Morphology, Molecular Phylogeny and Genome Content of *Bothriochloa* Focusing on Australian Taxa

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ALEX SUMADIJAYA

ABSTRACT

The study focuses on the genus *Bothriochloa* (Andropogoneae, Poaceae) in Australia. Despite morphological features separating this genus from the closely related two genera *Capillipedium* and *Dichanthium,* (the three hereafter will be called BCD), De Wet and Harlan introduced the compilospecies complex to show the interbreeding phenomena that occurred among species of these genera. This study was carried out to assess species/genus relatedness of the BCD complex using different evidences from morphology, molecular information and genomic content. Nineteen morphological characters were observed, three regions (*trnT-F, rps16 intron* and *3'trnK*) of chloroplast genome phylogenetic were used in phylogenetic reconstruction, and chromosome counting as well as flow cytometry for chromosome number and genome size were conducted during the study. Phylogenetic trees were constructed using MP with NJ for morphological data, and MP, RAxML, and BI for molecular data. Based on morphology, all three genera were separated as monophyletic units. *Bothriochloa* consisted of two clades. However, phylogenetic analyses based on chloroplast genomic regions reveal that *Bothriochloa* and *Dichanthium* are paraphyletic clades and only *Capillipedium* is resolved as a monophyletic clade. The concatenated data set has performed better than individual data sets in terms of resolution and support for clades. Flow-cytometry and chromosome counting only found diploid and tetraploid but not hexaplod species. TCS network reveals that tetraploidization followed different pathways from the ancestral diploid species. This study provided new insight onto the evolution of the chloroplast genome in the compilospecies and empirical evidence of species grouping of the compilospecies based on morphology.

DEDICATION

For Ivana & Clementine, two pillars of my life.

Such lonely days without you guys….

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I. INTRODUCTION

1. History, Taxonomic Overview, and Habit of *Bothriochloa*

Bothriochloa Kuntze, (tribe Andropogoneae, Poaceae) is a cosmopolitan grass genus of 32 to 35 species (Watson and Dallwitz 1992). Two genera closely related to *Bothriochloa* are *Capillipedium* Stapf with 14 to 17 species and *Dichanthium* Willemet that consisted of 16 to 21 species (Watson and Dallwitz 1992, Clayton et al. 2006). Despite their morphological differences, an alliance between these three genera has been recognized due to their ability to interbreed (De Wet and Harlan 1970b). The three genera share similar habitat requirement grassy warm region and open habitats. Morphologically all of them have similar general habit and inflorescence architectures (Watson and Dallwitz 1992, Clayton et al. 2006, Skendzic et al. 2007).

Kuntze (1891) was the first to describe *Bothriochloa* using *Bothriochloa anamitica* as the type species. *Bothrichloa* also has also synonyms, *Amphilophis* and *Gymnandropogon,* two taxa that were embedded in the genus *Andropogon*. *Andropogon* was first described in *Species Plantarum* vol. 2 (Linnaeus, 1753). Von Trinius (1832) described the subgenus *Amphilophis* in *Andropogon.* Nash (1901) recognized this subgenus as a separate genus, *Amphilophis*. Within *Andropogon*, Nees (1841) placed certain South African species in subgenus *Gymnandropogon* in his publication of the *Florae Africae Australioris Illustrationes Monographicae*. Dunthie (1882) then raised this subgenus to a genus level, *Gymnandropogon.* Roberty (1960) treated *Bothriochloa* and *Amphilophis* as sections of the genus *Dichanthium.* Currently, *Bothriochloa* is the legitimate generic name with *Amphilophis* and *Gymnandropogon* as synonyms (Watson and Dallwitz 1992, Shouliang and Phillips 2006).

Etymologically, the name of *Bothriochloa* is derived from the Greek words ***bothrion,*** 'the pit', and ***chloe*** 'culm', which refer to the pitted (foveolate) lower

glumes (Watson and Dallwitz 1992). *Bothriochloa* is diagnosed by the arrangement of racemes on a central axis of its inflorescence. Each raceme has more than eight joints. In some species, the inflorescence are as a panicle of racemes, the basal raceme does not branches beyond the 2nd degree, and the whole basal raceme with its secondary branches disarticulates as a single unit (Backer and Backhuizen van den Brink Jr. 1968, Watson and Dallwitz 1992, Clayton et al. 2006, Simon et al. 2012).

The morphological feature that unifies the closely related *Capillipedium* with *Bothriochloa* is the translucent mid-line between the thickened margins of the rachis and the pedicels, a condition termed 'canaliculate'. However, *Capillipedium* differs from *Bothriochloa* in having a panicle inflorescence that is composed of strongly branched racemes, the racemes never have more than nine joints, with joints disarticulating individually. *Dichanthium,* on the other hand, lacks the canaliculated character, and is separated as the next closest taxon to *Bothriochloa* (Simon et al. 2006, Backer and van den Brink Jr. 1968, Ohwi 1942, De Wet et al. 1961). Nevertheless, some authors united *Bothriochloa* and *Dichanthium* into a single genus of *Dichanthium* based on morphology. Gardner (1952) pointed out the unifying characters of these genera, *e.g.* the pedicelled spikelet being male or sterile, and when fertile, has much reduced awn; sessile spikelet with the upper floret being female or bisexual. Roberty (1960) merged *Bothriochloa* and *Dichanthium* based on the presence of foveolate fertile lower glumes and the "lack of canaliculation". This lack of canaliculation is inconsistent with Kuntze's (1891) description in which he underscored the presence of canaliculation in *Bothriochloa.*

2. The Compilospecies: an Interbreeding Complex

During the 1950's, researchers at the Experimental Agricultural Station of Oklahoma State University conducted extensive research on the improvement of the pasture quality of native and introduced grass of the tribe Andropogoneae with a main focus on *Bothriochloa* and *Dichanthium*. This represented a preliminary effort to find a correlation between polyploidy and morphological characters. As

part of that intensive research, De Wet and Harlan (1970a) conducted extensive greenhouse hybridization experiments to map out the interbreeding phenomena among the species of *Bothriochloa*, *Capillipedium*, and *Dichanthium* (referred to as BCD).

Harlan et al. (1962), Harlan and De Wet (1963), De Wet and Harlan (1970b) demonstrated that *Bothriochloa bladhii* (Retz.) S.T. Blake, formerly known as *Bothriochloa intermedia* (R.Br.) A. Camus, serves as the hub for gene flow from intergeneric hybridization among species of these three genera (De Wet and Harlan 1966). Although the previous specific epithet, *intermedia,* was well chosen for explaining intermediate forms produced from hybridization with other taxa, the name *B. bladhii* has priority. At the end, this hybrid complex involving *Bothriochloa*, *Capillipedium*, and *Dichanthium* (BCD, Figure 1) was named as the **compilospecies** to represent the existence of species complex as the taxonomic dilemma of BCD that is morphologically, but not reproductively distinct (Harlan and De Wet 1963, De Wet and Harlan 1970a).

Furthermore, De Wet and Harlan (1970a) proposed the grouping of these interbreeding taxa as sections under the genus *Dichanthium,* the earliest published generic name. Interestingly, this cross hybridization phenomenon happens only at the tetraploid level, both in greenhouse and in nature, and reproductive isolation occurred at the diploid and hexaploid levels (Celarier and Harlan 1955, De Wet et al. 1961). In nature, tetraploid *B. bladhii* was recorded to hybridize with *B. ewartiana* (Domin) C.E. Hubbard, *B. ischaemum* (L.) Keng, and *Capillipedium parviflorum* (R.Br.) Stapf. Interbreeding between *B. bladhii* and *C. parviflorum* possibly produces *Capillipedium spicigerum* S.T. Blake (Harlan et al. 1962). Diploid species of *Capillipedium* and *Dichanthium* are reproductively isolated and attempts to hybridize them failed (De Wet and Harlan 1966, 1970b), although Faruqi (1969) stated that artificial hybrids could be produced at the hexaploid level. Therefore, I hypothesize that interbreeding blurs demarcation among species of *Bothriochloa.*

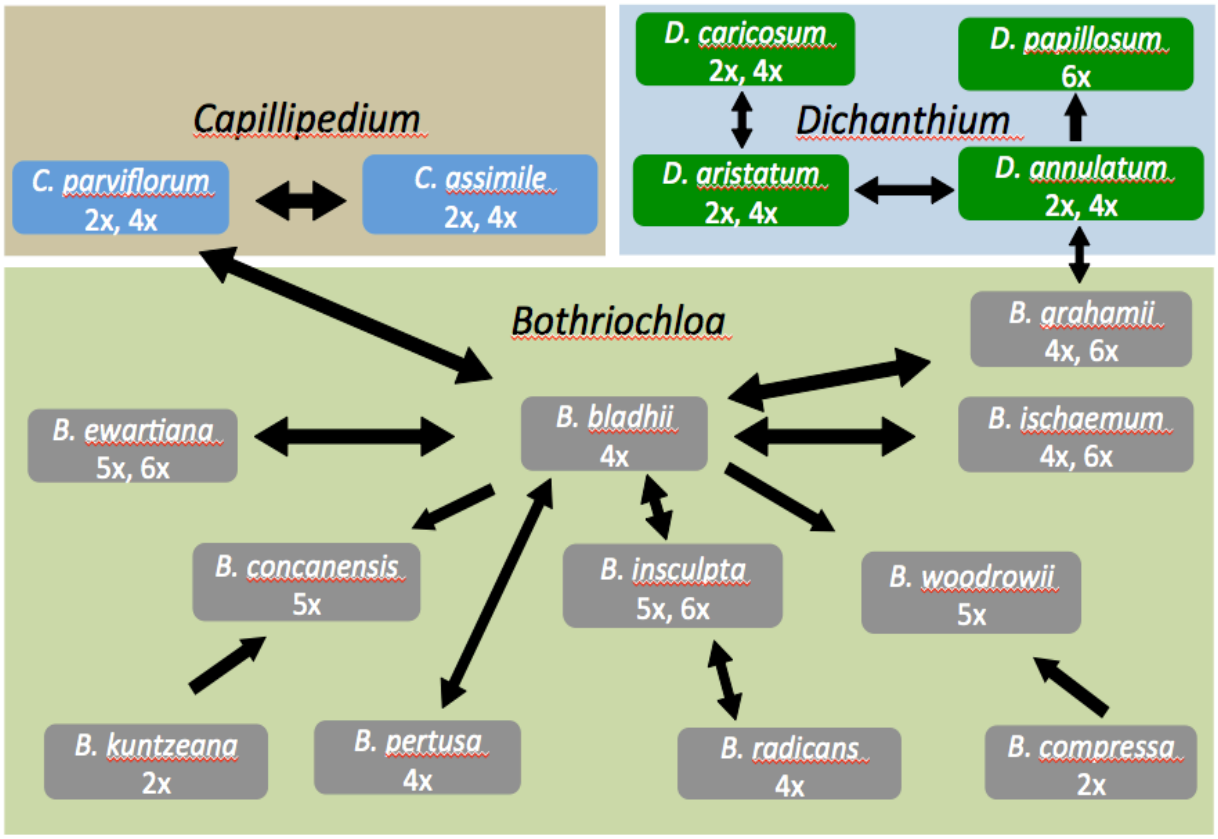

Figure 1. Compilospecies hybridization network among species of the three genera *Bothriochloa*, *Capillipedium,* and *Dichanthium* (Redrawn and revised from De Wet and Harlan, 1970*,* Apomixis, polyploidy, and speciation in *Dichanthium,* Evolution*,* Wiley Publisher). Ploidy level is noted as 2x, 4x, 5x and 6x.

3. Study Objective and Area of Focus

The overall objectives of this study is to assess the relationship among species of the *Bothriochloa, Capillipedium,* and *Dichanthium* (BCD) complex by using morphological characters to provide better insight into the compilospecies complex with a focus on the Australian taxa. In more detail, there are three points to be addresses: 1). To assess relationship of *Bothriochloa* to *Capillipedium* and *Dichanthium,* 2). To check the monophyly of *Bothriochloa* based on morphology, and 3). To evaluate the taxonomical relationship among the species of *Bothriochloa.* I decided to focus on the Australian species of the BCD complex for two reasons: 1). limited coverage provides a manageable starting point for this rather complex taxonomic problem, and 2.) availability of accessions for the

Australian taxa from the United States Department of Agriculture (USDA-GRIN) and the Department of Agriculture Fisheries and Forestry, Australia (DAFF).

Hubbard (1934) listed three species of *Bothriochloa* in Australia: *B. decipiens* (Hack.) C.E.Hubb (and *B. decipiens var. cloncurensis)*, *B. ewartiana,* and *B. erianthoides* (F. Muell) C.E.Hubb. Blake (1944) constructed the earliest identification key for the Australian *Bothriochloa,* and recognizing seven taxa: *B. erianthoides, B. biloba* S.T. Blake, *B. ambigua* S.T. Blake*, B. decipiens, B. decipiens var. cloncurensis, B. ewartiana*, and *B. intermedia.* Simon (1982) described a new species, *B. bunyensis* Simon. Later, he also recognized *B. glabra* as a subspecies of *B. bladhii* (Simon 1989).

As a comprehensive resource of Australian plant, Australian Plant Names Index (APNI) noted 10 valid species of *Bothriochloa.* Among these, six are natives with three being endemic (distributed in only part) of Australia, and the remaining four are naturalized species. Table 1 summarizes the history of species recognition in Australia and their geographical status.

II. MATERIAL AND METHODS

1. Species Used, Source of Materials and Seeds Germination

Six species of *Bothriochloa,* two species of *Capillipedium* and three species of *Dichanthium* were used in this study. Materials for these species were in the form of seeds and herbarium vouchers. The total number of herbarium vouchers examined and the number of seed samples obtain were 26. The species and the sources of the materials are noted in Table 2. Compilospecies network (Fig. 1) copyright permission was followed Wiley Publisher website (http://www.wiley.com/WileyCDA/Section/id-301464.html).

Table 1. Compilation of the *Bothriochloa* species in Australia based on different sources. Symbols are referring (+) present, (-) absent. Numbers refer to literatures 1). Hubbard (1934), 2). Blake (1944), 3). Simon (1982), 4). Simon (1989), 5). APNI (2014). Distribution (APNI: Australian Plant Names Index): A: Australia Capital Territory; NA: not available; Z: New South Wales; N: Northern Territory; Q: Queensland; S: Southern Australia; T: Tasmania; W: Western Australia; V: Victoria. Status; I: Invalid; E: Endemic; Nv: Native Nt: Naturalized; *N & T; partially naturalized in N & T.

Species	**1**	**2**	**3**	**4**	**5**	**Distribution (APNI)**	**Status (APNI)**
B. ambigua	-	+	-	-	-	-	I
B. biloba	-	+	-	-	+	Q, W	E
B. bladhii / B. intermedia / B. glabra	-	+	-	+	+	N, Q, W, Z	Nv
B. bunyensis	-	-	+	-	+	Q	E
B. decipiens	+	+	-	-	+	N, Q, W, Z	Nv
B. erianthoides	+	+	-	-	+	Q, Z	E
B. ewartiana	+	+	-	-	+	N, Q, S, W, Z	Nv
B. insculpta	-	-	-	-	+	Q, Z	Nt
B. macra	-	-	-	-	+	A, N, Q, S, T, V, Z	*N & T
B. pertusa	-	-	-	-	+	N, Q, W	Nt
B. saccharoides	-	-	-	-	+	A	Nt

In the case of seed materials, seeds were germinated in petri dish with a few drops of KNO_3 in the greenhouse. Germinated seeds were grown until seedlings reached five cm in height before transferring them to plastic container to reach maturity. After they produced flowers and seeds in the greenhouse, whole plants were collected for various purposes. Some specimens at the flowering stage were dried and pressed as herbarium vouchers. Data on morphological characters were derived from a combination of observations from these herbarium vouchers of germinated seeds and from descriptions in GrassBase (http://www.kew.org/data/grasses-db/sppindex.htm). Some taxa that naturally occur outside Australia were also added to provide a broader picture of the compilospecies.

Table 2. Species used in this study, seed accession and their geographic origins, and herbarium voucher examined. Accessions starting with PI were obtained from USDA-GRIN, and those with AUSTRCF were obtained from DAFF.

