	6 iii 2009	M2		X	X
B. montagnei	4741	13º 17’ N, 88º 52’ W	Playa al Puntilla, SAL	7 iii 2009	M2		X	X
B. montagnei	4745	13º 17’ N, 88º 52’ W	Playa al Puntilla, SAL	7 iii 2009	M2	X	X	X
B. tenella	2515	17 ° 58’ N, 67 ° 03’ W	La Parguera, PRI	20 iii1981	B1		B	
B. tenella	2722	20° 20’ N, 121° 47’ E	Deguey Is., PHL	10 v 1986	B1	B		
B. tenella	2756	17º 58’ 31”N, 62º 02’ 48” W	La Parguera, PRI	2 xi 1986	B1	B	B	B
B. tenella	2765	20° 54’ N, 121° 53’ E	Siayan I., Batanes, PHI	24 iv 1987	B1	B		
B. tenella	2815	16º 09’S, 145º 26’E	Bouncing Stones Beach, QLD, AUS	13 vi 1987	B1	B	B	B
B. tenella	2850	19º 07’ S, 146º 52’ E	Florence Bay, Magnetic I., QLD, AUS	4 vi 1987	B1	B		
B. tenella	2978	16º 49’ N, 88º 06’ W	Twin Cays, BLZ	15 vii 1989	B1	B		
B. tenella	2979	16º 49’ N, 88º 06’ W	Twin Cays, BLZ	15 vii 1989	B1	B		
B. tenella	2985	1° 04’ N, 103º 55’E	Batam I., IDN	17 vi 1989	B1	B		
B. tenella	2998	9º 23’ N, 79º 52’ W	Galeta, PAN	20 viii 1989	B1	B		B
B. tenella	3141	10º 59’ N, 64º 08’ W	Laguna de la Restinga, I. Margarita, VEN	13 iv 1991	B1	B	B	B

B. tenella	3417	25º 46’ N, 80º 01’ W	Miami R., Miami, FL, USA	14 vi 1994	B1	B		
B. tenella	3706	20º 53’ S, 115º 20’ E	Barrow I., WA, AUS	8 ii 1997	B1	B		B
B. tenella	3727	217º 52’ S, 177º 53’ E	Koro Levu, Viti Levu, FJI	4 vi 1997	B1	B		B
B. tenella	4116	26° 01’ N, 81° 44’ W	Rookery Bay, FL, USA	19 ix 2000	B1	B		
B. tenella	4311	23º 10’ S, 43º 36’ E	Angeva, MDG	22 iv 2003	B1	B		
B. tenella	4528	17° 44’ S, 168° 33’ E	Eton Beach, Efate Island, VUT	14 vi 2005	B1	B		
B. tenella	4552	29° 44’ S, 31° 05’ E	Umhlanga Rocks, KwaZulu, Natal, ZAF	17 viii 2005	B1	B		
B. tenella	4089	05° 49’ S, 118° 09’ E	Pulai Bai, Sandakan, Sabah, MYS	16 viii 2000	B1	B		
B. tenella	E743	27º 08’ S, 48º 32’ W	Ilha Joao de Cunha, Porto Belo, Santa Catarina, BRA		B1			
B. tenella	F150	29° 44’ S, 31° 05 E	Umhlanga Rocks, KwaZulu Natal ZAF	17 viii 2005	B1	B	B	
B. tenella	F154	28º 22’ S, 32º 25’ E	Rocktail Bay, Kwazulu Natal, ZAF (WPvR)	10 viii 2005	B1	B	B	
B. tenella	F33	--	03-418 (Indonesia) (Leiden)		B1	B		
B. tenella	F378	05º 17’ N, 163º 01’ E	Malem, Kosrae, FSM	7 ii 2006	B1	B		
B. tenella	F379	09º 56’ N, 123º 42’ E	Nan Madol, Pohnpei, FSM	4 ii 2006	B1	B		
B. tenella	F381	07º 19’ N, 151º 50’ E	Fefen, Chuuk, FSM	10 ii 2006	B1	B		
B. tenella	F905	22° 2' N, 80° 26' W	Rancho Luna (RL4), Cuba (J. Larrea)		B1			
B. tenella	F907	22° 2' N, 80° 26' W	Rancho Luna (RL6), Cuba (J. Larrea)		B1			
B. tenella	F912	22° 2' N, 80° 26' W	Rancho Luna (RL10), Cuba (J. Larrea)		B1			
B. tenella	G194	22° 2' N, 80° 26' W	Rancho Luna (RL2), Cuba (J. Larrea)		B1			
B. tenella	G196	--	J II, Cuba (J. Larrea)		B1			
B. tenella	G325	08º 47’ S, 115º 13’,E	Nusa Dua Beach, Bali, IDN		B1	B		
B. tenella	G327	02º 45’ S, 151º 05’ E	Put Put Village, Losuk, NI, PNG		B1			