Species	Seeds	Herbarium	Accession	Origin
A. glomeratus	+	+	AX 1	United States
A. gyrans	+	+	AX 2	United States
B. bladhii	+	+	AusTRCF105854	Australia
B. bladhii	+	+	PI 239164	Australia
B. bladhii	+	+	PI 300909	Australia
B. bladhii	+	+	PI 301375	Australia
B. decipiens	+	+	PI 239153	Australia
B. decipiens	+	+	PI 301294	Australia
B. decipiens	+	+	PI 257676	Australia
B. decipiens	+	+	PI 301290	Australia
B. ewartiana	+	+	PI 300723	Australia
B. ewartiana	+	+	PI 300724	Australia
B. insculpta	+	+	PI 301418	Mozambique
B. insculpta	+	+	AusTRCF320165	Australia
B. ischaemum	+	+	PI 300904	Thailand
B. ischaemum	+	+	PI 199861	Australia
B. macra	+	+	PI 301266	Australia
B. macra	+	+	PI 301267	Australia
B. macra	+	+	PI 301272	Australia
B. macra	+	+	PI 301273	Australia
C. parviflorum	+	+	PI 301780	China
C. spicigerum	+	+	PI 301775	Australia
C. spicigerum	+	+	PI 301378	Australia
D. annulatum	+	+	PI 302047	Australia
D. aristatum	+	+	PI 301994	Australia
D. sericeum	+	+	AusTRCF323612	Australia

2. Data analysis

Five vegetative and 14 reproductive characters (Table 3) were selected based on documented variability among *Bothriochloa* species. Characters states were coded as numeric values, either as bi-state or multistate (Table 3) and saved in a Nexus format using MacClade (Maddison and Maddison 2011) and Mesquite

(Madisson and Madisson 2015). All characters were treated equally and were unordered. Analyses were conducted using PAUP (Swofford 1997) with Neighbor Joining (BioNJ) Gascuel (1997). BioNJ was selected over UPGMA to exclude the molecular clock effect. Based on character substitution, Maximum Parsimony (MP) searched for the shortest tree. Heuristic search with Stepwise Addition was conducted with the random option and 100 replicates. Bootstrap (BS) search was conducted with 'Fast' stepwise addition, retaining group with >50% frequency, and with 100 replicates. Only support values above 50 were visualized with three confidence levels, high (85--100), moderate (75--84) and low (>50%).

Table 3. Vegetative and reproductive character along with their character states

		Character states		
	Characters	**1**	**2**	**3**
1	Culm habit	Erect	Decumbent	NA
2	Length	< 100 cm	> 100 cm	NA
3	Ligule	Ciliate	Eciliate	NA
4	Ligule tip	Truncate	Obtuse	NA
5	Inflorescence	Raceme	Panicle	NA
6	Infloresencence base	Hairy	Glabrous	Pilose
7	Espatheate raceme	Present	Absent	NA
8	Spikelet vestiture	Hairy	Glabrous	NA
9	Spikelet shape	Ellips	Lanceolate	Linear
10	Ratio sterile vs fertile	100%	<100%	NA
11	Translucent pedicel	Yes	No	NA
12	Pit presence	Present	Absent	NA
13	Pit number	1	2	NA
14	Lower Glume texture	Chartaceous	Coriaceous	Cartilagineous
15	Lower Glume base	Keeled	Round	NA
16	Lower Glume shape	Elliptic	Lanceolate	NA
17	Lemma surface	Pilose	Glabrous	NA
18	Lemma shape	Oblong	Lanceolate	NA
19	Anther	1	3	NA

NA: Not available

Furthermore, character optimization (ACCTRAN or DELTRAN) was also conducted in PAUP (Swofford 2003) to assess the best support for the topology. *Andropogon* was chosen as an outgroup since it has been shown consistency as a sister lineage to the BCD clade in phylogenetic analysis of sequence data from the *ndhf* region (Morrone et al. 2012).

III. RESULTS

Tree topology based on BioNJ (Figure 2) grouped the BCD together with 61% BS support. *Capillipedium* and *Dichanthium* appeared in a single lineage (<50% BS) sister to the *Bothriochloa* lineage. *Capillipedium* and *Dichanthium* species segregated into two separate clades with BS support 85% for *Dichanthium* and <50% BS support for the *Capillipedium* clade. Within *Capillipedium,* the two accessions of *C. spicigerum* did not group together but instead one of them, #378, appeared sister to the single accession of *C. parviflorum*. In the *Dichanthium* lineage (BS= 85), *D. aristatum* appeared sister to a *D. annulatum* plus *D. sericeum* clade.

The BioNJ analysis recovered two clades (**in bold**) in the monophyletic *Bothriochloa*: the **Bladhii** (**B1**) and the **Macra** (**M1**) clades (Fig. 2). The **B1 (**BS<50%**)** includes *B. bladhii, B. insculpta, B.* ischaemum #904. The three species appeared monophyletic, with *B. insculpta* sister to *B. bladhii* plus *B. ischaemum* #904. The **M1** clade comprised of *B. macra, B. decipiens, B. ewartiana,* and *B. ischaemum* #861. The clade is separate into two subclades, both received <50% BS support. One subclade consisted of two accessions of *B. ewartiana* (#723 and #724) and one accession of *B. ischaemum* #861. The two accessions of *B. ewartiana* did not group together, but instead one of them (*B. ewartiana* #724) appeared sister with *B. ischaemum* #861. The other subclade consisted of four accessions each of *B. macra* and *B. decipiens.* The *B. macra* accession formed a grade (*B. macra #*267 / *B. macra* #273 + #272/ *B. macra #*266) sister to the *B. decipiens* clade (BS=92).

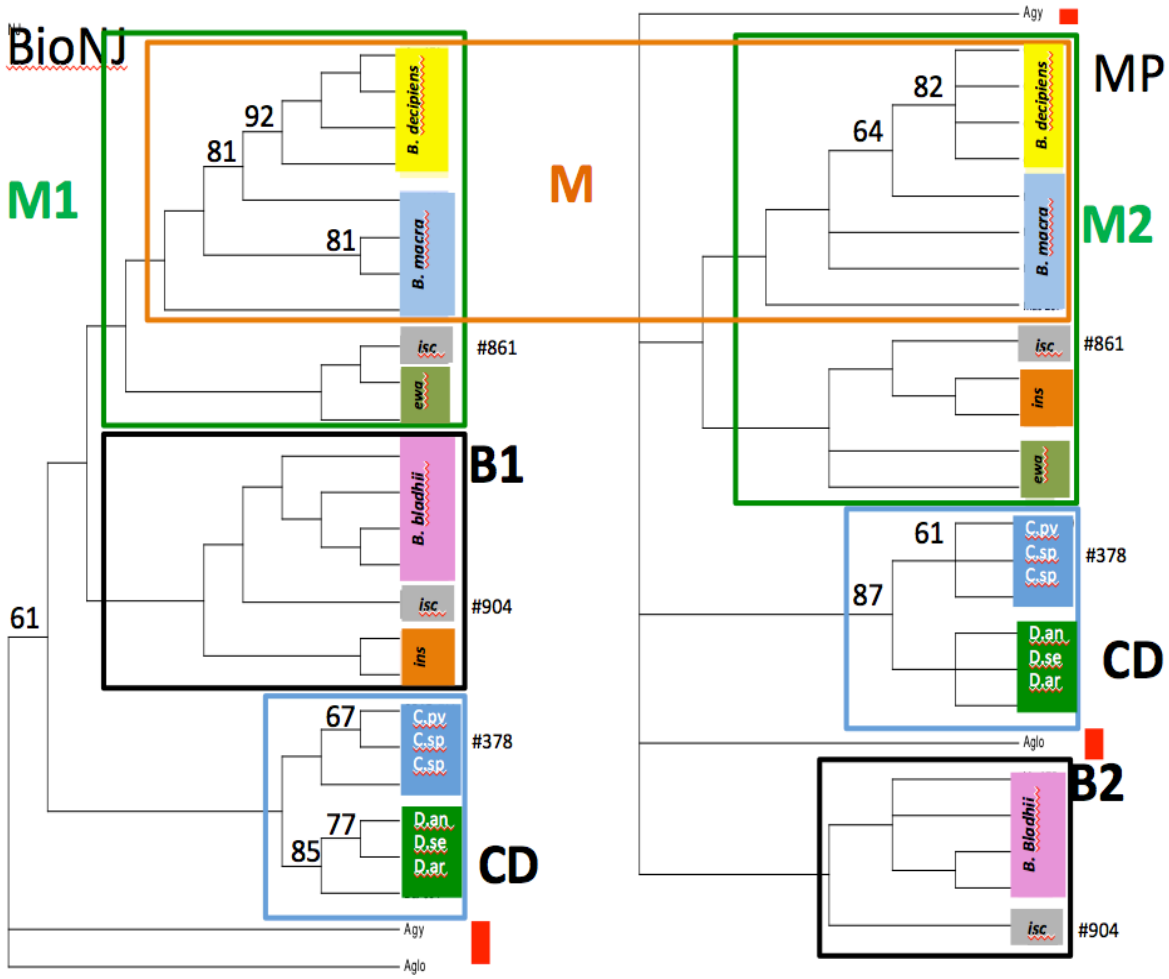

Figure 2. Neighbor Joining (BioNJ) tree (left) and Maximum Parsimony (MP) tree (right). Numbers above nodes represent BS support values. Species are color-coded. Colored bars on the right side of taxa represent *Capillipedium* (blue), *Dichanthium* (green) and the outgroup *Andropogon* (red). Colored boxes visualize the congruency between individual clusters on the two trees. Legend: ***Aglom***: *A. glomeratus*, ***Agy***: *A. gyrans*, ***isc***: *B. ischaemum*, ***ewa***: *B. ewartiana*, ***ins***: *B. insculpta*, ***C.pv***: *C. parviflorum*, ***C.sp***: *C. spicigerum*, ***D.an***: *D. annulatum*, ***D.ar***: *D. aristatum*, ***D.se***: *D. sericeum.*

The MP tree shows consistency index (CI=0.690), retention index (RI=0.410) and homoplasy index (HI=0.310). The tree showed a major basal polytomy of three clades: **Capillipedium+Dichanthium** (**CD,** BS=87%), **Macra** (**M2,** BS<50%) and **Bladhii** (**B2,** BS<50%**).** The composition of the *Capillipedium* (BS=61%), and *Dichanthium* (BS<50%**)** subclades was identical with that of the

BioNJ tree. The exception is that the accessions of each subclade appeared in a polytomy.

In the *Bothriochloa* clades (BS<50%), species composition is almost identical to that of the BioNJ. The only difference is the position of *B. insculpta*, as it appeared in the **B1** in the BioNJ whereas it appeared in the **M1** subclade in the MP (Fig. 2). The BS values supporting the sister relationship of *B. macra* #266 to the *B. decipiens* clade and the monophyly of the latter species decreased compare to the values in the BioNJ (Fig. 2).

Character optimization of ACCTRAN and DELTRAN (Fig. 3) was conducted to improve tree resolution and support in the MP tree. Both analyses did not improve on the resolution of the backbone topology since the basal polytomy remained unchanged. Further, the character optimization analyses did not resolve the genera *Capillipedium* and *Dichanthium* in one lineage as in the MP and BioNJ analyses (Fig. 2). The composition of the two *Bothriochloa* clades (**M3** and **B3**) were similar to that obtained in the BioNJ analysis, but the support for the sister relationship of B*. macra #*266 to the *B. decipiens* clade decreased.

IV. DISCUSSION

This morphological analysis is the first attempt to assess the species relationship in the compilospecies based on empirical approach. Previous studies were intuitive based on general observation and measurement without critical bioinformatics aspect (Sumadijaya and Veldkamp 2009, Sumadijaya and Veldkamp 2011, Roberty 1960). Based on selected reproductive characters, Roberty (1960) divided the genus *Dichanthium* into six sections with species of *Bothriochloa* distributed in three sections including section Bothriochloa.

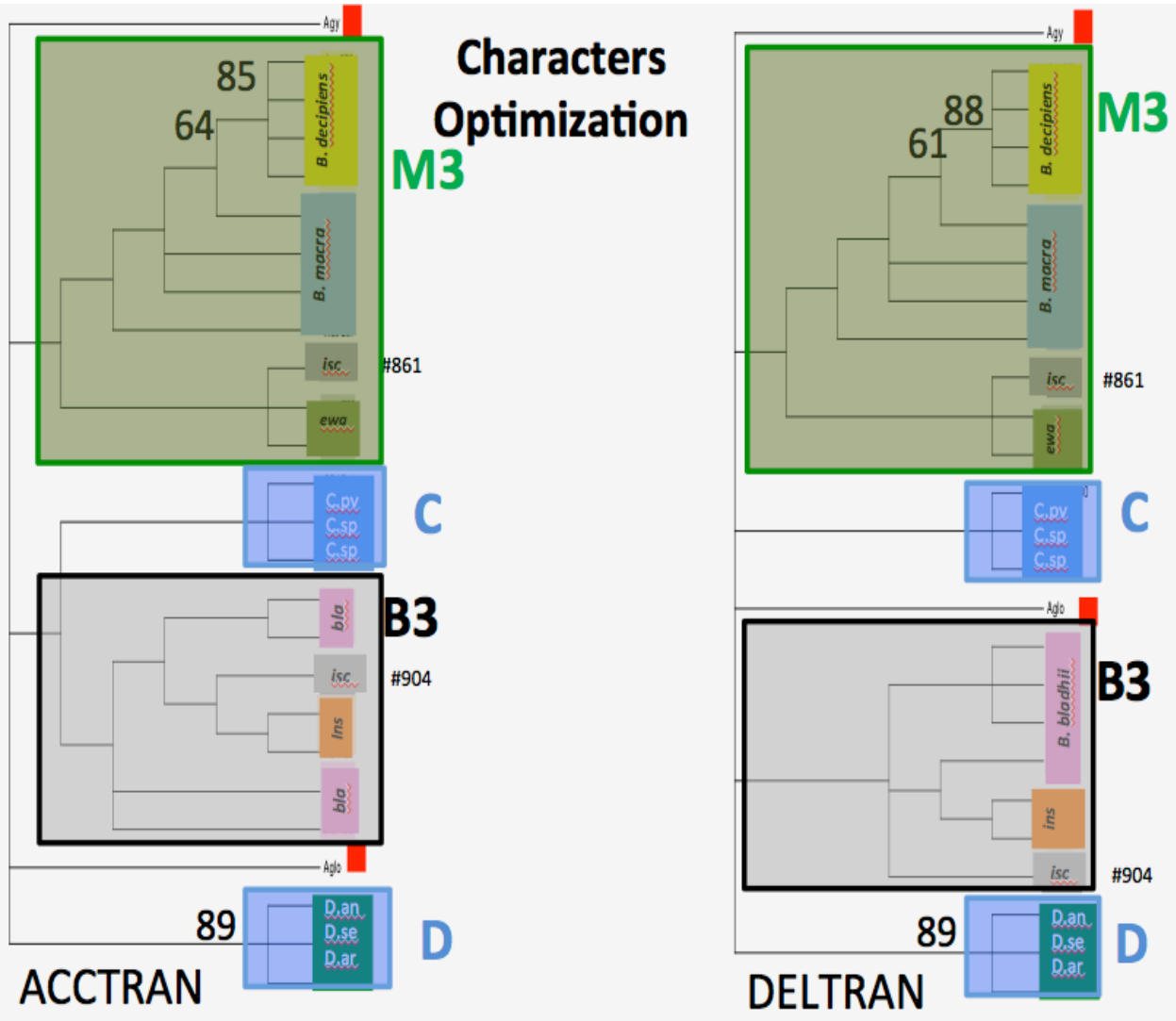

Figure 3. Character optimization of MP with ACCTRAN tree (left) and DELTRAN tree (right). Numbers above nodes represent BS support values. Species are color-coded. Colored bars on the right side of trees denote *Capillipedium* (blue), *Dichanthium* (green) and the outgroup *Andropogon* (red). Colored boxes visualize the congruency between individual clusters on the two trees. Legend: ***Aglom***: *A. glomeratus*, ***Agy***: *A. gyrans*, ***isc***: *B. ischaemum*, ***ewa***: *B. ewartiana*, ***ins***: *B. insculpta*, ***C.pv***: *C. parviflorum*, ***C.sp***: *C. spicigerum*, ***D.an***: *D. annulatum*, ***D.ar***: *D. aristatum*, ***D.se***: *D. sericeum.*

Based on the morphological character in both BioNJ and MP analyses, *Capillipedium* and *Dichanthium* were monophyletic genera and appeared in a single clade sister to each other. The BS support for this **CD** clade is 87% in MP and <50% in BioNJ. The relationships among these three genera have been

inconsistently resolved. The topology from this morphological study is in agreement with that obtained in the phylogenetic study of Teerawatananon et al. (2009) based on combined sequences from *trnL–F, atpβ–rbcL* and ITS regions. Mathews et al. (2002) using *phyB*, GBSSI, and *ndhF* resolved *Dichanthium* as sister lineage to a clade that included *Bothriochloa* and *Capillipedium*. On the other hand, Guissani et al. (2001) and Moronne et al. (2012) have shown a topology of *Bothriochloa* plus *Dichanthium* sister to *Capillipedium* based on *ndhF* sequences. These molecular phylogenetic studies are based on single species per genus, and thus robustness of the relationship is in question.

Capillipedium is represented in Australia by two species, *C. spicigerum* and *C. parviflorum*. In the **CD** clade of BioNJ, *C. spicigerum* appeared paraphyletic with *C. parviflorum* nested between its two accessions. This situation may be due to the small sampling available for these species. Thus, adding more accessions may refine the topology of the genus.

The genus *Dichanthium* comprises three species in Australia: *D. annulatum*, *D. aristatum* and *D. sericeum*. Although in the MP analysis the three species appeared in a polytomy (BS<50%), the BioNJ analysis (BS=85%) resolved *D. aristatum* sister to a clade containing *D. annulatum* plus *D. sericeum* (BS=77%). Similar relationship was also recovered in the molecular phylogenetic study (Sumadijaya, Chapter 2).

The genus *Bothriochloa* lacks of comprehensive intrageneric taxonomic treatment. The name of *Bothriochloa* was derived from the presence of 1-3 pits in the fertile glume of the spikelet, and this feature was regarded as an apomorphic morphological character. The pits, however, do not serve as a consistent character for species delimitation. Furthermore, pits disappeared in hybrid offspring of *B. bladhii* and *D. annulatum*, but not in *B. bladhii* and *B. ischaemum* hybrids. Therefore, the pit is not an informative character in tree reconstruction and consequently was not included in the BioNJ and MP analyses. The overall topology of the *Bothriochloa* clade resolved in this morphological study shows a division of the five Australian species into two major clades. The composition of

the two clades in both BioNJ and MP analyses (BS<50%) are almost identical. These two major clades correspond to the two clades recovered by the molecular phylogenetic study (Sumadijaya, Chapter 2). Due to the small sample for the genus, we will tentatively refer to them as the **Macra** (**M1** and **M2**) and **Bladhii** (**B1** and **B2**) clades without any formal taxonomic recognition at this point.

Clade **Bladhii (B1)** has three species: *B. bladhii, B. insculpta* and *B. ischaemum* # 904. Although Faruqi (1969) mentioned the plasticity of *B. bladhii* in its interbreeding with other species, this species is resolved as a monophyletic unit in BioNJ and MP analyses. *Bothriochloa insculpta* also appears as monophyletic in both analyses. The difference is that *B. insculpta* emerges sister to clade *B.bladhii* plus *B. ischaemum* #904 in the **Bladhii** clade in the BioNJ analysis, whereas this species appeared sister to *B. ischaemum* #861 in **Macra** clade in the MP analysis. This difference in topology represents soft incongruence since the BS support for these relationships were <50%. Inconsistency of the placement of *B. ischaemum* could plausibly be caused by character state variations in spikelet shape, ligule hair, inflorescence architecture, and spikelet ratio. Matakis et al. (2011) mentioned that genetic variability in *B. ischaemum* was contributed by polymorphism of microsatellite loci.

Clade **Macra (M1)** consisted of *B. decipiens, B. macra, B. ewartiana* and *B. ischaemum* #861) in the BioNJ, whereas the clade also included *B. insculpta* in MP analysis (Fig. 2). With the exception of *B. ischaemum*, these species are native or endemic to Australia. The presence of *B. ischaemum* in the macra clade in the MP analysis is in agreement with Faruqi (1969) classification where he recognized the close morphological relationship between *B. ewartiana* and *B. ischaemum* in his identification key.

Bothriochloa macra appeared consistently as a paraphyletic group with *B. decipiens* nested within it in both BioNJ and MP analyses. These two species are sympatric in East Australia (Queensland, NSW, and Victoria based on ALA (Atlas of Living Australia), *(*http://spatial.ala.org.au/webportal//#), but their possible

interspecific hybridization and chromosome number have not been reported. *Bothriochloa decipiens* accessions form a well-defined clade with strong support in BioNJ (BS=92%) and in MP (BS=82%). Morphologically, the single anther of *B. decipiens* sets it apart from *B. macra* that has three anthers.

V. CONCLUSION

The 19 vegetative and reproductive characters on which BioNJ and MP analyses were based represent the first empirical study on the compilospecies The analyses resolved the three genera, *Bothriochloa, Capillipedium,* and *Dichanthium* as distinct individual monophyletic units. This was resolved despite reported intergeneric hybridization among them. The study also showed the close relationship between *Capillipedium* and *Dichanthium,* where the two emerged as sister clades in one well-supported lineage. The relationship between *Capillipedium* and *Dichanthium* has been previously disputed. Tree topology based on BioNJ and MP analyses are highly congruent. The structure of the *Bothriochloa* lineage in both analyses was highly similar, resolving two clades that may represent nuclei for future sectional classification of the genus. The analyses also identified core species in each subclade of *Bothriochloa*. These are *B. bladhii* plus *B. ischaemum* #904 of the **B** subclade and *B. macra* plus *B. decipiens* of the **M** subclade.

The hypothesis is refuted since the interbreeding does not blur the demarcation among genera and species. The possibilities are either non-viable hybrids or dominant alleles effect.

This study provided new insight into the relationship among species of the compilospecies complex in general and on the Australian taxa in particular. Incorporating other species of *Bothriochloa* beyond Australia will refine the overall picture of the classification of this genus.

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CHAPTER II - MOLECULAR PHYLOGENETIC STUDY

I. INTRODUCTION

Some broad scope studies on Andropogoneae, Panicoideae, and Paniceae included a small representation from *Bothriochloa*, *Capilipedium*, and *Dichanthium* (BCD). Mathews et al. (2002) showed that *Dichanthium aristatum* is a sister lineage to the clade that included *Bothriochloa odorata* and *Capillipedium parviflorum* based on a molecular phylogeny using *phyB*, GBSSI, and *ndhF*. Guissani et al. (2001) and Moronne et al. (2012) found out that *Bothriochloa bladhii* was sister to *Dichanthium aristatum*. These two taxa were in one clade sister to *Capillipedium parviflorum* based on *ndhF* sequences. Teerawatananon et al. (2009) constructed a phylogenetic tree based on nucleotide sequences from the combined regions *trnL–F, atpβ–rbcL* and ITS that resolved *Dichanthium theinlwinii* and *Capillipedium assimile* as sister to each other, with *Bothriochloa pertusa* as their next closest taxon. Therefore, using different genomic regions and different species show different phylogenetic results. However, these studies were not focused on the compilospecies and sampled only a single species per genus. Therefore a reliable relationship among the three genera will require a denser sampling to resolve finer details of genus/species taxonomic relationships.

Fourteen species of *Bothriochloa, Capillipedium* and *Dichantium* were included in the study of Skendzic et al. (2007) using the nuclear (ITS) and the chloroplast (*trnL-F*) regions. In both partitioned and combined data sets, BCD was resolved as a monophyletic clade with 1.0 Posterior Probability (PP) support for ITS. However, a species from another genus, *Euclasta condylotricha*, was nested within the clade containing most *Bothriochloa.* The exception here is *B. grahamii* that appeared as a separate lineage in a polytomy with *C. venustum* and *D. annulatum.* Their *trnL-F* analysis although recovered the BCD clade (BS=82%, PP=1.0), it resulted in a complete basal polytomy for all members of the BCD genera. The tree based on concatenated data sets also recovered the BCD group (BS=77%, PP=1.0) and demonstrated the monophyly of *Bothriochloa*, with the

exception of *B. grahamii* that appeared sister to a polytomy of *Capillipedium* and *Dichanthium*. Although they sampled three genera, the study did not address the compilospecies issue. Estep et al. (2014) in a study of polyploid evolution in the tribe Andropogoneae showed *B. bladhii* as the core of the compilospecies and noted that its genome has similarity with the genomes of *Capilipedium* and *Dichanthium.* They emphasized the importance of allopolyploidy in the defining the relationship within the compilospecies complex.

The objective of this study is to use chloroplast genomic regions to reconstruct phylogenetic trees with the focus on the Australian taxa as a starting point for a detailed understanding of the phylogenetic relationship among the compilospecies globally.

II. MATERIAL AND METHODS

1. Taxon sampling and DNA sequencing

Eight species of *Bothriochloa*, two species of *Capillipedium* and three species of *Dichanthium,* with total 28 accessions were sampled for the study. Two species of *Andropogon* were chosen as outgroup based on the phylogenetic affinity of this genus to BCD (Morrone et al. 2012). The species used and the sources of the material are listed in Table 4. When available, fresh leaf material was used to obtain high quality genomic DNA. Otherwise, leaf tissue was removed from herbarium specimens and used for DNA isolation. Genomic DNA isolation followed the CTAB protocol of Doyle and Doyle (1990). The Polymerase Chain Reaction (PCR) was used for amplifying the regions. A 25 µl PCR reaction was prepared by mixing 2.5 µl 25mM $MgCl_2$ (New England Biolabs), 2.5 µl Thermopol buffer (New England Biolabs), 5 µl dNTP 1.25 uM, 1 µl of each primer for chloroplast (Taberlet et al. 1991), and for nuclear (White et al. 1990), 0.2 µl *Taq polymerase* (ABI), genomic DNA, and then adjusted with ddH_2O. More genomic DNA was necessary for successful amplification from herbarium specimens. PCR amplification followed a modification of Woods et al. (2005) protocol using 30 cycles (*trnT-F*) and a modified Dunthie and Dunthie (1999) protocol for *rps 16 intron* using 35 cycles for

3'trnK (Liang and Hilu 1996); other components of the protocol remain the same. The temperature profile consisted of an initial denaturation step at 94°C for 240 s followed by 30 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 52°C for 30 s, and an extension step at 72°C for 60 s. The final extension was set at 72°C for 300 s. PCR products were first run on an agarose gel, the DNA fragment was excised, and cleaned using Promega Wizard® SV Gel and PCR Clean-Up System. The sequence reaction was set up using an ABI kit (BigDye® Terminator v3.1 Cycle Sequencing Kit) and following their protocol. Sequencing was conducted at the Duke IGSP Sequencing Facility.

Sequence phenograms were checked by 4Peaks (http://nucleobytes.com/index.php/4peaks), and then manually aligned using Quickalign (Müller and Müller 2003) and PhyDe (Müller et al. 2006). Gaps (insertions/deletions, indels) were introduced at the cost of two or more substitution and coded as additional characters using Simmons and Ochoterena (2000) Simple Coding option in SeqState (Muller 2005). Absence and presence of gaps was coded as 0 or 1, respectively, and converted into R and Y states that represent purine and pyrimidine.

The initial target was to examine the phylogenetic suitability of six chloroplast regions: *trnT-F, rps 16 intron, 3'trnK, trnH-psbA, rpl 16 intron* and *petA-psbJ*. The presence of nucleotide variation and indels were two important factors determining potential effectiveness of region in phylogenetic reconstruction and consequently in selecting them for the study. The *trnT-F*, *rps 16 intron*, and *3'trnK* regions were selected due greater variation in both nucleotide substitution and indels. However, the *rps 16 intron* region was difficult to amplify from some of the accessions, and a single accession failed to amplify the *3'trnK* region in spite of repeated effort (Table 4). Consequently, two approaches (Region Focus and Taxa-Focus) were needed to handle the missing data issue. In the Region-Focus approach, 26 accessions were selected based on the presence of all three genomic regions. In the Taxa-Focus approach, 30 accessions were included that

Table 4. List of accessions used in the study. Red outline refers to the REGION FOCUS, and shaded green refers to TAXA-FOCUS approaches. Symbol (+) refers to success; (-) refers to fail.

No	Accessions	trnTF	3'trnK	rps 16
1	*B. bladhii* 164	+	+	+
2	*B. bladhii* 375	+	+	+
3	*B. bladhii* 909	+	+	+
4	*B. bladhii* A854	+	+	+
5	*B. ewartiana* 723	+	+	+
6	*B. ewartiana* 724	+	+	+
7	*B. macra* 266	+	+	+
8	*B. macra* 267	+	+	+
9	*B. macra* 272	+	+	+
10	*B. macra* 273	+	+	+
11	*B. insculpta* 165	+	+	+
12	*B. insculpta* 418	+	+	+
13	*B. ischaemum* 861	+	+	+
14	*B. ischaemum* 904	+	+	+
15	*B. decipiens* 153	+	+	+
16	*B. decipiens* 290	+	+	+
17	*B. decipiens* 294	+	+	+
18	*B. decipiens* 676	+	+	+
19	*D. aristatum* 994	+	+	+
20	*D. annulatum* 047	+	+	+
21	*D. sericeum* 612	+	+	+
22	*C. spicigerum* 378	+	+	+
23	*C. spicigerum* 775	+	+	+
24	*C. parviflorum* 780	+	+	+
25	*B. bunyensis* 354	+	-	-
26	*B. bunyensis* 3525	+	+	-
27	*B. erianthoides* 705	+	+	-
28	*B. erianthoides* 26164	+	+	-

REGION FOCUS

TAXA FOCUS

have the *trnT-F* and *3'trnK* regions only. As such, seven data sets were constructed, five partitioned data sets and two concatenated ones. The data sets from the Region Focus approach have more sequence characters but fewer species compare with the data sets from the Taxa-Focus approach. For each

approach, individual data sets were analyzed and the trees were compared with their respective concatenated data sets to evaluate topology, support and tree resolution.

2. Sequence data analysis

The partitioned and concatenated data sets were analyzed using Maximum Parsimony (MP), Rapid Accelerated Maximum Likelihood (RAxML), as well as by the Bayesian Inference (BI) to construct the maternal trees. MP analysis was performed in PAUP (Swofford 2003) with heuristic search and Stepwise Addition conducted with the random option and 100 replicates. Characters were equally weighted. A majority rule tree was selected from the shortest trees. A bootstrap (BS) search with 100 replicates was conducted with 'Fast' stepwise addition retaining groups with frequency of >50%. To evaluate the possibility of combining data sets from the different chloroplast regions, a Partition Homogeneity Test (PHT) was conducted in PAUP (Swofford 2003). The test failed to reach completion after more than seven days of computing time and thus it was terminated. Nevertheless, the PHT test has been noted to overestimate incongruence, and consequently data sets are most often combined regardless of the outcome (Barker and Lutzoni 2002, Darlu and Lecointre 2002).

The RaxML analysis was conducted in the CIPRES portal (Miller et al. 2010), using the BlackBox option. Two species of *Andropogon* (*A. glomeratus* and *A. gyrans*) were selected as outgroup species based on published work that demonstrated the close phylogenetic affinity to the compilospecies genera. The analysis ran for a maximum of 24 hours. Bipartition tree files were downloaded to visualize the topology with values above 50% BS support using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

In the Bayesian Inference (BI), the nexus file of the data sets was first edited in PAUP (Swofford 2003) to introduce ModelTest commands, and then the data set was executed in JModeltest v.3.7 (Muller 2005) to select the most appropriate model for the BI analysis. The ModelTest result showed different chloroplast

regions have different models. The BI analysis was conducted using BEAUTI v.1.6 and BEAST v.1.6 by using 10,000,000 generations. The first 1,000,000 were discarded as burn-in. The quality of the result was checked using Tracer (Rambaut et al. 2014). TreeAnnotator (http://beast.bio.ed.ac.uk/TreeAnnotator) was used to construct the tree. Phylogenetic trees with PP support were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and values above 0.95 were considered as the best confidence level. All supporting values were noted on the branches as MP BS / RAxML BS / BI PP. PhyDesign (Lopez-Giraldez and Townsend 2011, Mayrose et al. 2004, Pond et al. 2005) was used to compute the phylogenetic informativeness of each plastid region, as well as those of the concatenated data sets. TCS, a computer program to build phylogenetic network in estimating genealogies by statistical parsimony (Clement et al. 2000) was used to map the pattern of nucleotide mutations between pairs of sequences of these three genera. The assessment visualized the homogeneity sequences using default settings (95% parsimony probability and indels coded as missing data).

III. RESULT

1. Nuclear genome

The amplification of the nuclear genomic regions ITS and 5S generated inconsistent results, producing either faint or no amplicons despite following Skendzic et al. (2007) procedure. I modified the PCR protocol by adjusting the annealing temperature and DNA template concentration but without success. Therefore, these two genomic regions were dropped from the study.

2. Plastid genome

A. Region-Focus Analyses

Data sets and tree statistic (number of character, number of variable and non variable character, Parsimony Informative (PI) characters, number of most parsimonious, tree length, consistency index (CI=0.771), retention index (RI= 0.833**)** and homoplasy index (HI=0.229) are reported in Table 5 for analyses based

on partitioned and concatenated data sets. Three adenine poly-nucleotide regions were excluded from the analysis. These regions were at position 84-95 and 760-768 (8-12 nucleotides in each position) and 422-433 (8-9 nucleotides). Summaries of the results are shown in Table 5 for Region-Focus.