B. tenella	G366	08º 47’ S, 115º 13’ E	Nusa Dua Beach, Bali, IDN	26 iv 2013	B1			
B. tenella	G385	2° 49’ S, 151° 2’ E	Sicaciu, NI, PNG	7 vi 2013	B1			
B. tenella	G389	3º 39’S, 152º 26’ E	Namatanai, NI, PNG	5 vi 2013	B1			
B. tenella	G99	--	French Antilles (R. Lewin)		B1	B		
B. tenella	G397	33º 14’ N, 126º 35’ E	Jeju Is., KOR	20 vi 2012	B1			
B. tenella	G409	--	Satun Province, THA	11 iii 2014	B1			
B. tenella	2905	08º 30’ N, 124º 18’ E	Initao, Misamis Oriental, PHI	14 iii 1988	B2	B		
B. tenella	2751	8° 31’ N, 124° 18’ E	Initao, Misamis Oriental, PHI	25 x 1986	B3	B	B	B
B. tenella	2904	13º 34’ N, 124º 14’ E	Talisoy, Virac, Catanduanes, PHI	14 v 1988	B4	B		B
B. tenella	2908	08º 30’ N, 124º 18’ E	Libertad, Initao, Misamis Oriental, PHI	9 vi 1988	B5	B		
B. tenella	2767	21° 06’ N, 121° 57’ E	Y’Ami I., Batanes, PHI	25 iv 1987	B6	B		
B. tenella	E616	07º 36’ N, 134º 36’ E	Palau (Y. Hara)	21 iii 2001	B7			
B. tenella	F913	22° 2' N, 80° 26' W	Rancho Luna (RL11), Cuba			B		
B. tenella	G335	03º 28’S, 152º 13’,E	Karu, NI, PNG	4 vi 2013		B	B	
B. tenella	3655	13º 02’ S, 38º 40’ W	Ilha do Itaparica, Bahia, BRA	11 xi 1996	C1	C	C	C
B. tenella	4434	20º 44’ S, 43º 59’ E	River Sangara, Belo sur Mer, MDG	27 v 2004	C1	C		
B. tenella	D533	25º 17’ S, 152º 49’ E	Hervey Bay, QLD, AUS		C1	C		
B. tenella	F374	07º 27’ N, 151º 53’ E	Weno I., Chuuk, FSM	11 ii 2006	C1	C		
B. tenella	F375	06º 58’ N, 158º 13’ E	Nett Point, Pohnpei FSM	5 ii 2006	C1			
B. tenella	G302	--	Kenya (O. De Clerck)		C1	C		
B. tenella	G310	2º 58’ S, 150º 53’ E	Dyaul Island, NI, PNG	8 vi 2013	C1		C	
B. tenella	G314	2º 58’ S, 150º 53’ E	Dyaul Island, NI, PNG	8 vi 2013	C1			
B. tenella	G391	2º 58’ S, 150º 53’ E	Dyaul Island, NI, PNG	8 vi 2013	C1			
B. tenella	G392	2º 58’ S, 150º 53’ E	Dyaul Island, NI, PNG	8 vi 2013	C1			
B. tenella	G394	2º 58’ S, 150º 53’ E	Dyaul Island, NI, PNG	8 vi 2013	C1			
B. tenella	G395	2º 58’S 150º 53’E	Dyaul Island, NI, PNG	8 vi 2013	C1			
B. tenella	G396	2º 58’ S, 150º 53’ E	Dyaul Island, NI, PNG	8 vi 2013	C1			

B. tenella	2764	8° 31’ N, 124° 18’ E	Initao, Misamis Oriental, PHI	13 v 1987	C1	C		C
B. tenella	3079	9° 34’ N, 123° 10’ E	Talabong, Negros Oriental, PHI	19 vii 1990	C1	C		
B. tenella	4440	20º 44’ S, 43º 59’ E	Chenal d’Ampanarata , Belo sur Mer, MDG	27 v 2004	C1	C		
B. tenella	4662	07º 26’ N, 151º 53’ E	Peniyak Village, Weno I., Chuuk, FSM	11 ii 2006	C1	C	C	
B. tenella	4664	07º 26’ N, 151º 53’ E	Peniyak Village, Weno I., Chuuk, FSM	11 ii 2006	C1	C	C	
B. tenella	4081	06° 53’ S, 116 ° 42’ E	Sikuati beach, Sabah, MYS	13 viii 2000	C2	C		
B. tenella	4450	17º 05’ S, 49º 48’ E	Anafialy, I. St. Marie, MDG	22 v 2004	C3	C		
B. tenella	3807	20° 18’ S, 118 ° 36’ E	Port Hedland, WA, AUS	9 xii 1997	C4	C		C
B. tenella	4229	10º 41’ S, 142º 31’ E	Pajinka, Cape York, QLD, AUS	19 vi 2002	C5	C		C
B. tenella	4096	05° 49’ S, 118 ° 09’ E	Sandakan, Sabah, MYS	16 viii 2000	C6	C		C