Table 5. The summary of the analyses of three regions of individual data sets of *trnT-F, rps 16 intron, 3'trnK* and concatenated data sets. Included are alignments statistic and substitution models for each region, and support for clades. N: not resolved, (**p):** paraphyletic, (-): BS <50% or PP<0.95.

	trnT-F	***rps 16 intron***	***3'trnK***	**3-regions concatenation**
Characters	**1681**	**779**	**477**	**2937**
Substitution	**62**	**14**	**8**	**84**
Indels	**20**	**8**	**6**	**34**
Tree number/ length	**696/131**	**16/11**	**1/8**	**701/110**
Substitution Model	F81 + G +I	F81	F81	F81 + G + I
B. bladhii	72%/68%/-	-/-/-	65%/73%/0.99	65%/82%/0.99
B. decipiens	92%/82%/-	-/56%/- (**p)**	-/81%/-**(p)**	54%/93%/-
B. ewartiana	97/97/-	-/88%/-	59%/96%/0.99	97%/100%/0.99
B. insculpta	N	77%/91%/0.99	60%/70%/0.99	-/99%/0.99
B. ischaemum	-/64%/-	68%/73%/0.99 (p)	N	-/85%/-
B. macra	72%/64%/0.99	-/-/-	N	-/79%/0.99
Capillipedium	98%/98%/0.99	74%/78/%0.99	N	95%/100%/0.99
Dichanthium **(p)**	-/60%/-	57%/69%/-	55%/75%/0.99	-/81%/0.98

Based on partitioned data sets of the three genomic regions, none of the three analyses (MP, RAxML and BI) recovered *Bothriochloa* and *Dichanthium* as monophyletic lineages (Fig. 4, appendix with MP and BI trees). *Capillipedium,* on

the other hand, formed a single clade with the *trnT-F* and *rps 16 intron* sequence data while it appeared as a grade in the *3'trnK* based tree with all three analyses (Fig. 4, appendix with MP and BI trees). The tree based on the concatenated data set again did not resolved *Bothriochloa* and *Dichanthium* as monophyletic lineages, but also recovered *Capillipedium* in a single clade (Fig. 4, appendix with MP and BI trees). In both partitioned and concatenated analyses, *B. ewartiana* was monophyletic (Fig. 4, appendix with MP and BI trees). The phylogenetic position of *B. ewartiana* is inconsistently resolved in the tree based on the individual genomic regions. *Bothriochloa bladhii* is monophyletic in all trees except the on *trnT-F-* based tree. With the exception of the tree based on the *3'trnK* data set, *Capillipedium* and *B. decipiens* clades were sister to each other. There is a general trend showing phylogenetic affinities between *B. macra* and *B. insculpta.* Another trend reflected phylogenetic affinity between *B. ischaemum* and *Dichanthium.*

Based on the per site informativeness results (Fig. 5), the concatenated data sets included more phylogenetic information than each of the individual data sets. Of the individual chloroplast regions, *trnT-F* (black) has the most information followed by the *rps 16 intron* (blue), and then the *3'trnK* (green).

B. Taxa-Focus Analyses

Data sets and tree statistic (number of character, number of variable and non variable character, Parsimony Informative (PI) characters, number of most parsimonious, tree length, are reported in Table 6 for analyses based on partitioned and concatenated data sets. Concatenated data set has consistency index (CI=0.771), retention index (RI= 0.614**)** and homoplasy index (HI=0.229). Three adenine poly-nucleotide regions were excluded from the analysis due to no variation available though could skew the reading of the next nucleotide after this region. These regions were at position 84-95 and 760-768 (8-12 nucleotides in each position) and 422-433 (8-9 nucleotides). A summary of Taxa-Focus statistic is reported in Table 6.

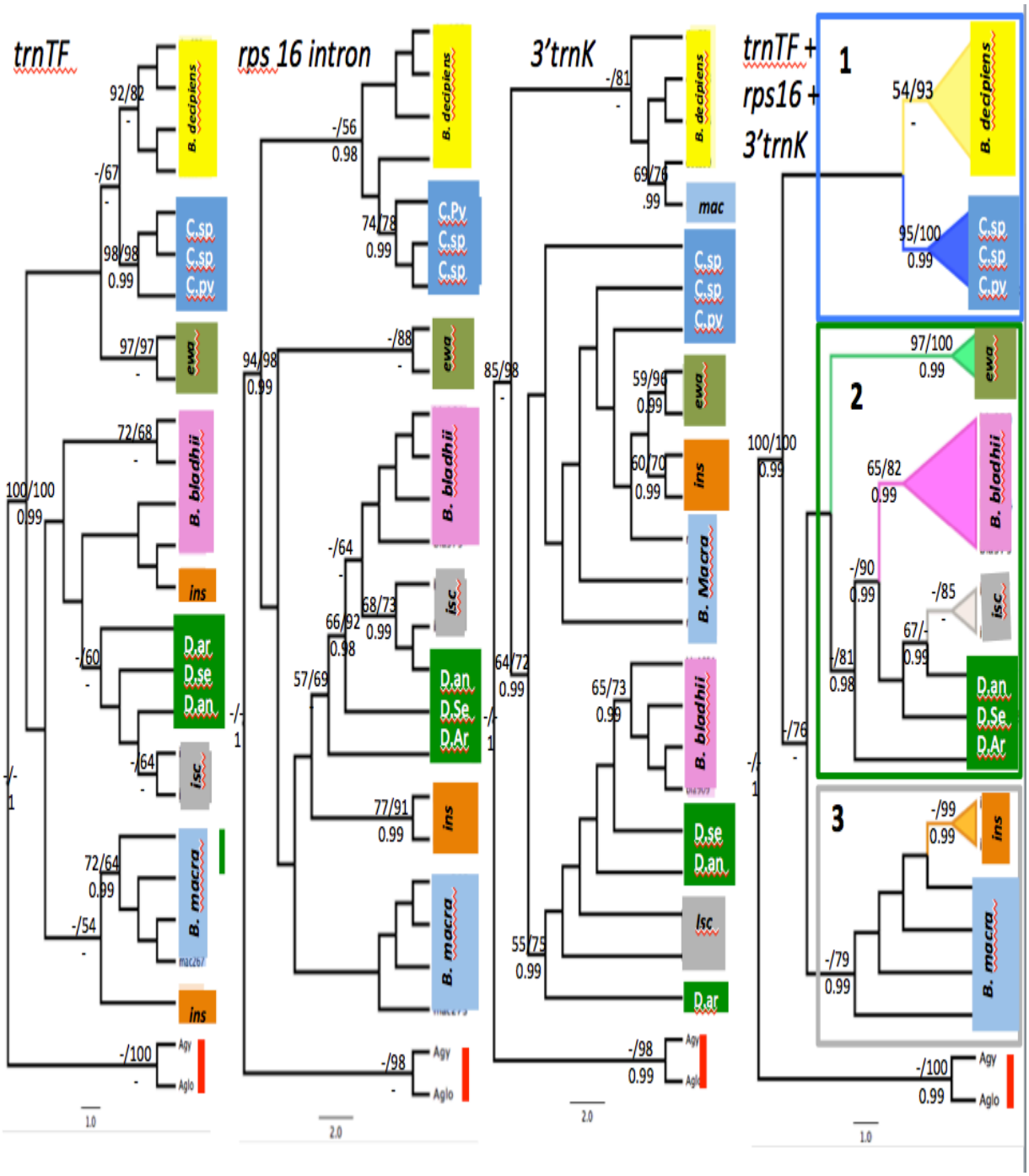

Figure 4. Phylogenetic trees derived from the REGION FOCUS are compared side by side: trnT-F (Left), 3'trnK (Middle left), rps 16 intron (Middle right) and concatenated data sets (Right). Outlines refer to the clades (1, 2, and 3). Values above the nodes refer to MP BS/RAxML BS. Values under the node denote BI PP. Legend: ***Aglom***: *A. glomeratus*, ***Agy***: *A. gyrans*, ***isc***: *B. ischaemum*, ***ewa***: *B. ewartiana*, ***ins***: *B. insculpta*, ***C.pv***: *C. parviflorum*, ***C.sp***: *C. spicigerum*, ***D.an***: *D. annulatum*, ***D.ar***: *D. aristatum*, ***D.se***: *D. sericeum.*

The Taxa-Focus data sets encompasses the *trnT-F* and *3'trnK* region and a total of 13 species and 30 accession including the outgroup species, with two additional species (4 accessions). The *trnT-F* based tree as well as the concatenated based tree recovered three major clades refers to here as clade 1, 2, and 3. Support for these clades was low or lacking and their position in the tree varied (Fig. 6,

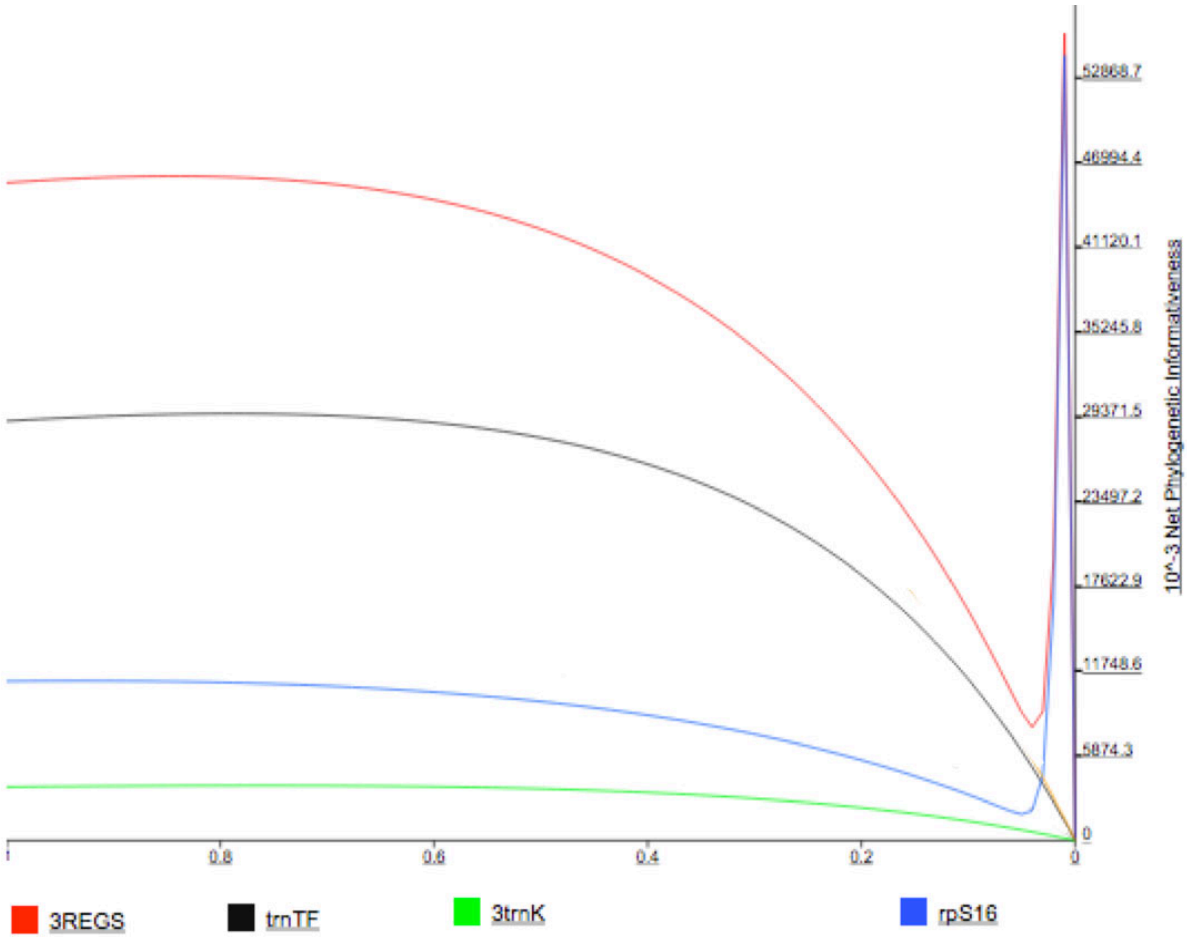

Figure 5. Sites informativeness curve generated by PhyDesign for the *trnT-F, rps 16 intron, 3'trnK* regions individually and their concatenated data set. The X-axis represents the relative age and the Y-axis represents informativeness.

appendix MP and BI). However, the tree based on *3'trnK* data resulted in a grade with *B. decipiens* and one accession of *B. macra* (#266) sister to the remaining taxa. Support for the backbone of this tree was very low. *Bothriochloa decipiens* is recovered monophyletic for the most part of the analyses (Fig. 6, appendix with MP

and BI). Clade 1 consisted of *B. decipiens* plus *Capillipedium* sister to *B. ewartiana* in both *trnT-F* and concatenated data sets. Clade 2 consisted of *B. bladhiii, B. ischaemum, B. bunyensis,* and *Dichanthium.* However, the structure of the clade differs from the trees based on the *trnT-F* and concatenated data sets. *Bothriochloa bladhii* is recovered as a monophyletic lineage with the concatenated and *3'trnK* data sets but not with the *trnT-F* data set.

Table 6. The summary of the analysis of individual regions of *trnT-F, 3'trnK* and concatenated data sets for the Taxa-Focus approach. Statistic and substitution models of each region, support for monophyly and clades. Information includes of statistic and substitution models of each region, support for monophyly and clades. N: not resolved, (**p):** paraphyletic, (-): BS <50% or PP<0.95.

	trnT-F	***3'trnK***	***trnT-F + 3'trnK***
Character	**1681**	**477**	**2158**
Substitutions	**62**	**8**	**70**
Indels	**20**	**6**	**26**
Tree number /length	**¥/91**	**126/13**	**1188/113**
Substitution Model	F81 + G + I	F81	F81 +G
B. bladhii	N	55%/70%/-	65%/74/0.97
B. decipiens	-/77%/-	-/78%/-	55%/93%/-
B. ewartiana	96%/97%/0.97	-/95%/-	100%/100%/0.99
B. insculpta	N	61%/72%/-	55%/74%/0.99
B. ischaemum	-/55%/-	-/63%/- **(p)**	-/54%/0.99
B. macra	66%/55%/0.99	N	-/53%/- **(p)**
B. bunyensis	-/56%/-	N	-/69%/-
B. erianthoides	-/80%/-	N	-/74%/0.96
Capillipedium	97%/99%/0.99	N	98%/99%/0.99
Dichanthium (**p**)	-/63%/-	N	-/51%/-

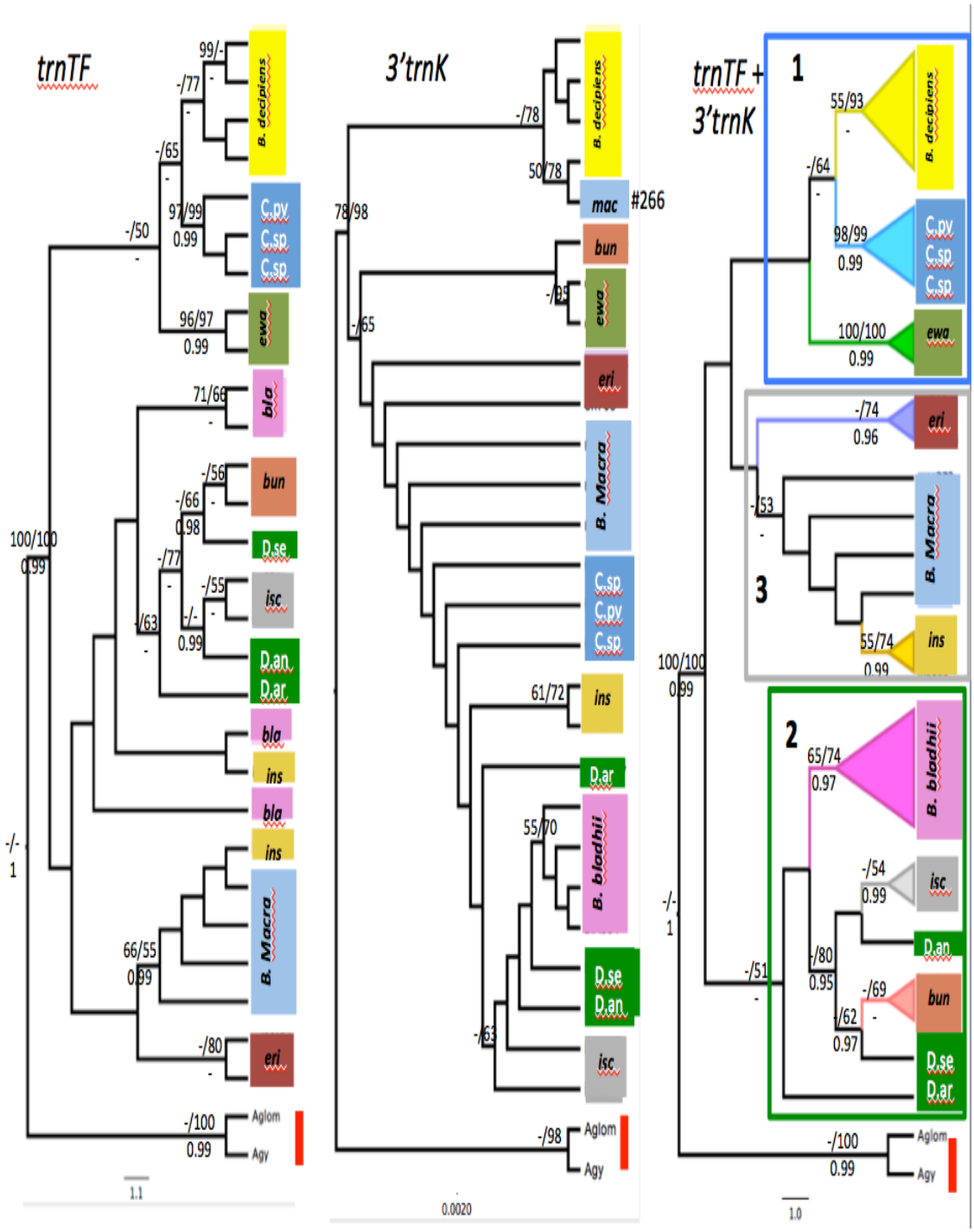

Figure 6. Phylogenetic trees derived from the TAXA-FOCUS trees are compared side by side. The trees are *TrnT-F* (Left), *3'trnK* (Middle) and concatenated data sets (Right). Outlines refer to the clades (1, 2, and 3). Values above the nodes refer to MP BS/RAxML BS. Values under the node refer to BI PP. Legend: ***Aglom***: *A. glomeratus*, ***Agy***: *A. gyrans*, ***bun:*** *B. bunyensis*, ***eri:*** *B. erianthoides*, ***isc***: *B. ischaemum*, ***ewa***: *B. ewartiana*, ***ins***: *B. insculpta*, ***C.pv***: *C. parviflorum*, ***C.sp***: *C. spicigerum*, ***D.an***: *D. annulatum*, ***D.ar***: *D. aristatum*, ***D.se***: *D. sericeum.*

Clade 3 consisted of *B. macra, B. insculpta,* and *B.erianthoides*. *Bothriochloa macra* accessions formed a grade, *B. erianthoides* appeared in a clade, while *B. insculpta* formed a clade in concatenated data set but only one of the two accession appeared in clade 3; the other accession appeared in clade 2.

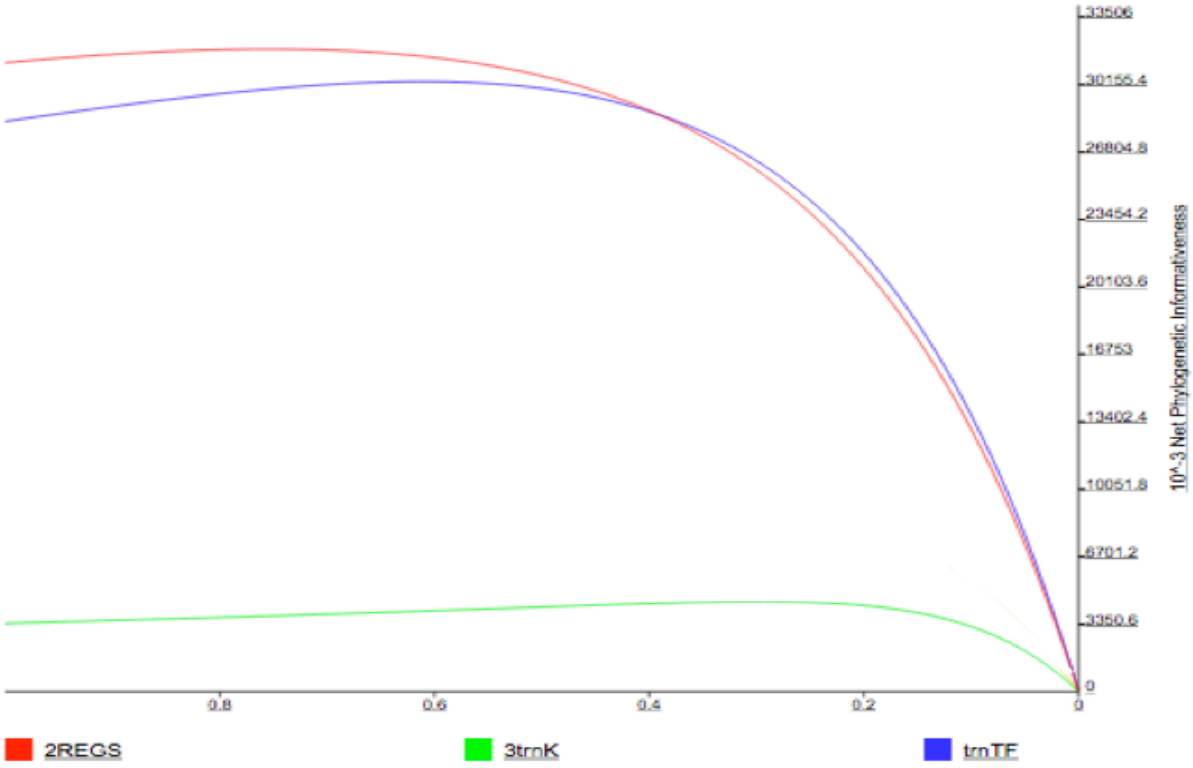

Figure 7. Sites informativeness curve generated by PhyDesign for the *trnT-F* and *3'trnK* regions and their concatenations. The X-axis represents the relative age and the Y-axis represents informativeness.

In both Region-Focus and Taxa-Focus approaches, the MP and BI analyses of the partitioned data sets resulted in some rearrangements but those changes did not receive significant BS and PP support. However, analysis of the concatenated data sets with BI and MP resulted in topologies that were highly congruent with that derived from the RAxML analysis. In general, support for lineages was higher with concatenated data sets than with the individual regions.

Per sites informativeness for the two regions was similar to that of the Region-Focus (Fig. 7). The concatenated data set (red) has higher per site and net informativeness compared with informativeness in either one of the two regions.

The *3'trnK* region displayed the lowest amount of phylogenetic information across the history of the compilospecies probably due to low degree of nucleotide substitution.

IV. DISCUSSION

Compared with morphological analyses where the *Bothriochloa, Capillipedium* and *Dichanthium* genera were recovered as monophyletic units, the molecular phylogenetic analyses based on the partition and concatenated data sets only recovered *Capillipedium* as a monophyletic genus. Both *Bothriochloa* and *Dichanthium* are paraphyletic. Since the concatenated data sets resulted in the most robust phylogeny, further discussion will focus on the phylogeny derived from them. Knowing that the three genomic regions are derived from the chloroplast genome, which is maternally inherited, the discussion of relationship of the taxa will reflect the maternal phylogeny for the compilospecies.

What stands out is that the topology of the tree based on the concatenated data sets is highly congruent with the species relationship and pattern of hybridization in the compilospecies (Fig. 8) proposed by De Wet and Harlan (1970). In the Region-Focus analyses*, B. bladhii, B. ewartiana, B. ischaemum* appeared in one clade, which is similar to the pattern of species hybridization noted in the compilospecies. This clade also included all three species of *Dichanthium*. Members of the genus *Dichanthium* were proposed by De Wet and Harlan (1970) to hybridize with species of *Bothriochloa* through *B. grahamii.* This latter species is not in Australia and thus was not included in the study.

In clade 1, *Capillipedium* is sister to *B. decipiens*. De Wet and Harlan (1970) showed that *Capillipedium* hybridizes with *Bothriochloa* through *C. parviflorum* and *B. glabra* (a synonym of *B. bladhii*). However, they did not include *B. decipiens* in the compilospecies. This finding points to a link between *Capillipedium* and *Bothriochloa* through *B. decipiens,* a new, most parsimonious hypothesis that needs to be verified by interspecific hybridization. A similar situation relates to the placement of *B. macra* and *B. insculpta* in the tree is

evident. *Bothriochloa macra* was not included in the compilospecies network of De Wet and Harlan (1970); their network points to a link between *B. insculpta* and *B. bladhii.* *Bothriochloa macra* appeared paraphyletic with B. *insculpta* nested within it, a relationship that is not in disagreement with the phylogeny since they did not include *B. macra.*

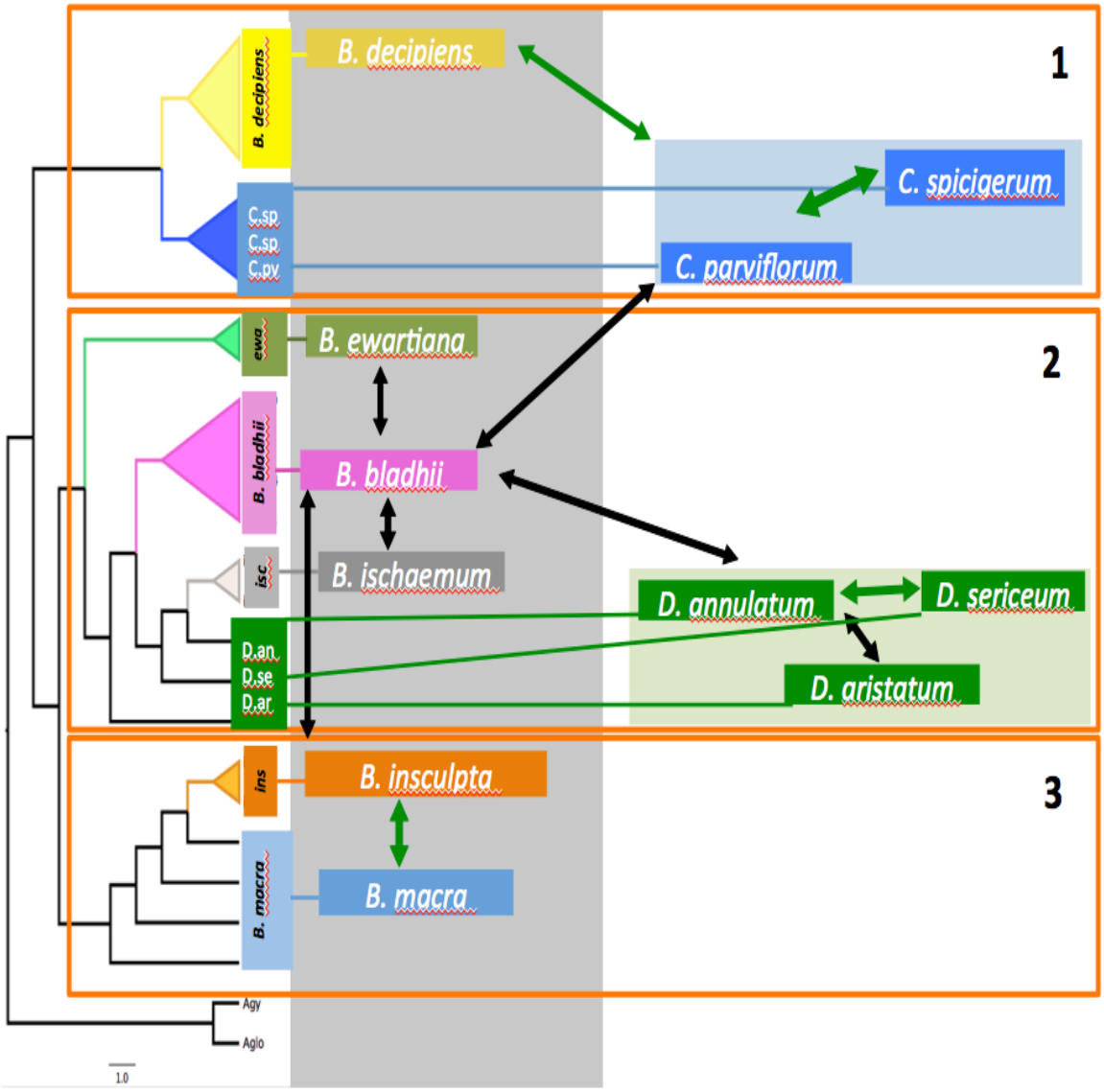

Figure 8. Phylogeny based on RAxML analysis Region-Focus approach is compared side by side with the modified network of the compilospecies (right). Shaded areas correspond to *Bothriochloa* (B) in grey, *Capillipedium* (C) in blue, and *Dichanthium* (D) in green. Orange outlines are the clade separation. Black arrows refer to gene flow within the compilospecies. Green arrows refer to new connections revealed by the tree. Legend: ***Aglom***: *A. glomeratus*, ***Agy***: *A. gyrans*, ***isc***: *B. ischaemum,* ***ewa***: *B. ewartiana,* ***ins***: *B. insculpta,* ***C.pv***: *C. parviflorum,*

***C.sp**: C. spicigerum, **D.an**: D. annulatum, **D.ar**: D. aristatum, **D.se**: D. sericeum.*

The Taxa-Focus (Fig. 9) approach also added the Australian endemics species *B. bunyensis* and *B. erianthoides*. The tree topology from this approach using the concatenated data set is almost identical with that based on the concatenated data set of the Region–Focus approach. The exception is the shift in the position of *B. ewartiana* from clade 2 to clade 1 as sister to *Capillipedium* plus *B. decipiens,* but support for the new placement of *B. ewartiana* is lacking and thus it represents soft incongruence. *Bothriochloa erianthoides* appeared sister to the *B. macra* plus *B. insculpta* clade. *Bothriochloa bunyensis* on the other hand appeared sister to *D. sericeum* (RAxML BS=62%, PP=0.97) in a clade consisted of *B. bladhii*, *B. ischaemum,* and *Dichanthium.* These phylogenetic associations may reflect their chloroplast maternal inheritance, but information on their potential hybridization is not available.

1. Integration of Phylogenetic tree, Nucleotide substitution network and Compilospecies complex of De Wet and Harlan (1970)

Figure 8 was constructed to integrate phylogenetic relationships and the compilospecies relationship envisioned in De Wet and Harlan (1970). In clade 1 (Fig. 8), the compilospecies network showed *Capillipedium* species hybridizing with *B. bladhii.* In contrast, our phylogeny revealed phylogenetic relationship between *Capillipedium* and *B. decipiens* and then a sister relationship of the two to remaining species. Our network analysis demonstrates that *B. decipiens* is the ancestral species in the compilospecies complex (Fig. 9). *Bothriochloa decipiens* is not included in De Wet and Harlan (1970) study and data are not available on potential hybridization of *B. decipiens* with other compilospecies, therefore this finding represents a new hypothesis that need to be tested. The phylogeny points to the *Capillipedium* species as potential ancestral chloroplast genome donors to remaining compilospecies including *B. bladhii,* which is in partial agreement with their compilospecies concept.

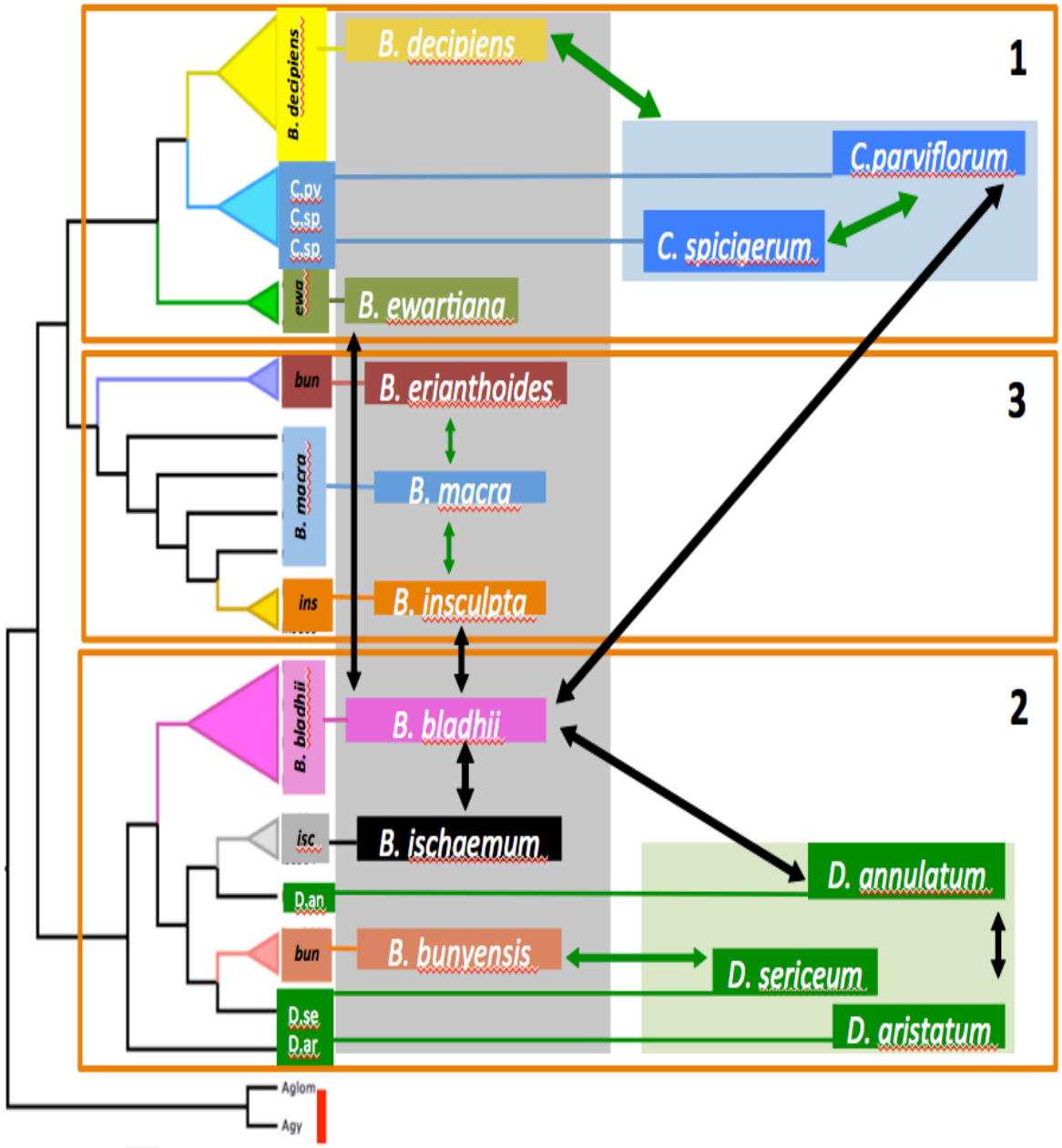

Figure 9. Phylogeny based on RAxML analysis Taxa-Focus approach is compared side by side with the modified network of the compilospecies(right). Shaded areas correspond to *Bothriochloa* (B) in grey, *Capillipedium* (C) in blue, and *Dichanthium* (D) in green. Orange outlines refer to clade separation. Black arrows refer to gene flow based on compilospecies. Green arrows refer to new connections revealed by the tree. Legend: ***Aglom***: *A. glomeratus*, ***Agy***: *A. gyrans*, ***bun:*** *B. bunyensis*, ***eri:*** *B. erianthoides*, ***isc***: *B. ischaemum*, ***ewa***: *B. ewartiana*, ***ins***: *B. insculpta*, ***C.pv***: *C. parviflorum*, ***C.sp***: *C. spicigerum*, ***D.an***: *D. annulatum*, ***D.ar***: *D. aristatum*, ***D.se***: *D. sericeum.*

In clade 2 (Fig. 8), the grouping of *B. ewartiana, B. bladhii, B. ischaemum* and *Dichanthium* is also reflected in De Wet and Harlan (1970) compilospecies network. They showed *B. bladhii* linked to these three *Bothriochloa* species directly, and indirectly to *Dichanthium* through *B. grahamii* (not included in this study). Therefore, the structure and composition of clade 2 is in agreement with De Wet and Harlan (1970) proposed patterns of hybridization. Since this is a maternal phylogenetic tree, it does not reflect the proposed interbreeding between *B. bladhii* and other *Bothriochloa* and *Dichanthium* species. The TCS network (Fig. 10) revealed that B. *ischaemum* and *D. annulatum* on one hand and *B. ewartiana* on the other hand are connected to *B. bladhii* through *B. decipiens.* This kind of relationship is similar to the one proposed by De Wet and Harlan (1970), differing only by the presence of *B. decipiens* in our study and is lacking from their study. Therefore, the addition of *B. decipiens* resulted in a modification of their compilospecies network.

In clade 3 (Fig. 8), *B. macra* was not included in De Wet and Harlan (1970) network. However, they showed that *B. insculpta* interbreed with *B. bladhii.* The phylogeny showed that *B. insculpta* is nested within *B. macra*. The placement of *B. macra* could be proposed into compilospecies complex. This path of interbreeding is not in disagreement with the compilospecies since the *B. macra* plus *B. insculpta* clade is sister to clade 2 that includes B. *bladhii* in maternal phylogeny. Further, based on the TCS network (Fig. 10), *B. decipiens* is connected *B. bladhii* and also to *B. macra* then to *B. insculpta,* which is in agreement with De Wet and Harlan (1970) network.

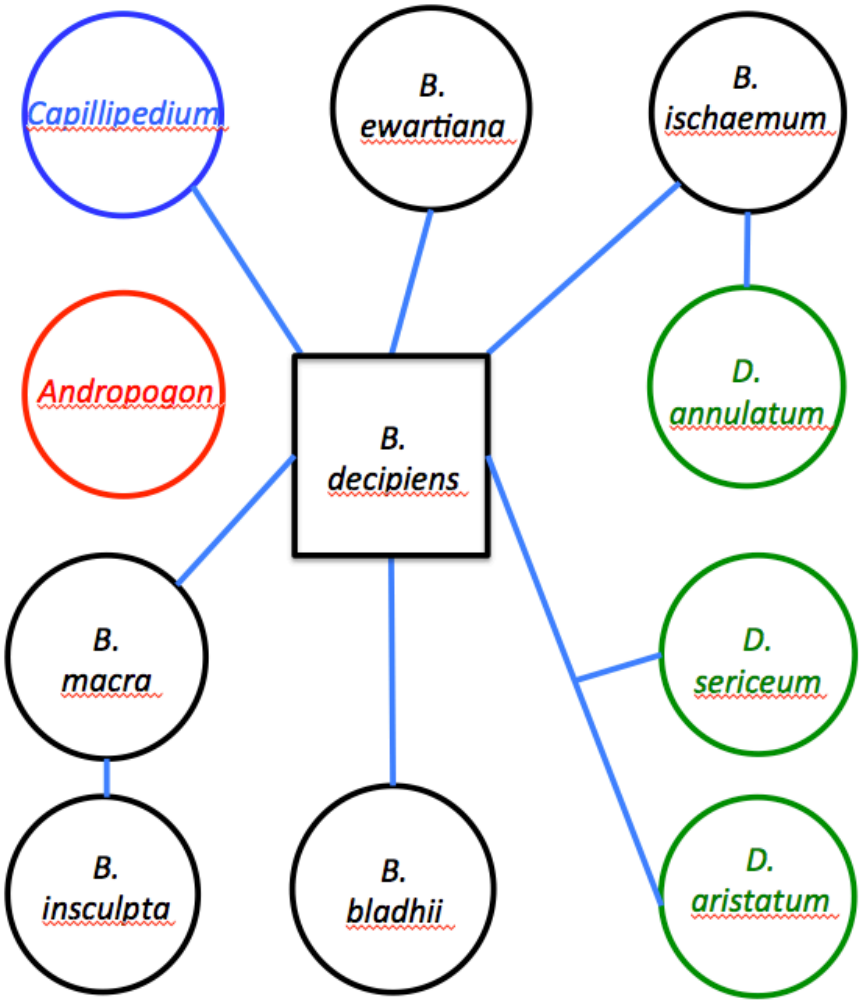

Figure 10. TCS define network based on concatenated of the Region-Focus (*trnT-F + rps 16 intron + 3'trnK*) data set with 95% parsimony, with gaps treated as missing data. Square indicates ancestral, Outgroup (*Andropogon*) in red, *Capillipedium* in blue and *Dichanthium* in green.

In the Taxa-Focus network (Fig. 9), the *rps 16 intron* was eliminated and *B. bunyensis* and *B. erianthoides* were added. The *rps 16 intron* although has less phylogenetic informativeness than the *trnT-F* but it is by far more informative than the *3'trnK*. The exclusion of the *rps 16 intron* and the addition of the two *Bothriochloa* species resulted in some changes in the network. In this case, the newly added species *B. bunyensis* emerged as an ancestral species to remaining taxa since it is the first that connect to the outgroup *Andropogon.* This relationship

was not apparent in the phylogenetic tree based on the same concatenated data set (Fig. 9). *Bothriochloa bunyensis* has a significant amount of missing data, a factor that might have effected its placement. In addition, *B. insculpta* and *B. bunyensis* appeared as two potential central species in the network. The pattern of links among the species in the Taxa-Focus TCS network is not congruent with the pattern of species relationship of the corresponding phylogenetic tree (Fig. 11). The noted differences between the Region-Focus TCS network and the Taxa-Focus network may be due to both the missing data in B. *bunyensis* and the addition of the two *Bothriochloa* species. Whereas, the difference between the TCS of the Region-Focus network and the corresponding phylogeny may be possible due to the sensitivity of TCS to the missing data in B. *bunyensis*.

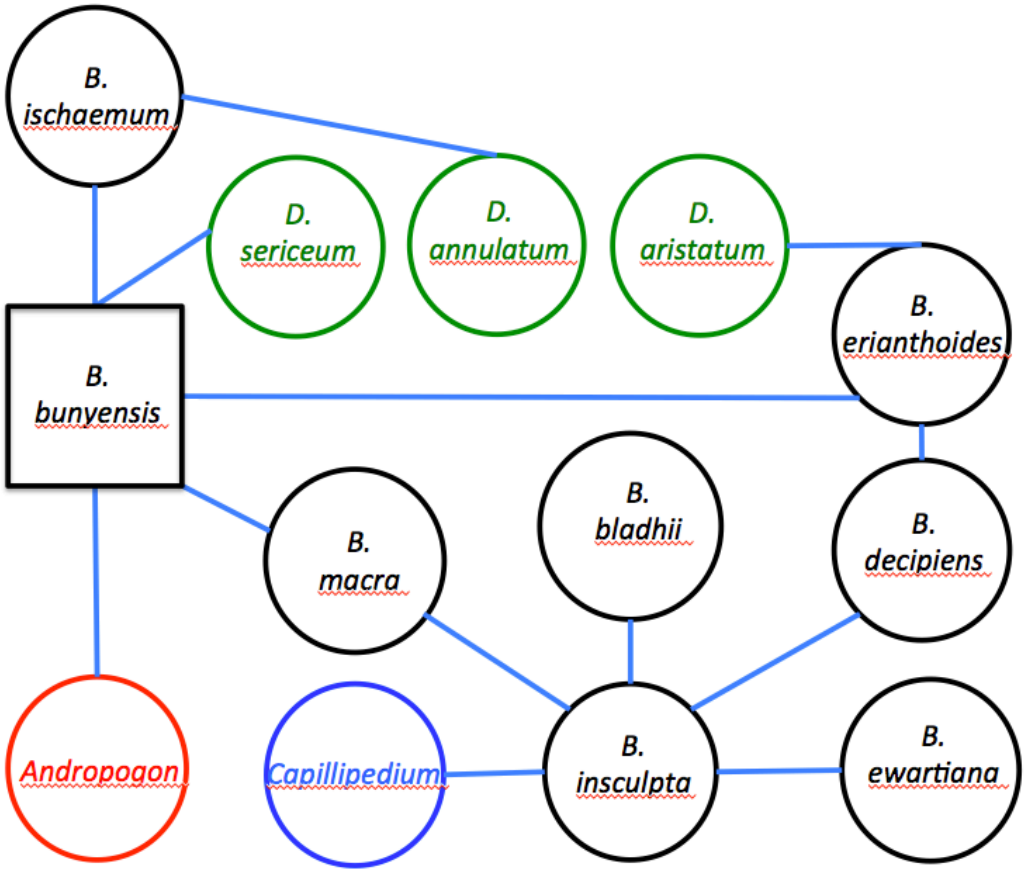

Figure 11. TCS network based on concatenated of the Taxa-Focus (*trnT-F + 3'trnK*) of the region Focus approach with 95% parsimony and gap treated as missing data. Square refers to ancestral, outgroup (*Andropogon*) in Red, *Capillipedium* in blue and *Dichanthium* in green.

2. Other regions of *trnH-psbA, rpL 16 intron*, and *petA-psbJ*

Although *trnH-psbA* is a commonly used region in plant systematics, in this study this region exhibited with only single substitution and no indels over a 500 nucleotide sequence. The *rpL 16 intron* (500 bp) and *petA-psbJ* (300 bp) regions could not be aligned properly. The lack or low amount of variation in these regions from is the main factor for excluding them from further analysis. Low rates of nucleotide substitution in Andropogoneae has been mentioned by Mason-Gamer et al. (1998) for GBSSI, Spangler et al. (1999) for *ndhF*, Lukens and Doebley (2001) for *tb1,* and Mathews et al. (2002) for PHYB.

V. CONCLUSION

In these chloroplast-based phylogenies, only *Capillipedium* appear monophyletic, whereas both *Bothriochloa* and *Dichanthium* are paraphyletic. This is in contrast with the results of the morphological analyses where all three genera were recovered as monophyletic units. Therefore, hybridization does not seem to blur the morphological species boundaries, and the species relationship in the molecular phylogeny provided insight into the maternal pattern of species relationship within the context of hybridization. When the topology of the trees based on concatenated data set is compared with the TCS network and the De Wet and Harlan (1970) network, a high degree of congruent is noted. However, the addition of species in our study that were missing from the De Wet and Harlan (1970) work provided new understanding of the compilospecies complex in the form of a hypothesis that can be tested by hybridization. The results of the study can benefit greatly from the inclusion of markers from the bipaternally inherited nuclear genome.

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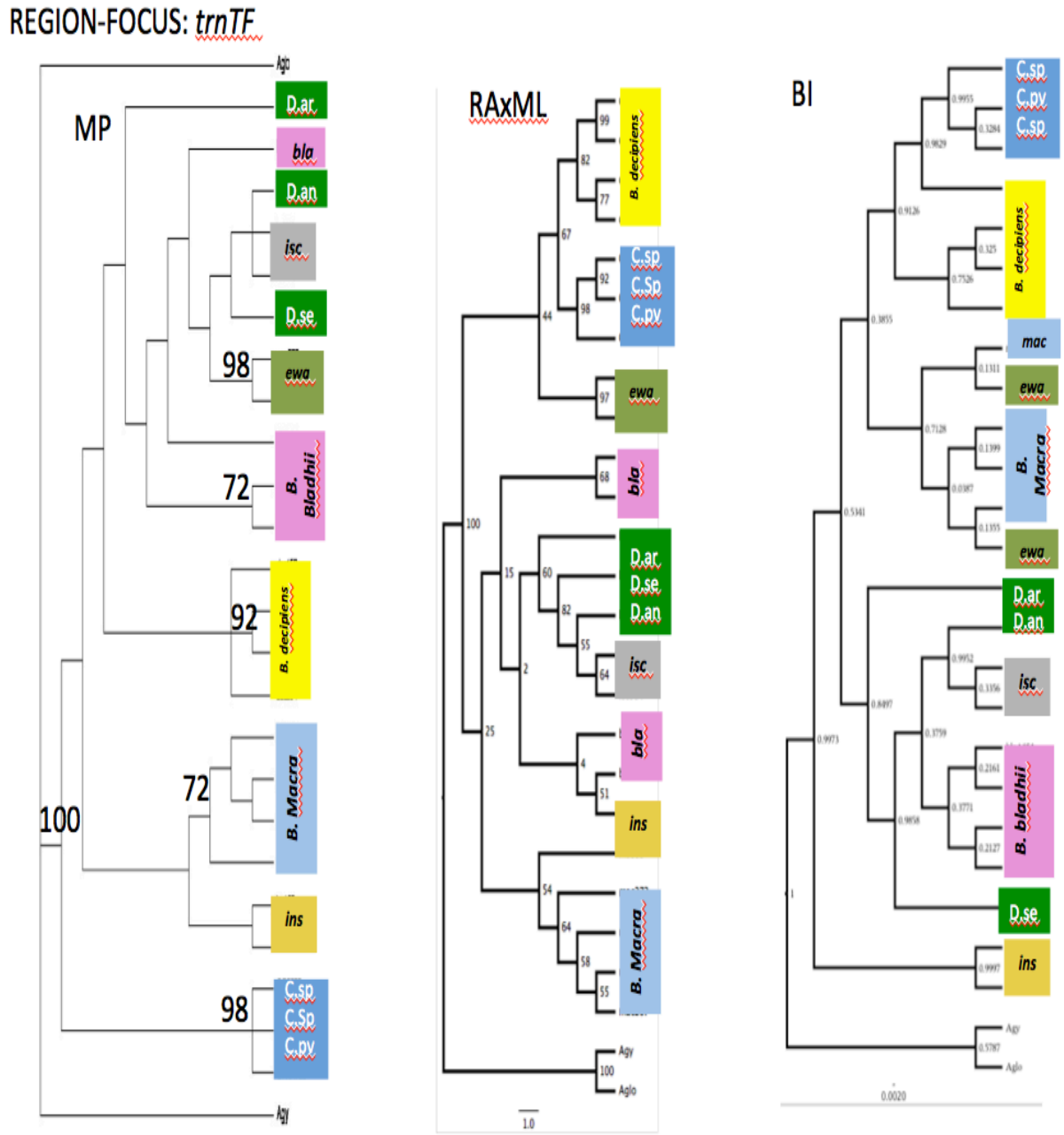
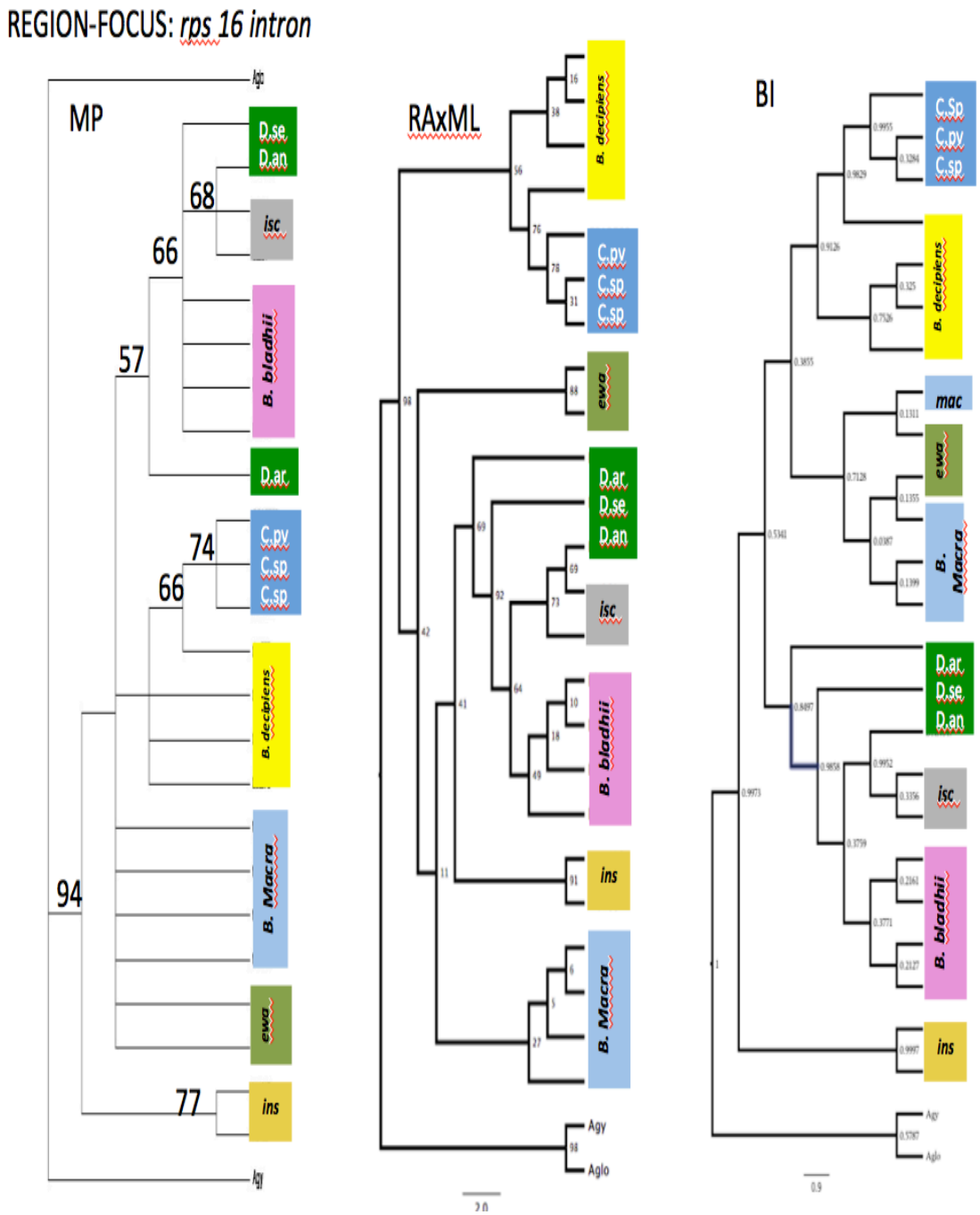
VII. APPENDICES

Appendix A. Region Focus *trnT-F*



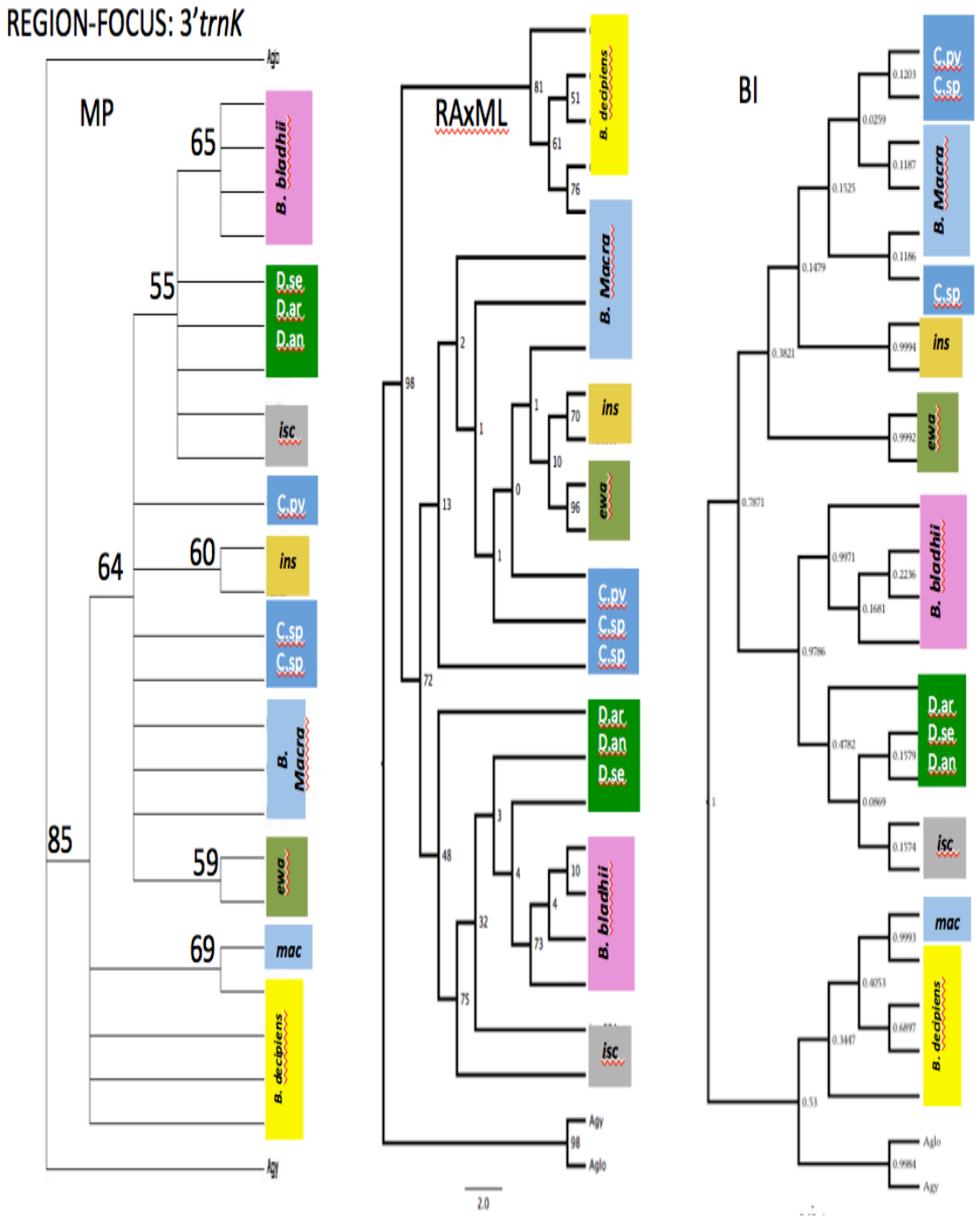
Legend: ***Aglom***: *A. glomeratus*, ***Agy***: *A. gyrans*, ***isc***: *B. ischaemum*, ***ewa***: *B. ewartiana*, ***ins***: *B. insculpta*, ***C.pv***: *C. parviflorum*, ***C.sp***: *C. spicigerum*, ***D.an***: *D. annulatum*, ***D.ar***: *D. aristatum*, ***D.se***: *D. sericeum*.

Appendix B. Region Focus *rps16 intron*



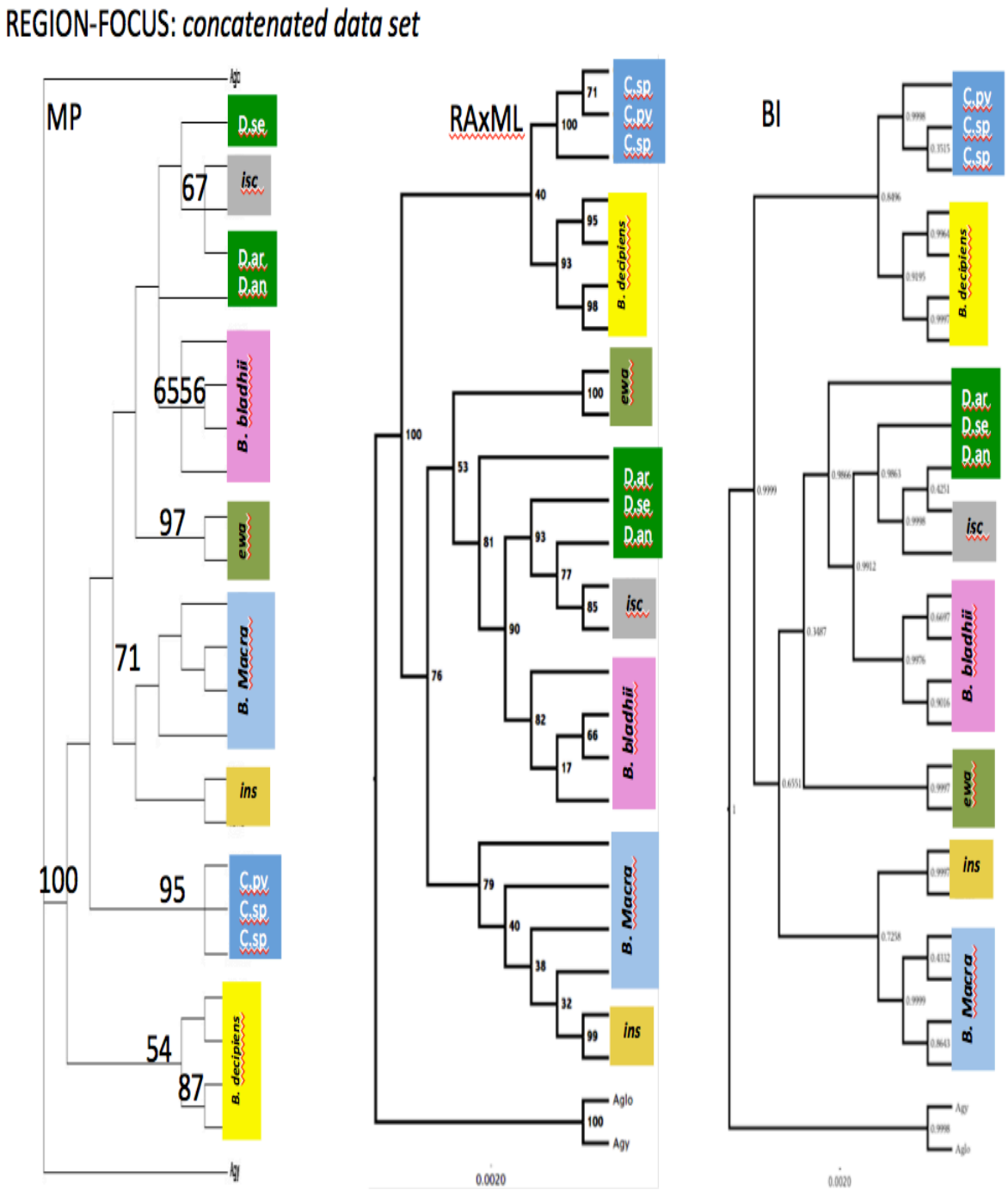
Legend: ***Aglom***: *A. glomeratus*, ***Agy***: *A. gyrans*, ***isc***: *B. ischaemum*, ***ewa***: *B. ewartiana*, ***ins***: *B. insculpta*, ***C.pv***: *C. parviflorum*, ***C.sp***: *C. spicigerum*, ***D.an***: *D. annulatum*, ***D.ar***: *D. aristatum*, ***D.se***: *D. sericeum.*

Appendix C. Region Focus *3'trnK*



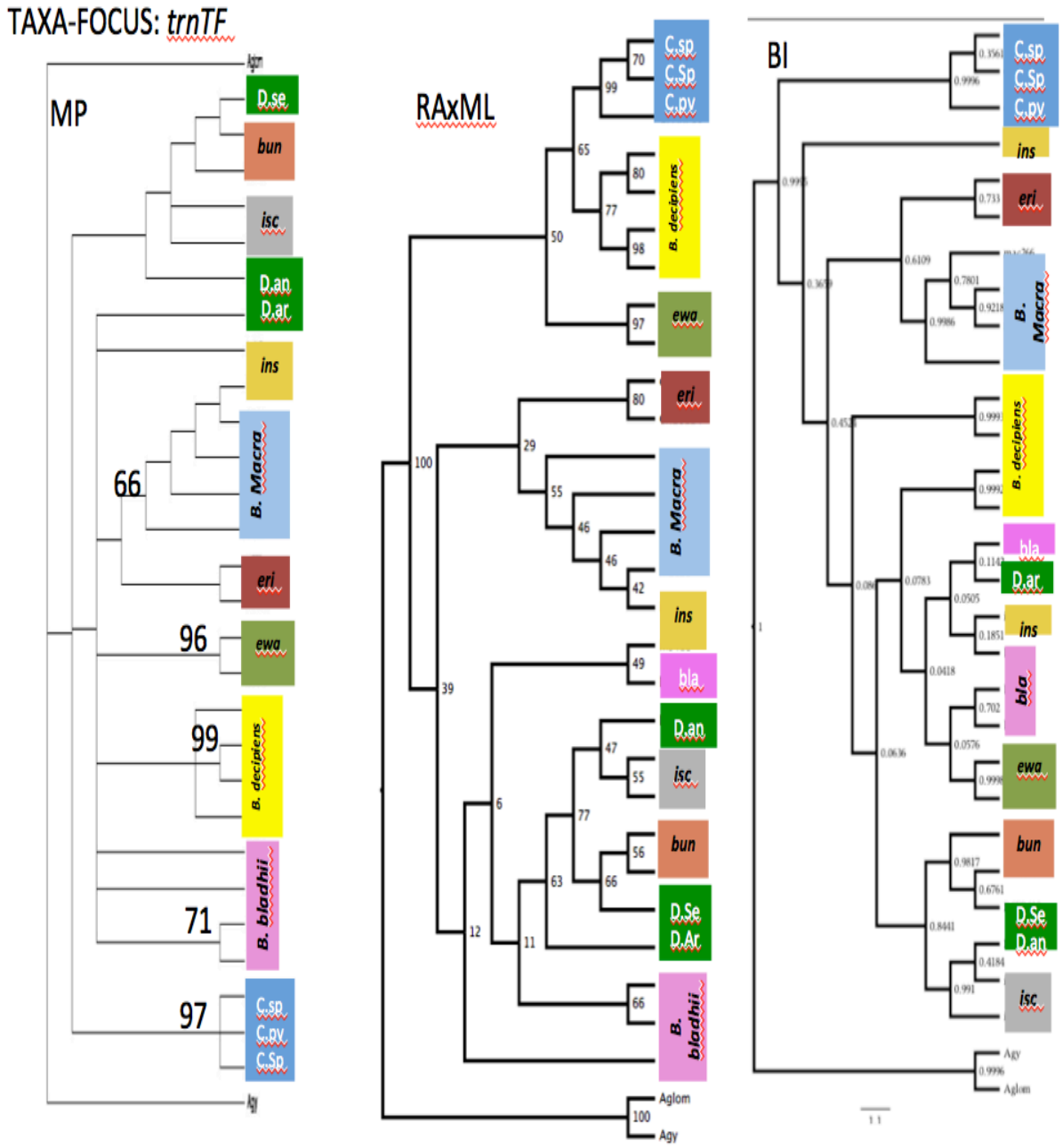
Legend: ***Aglom***: *A. glomeratus*, ***Agy***: *A. gyrans*, ***isc***: *B. ischaemum*, ***ewa***: *B. ewartiana*, ***ins***: *B. insculpta*, ***C.pv***: *C. parviflorum*, ***C.sp***: *C. spicigerum*, ***D.an***: *D. annulatum*, ***D.ar***: *D. aristatum*, ***D.se***: *D. sericeum.*

Appendix D. Region Focus concatenated data set



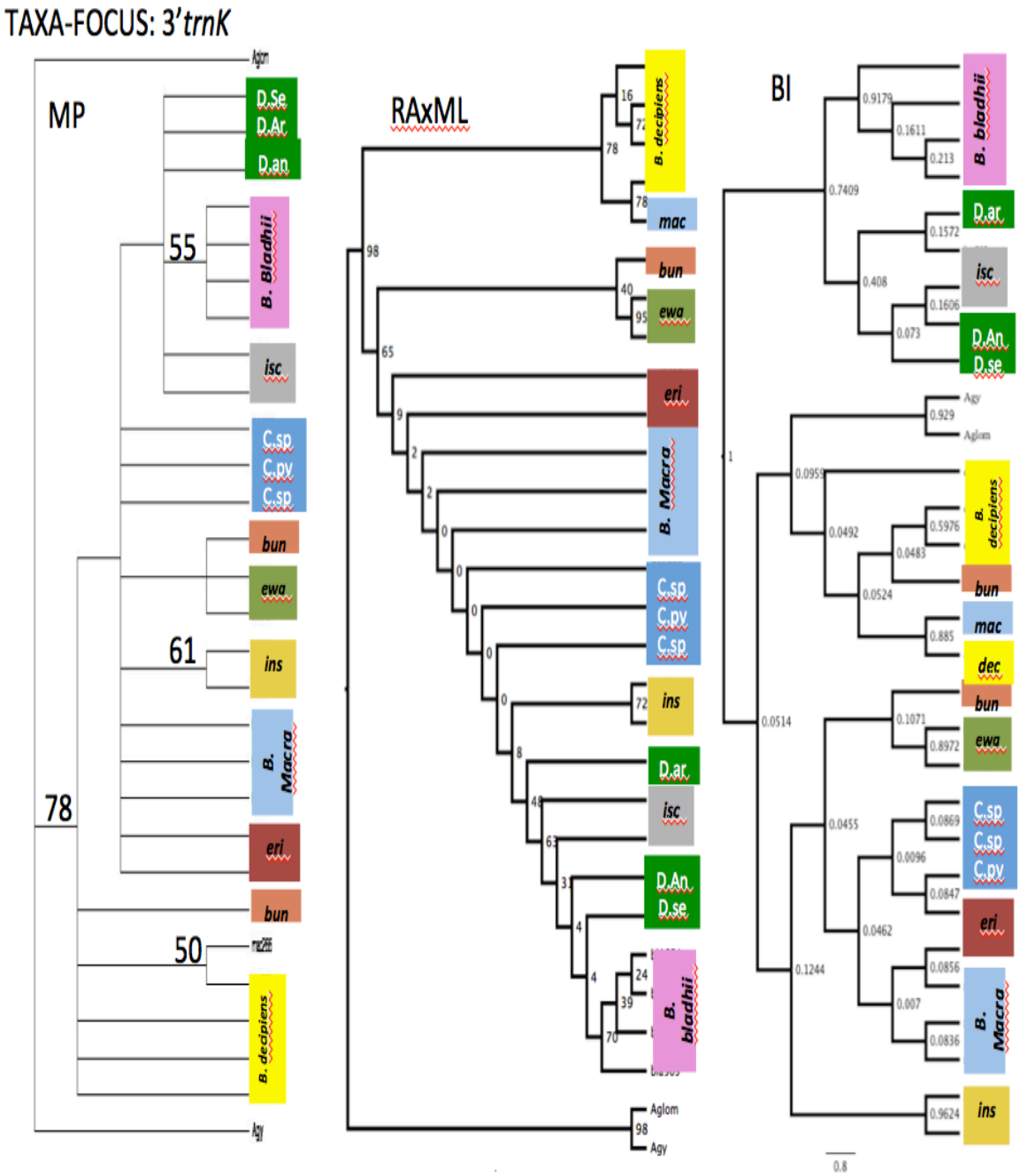
Legend: ***Aglom***: *A. glomeratus*, ***Agy***: *A. gyrans*, ***isc***: *B. ischaemum*, ***ewa***: *B. ewartiana*, ***ins***: *B. insculpta*, ***C.pv***: *C. parviflorum*, ***C.sp***: *C. spicigerum*, ***D.an***: *D. annulatum*, ***D.ar***: *D. aristatum*, ***D.se***: *D. sericeum*.

Appendix E. Taxa Focus *trnT-F*



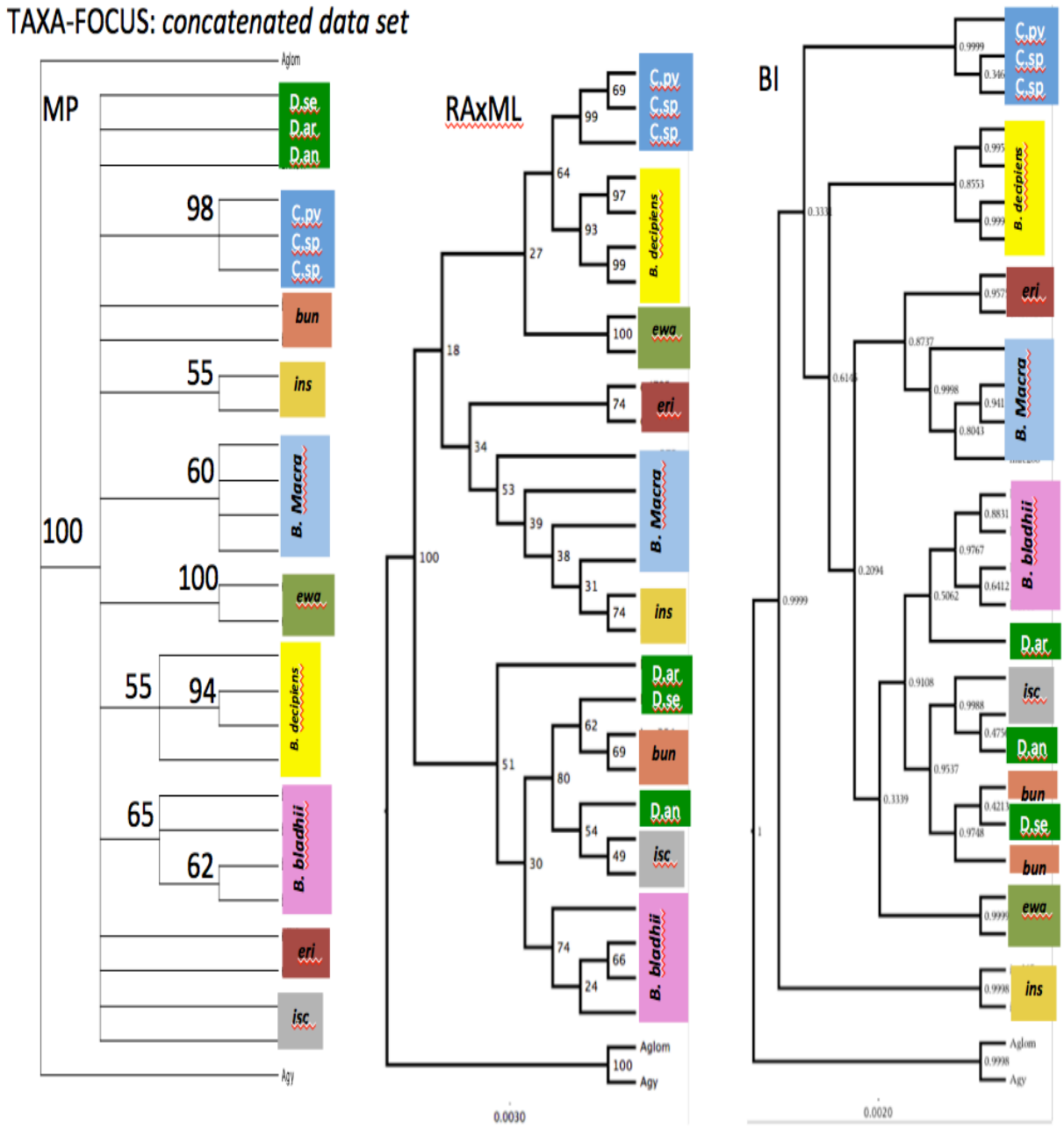
Legend: ***Aglom***: *A. glomeratus*, ***Agy***: *A. gyrans*, ***bun:*** *B. bunyensis*, ***eri:*** *B. erianthoides*, ***isc***: *B. ischaemum*, ***ewa***: *B. ewartiana*, ***ins***: *B. insculpta*, ***C.pv***: *C. parviflorum*, ***C.sp***: *C. spicigerum*, ***D.an***: *D. annulatum*, ***D.ar***: *D. aristatum*, ***D.se***: *D. sericeum.*

Appendix F. Taxa Focus *3'trnK*



Legend: ***Aglom***: *A. glomeratus*, ***Agy***: *A. gyrans*, ***bun:*** *B. bunyensis*, ***eri:*** *B. erianthoides*, ***isc***: *B. ischaemum*, ***ewa***: *B. ewartiana*, ***ins***: *B. insculpta*, ***C.pv***: *C. parviflorum*, ***C.sp***: *C. spicigerum*, ***D.an***: *D. annulatum*, ***D.ar***: *D. aristatum*, ***D.se***: *D. sericeum.*

Appendix G. Taxa Focus concatenated data set



Legend: ***Aglom***: *A. glomeratus*, ***Agy***: *A. gyrans*, ***bun:*** *B. bunyensis*, ***eri:*** *B. erianthoides*, ***isc***: *B. ischaemum*, ***ewa***: *B. ewartiana*, ***ins***: *B. insculpta*, ***C.pv***: *C. parviflorum*, ***C.sp***: *C. spicigerum*, ***D.an***: *D. annulatum*, ***D.ar***: *D. aristatum*, ***D.se***: *D. sericeum.*

CHAPTER III – CHROMOSOME NUMBER AND GENOMIC CONTENT

I. INTRODUCTION

The best way to assess the ploidy level of a taxon is by counting the chromosomes. The number of chromosomes for a single species can vary greatly in the cases of aneuploidy and eupolyploidy. Polyploidization is the most common and important process that can give rise to a new species (Soltis and Soltis 1999, Levy and Feldman 2002, Madlung 2013). Polyploids could arise from chromosome doubling in somatic cells as autopolyploidy. However, autopoliploidy more commonly arises from cytologically unreduced gametes (De Wet 1971). Polyploidy can associate with hybridization resulting in allopolyploidy. *Bothriochloa* has a basic chromosome number *x* = 10 with 2n = 20, 30, 40, 50, and 120. The ploidy level of this genus may be 2x, 4x, 5x, 6x, or 12x, and aneuploidy is common. Table 7 lists the different number of chromosomes of various *Bothriochloa*, *Capillipedium* and *Dichanthium* (BCD) species based on previous chromosome counts (Gould 1956, Singh and De Wet 1960, Cheeda and Harlan 1961, Watson and Dallwitz 1992). *Bothriochloa bladhii* and *B. ischaemum* show the greatest variation in chromosome level for the sporophytic stage, followed by *B. insculpta*. Surprisingly, there are no chromosome counts reported for *B. ewartiana, B. macra,* and *B.decipiens.*

Hybridization that occurs among the BCD taxa leads to the reasonable assumption that variation in chromosome number in *Capillipedium* and *Dichanthium* are expected. *Capillipedium spicigerum* (2n= 40) resembles a hybrid between *B. intermedia* (2n= 40, now considered as *B. bladhii*) and *C. parviflorum* (2n= 40) (Harlan et al. 1962). However, *B. intermedia* x *C. spicigerum* hybrids resulted in 2n= 47, whereas *B. intermedia* (2n=40) x *C. parviflorum* (2n=40) resulted in 2n= 40 hybrids (De Wet et al. 1961). The chromosome count database (Rice et al. 2014) provides the latest compilation of chromosome numbers for these three genera (Table 7).

Estep et al. (2014), in their study of the Andropogoneae, summarized the congruency between their work on the phylogeny of the BCD group with the hybridization experiments of De Wet and Harlan (1970). They found that tetraploidization and hexaploidization occurred during lineage separation. Several of *B. bladhii* accessions were found to bear *Capillipedium* and *Dichanthium* genomes, which explains the hybridization and introgression leading to the compilospecies situation.

Table 7. Recorded chromosomes number of various species of *Bothriochloa*, *Capillipedium*, and *Dichanthium* based on chromosome number database Rice et al. (2014) (http://ccdb.tau.ac.il/).

Species	Sporophytic (2n) / **Gametophytic (n)**
Bothriochloa bladhii	30, 34-44, 50, 60, c.70, 80
Bothriochloa insculpta	50,60
Bothriochloa ischaemum	30, 31, 36, 40, 42, 45, 50, 60
Capillipedium parviflorum	20, 40, 60
Capillipedium spicigerum	40
Dichanthium aristatum	20, 40, 50, 60
Dichanthium annulatum	20, 40, 60
Dichanthium sericeum	**5**

The objectives of this study are to count the chromosomes and estimate genomic contents using Flow cytometry to provide more information on the polyploidy level in the compilospecies. The information will be interpreted in the context of phylogeny and proposed network of hybridization. Flow cytometry is the fast, easy and robust way to determine genomic content and to infer polyploidy levels (Doležel et al. 1994, Doležel et al. 2007, Loureiro et al. 2008).

II. MATERIALS AND METHODS

1. Chromosomal counting

The materials were derived from fresh meristematic leaf sample of 10 species from USDA –GRIN. Cytological preparations were made to determine the ploidy level in root tip meristematic cells from both mature plants and seedlings. Pollen from developing anthers was also examined for chromosome number. Preparations from mature plants and pollen failed but chromosome were observed from mitotic squashes of root tips collected from germinated seeds. Leaf materials were treated at room temperature (20--25 °C) for two hour with paradichlorobenzena to stop spindle fiber formation and separate homologous chromosomes. Fixation followed the pretreatment by placing seedings in a fixative solution of ethanol:acetic acid glacial (3:1) for 20 minute. Samples were then macerated with HCl:ethanol 70% (1:1) for 5 minute at room temperature, placed in Basic Fuchsin for 10 minutes, and stained in 45% acetocarmine. Then, the root tips were cut and put on a slide. Observation and chromosome counting were conducted under a microscope (Olympus CHT, Ch-2) with 400x magnification.

2. Flow cytometry

Flow cytometry buffer was made by mixing 882 mg sodium citrate, 419 mg MOPS, 915 mg MgCl2, 0.1 ml Triton X-100, and 250 ml distilled water. Meristem tissue from the basal portion of young folded leaves was chopped using a razor blade on petri glass after adding the buffer. The chopped materials were double filtered using two cylinders (250 um on the top and 63 um on the bottom) into a 1.5 ml tube and placed on ice. Two hour before flow-cytometry, 0.5 ml of Ribonuclease A (40 mg RNAse in 50 ml chopping buffer) was added. The digestion of RNA occurred at room temperature. One hour before the flow cytometry, 0.125 ml of propidium iodide (PI) stain (6.0 mg PI in 15 ml chopping buffer) was added to the sample and kept on ice.

These samples were run at the Virginia-Maryland Regional College of Veterinary Medicine Flow Cytometry Core Facility using their BD FACSCallibur Flow cytometer. FlowJo software (Treestar Inc.) was used for further analysis. The graph of the FlowJo result consists of three peaks, two minor one and a major peak. The major peak represents the diploid cell count. The first minor peak on left of the major peak is caused by over-chopping of the leaf, while the third peak on the right of the major one corresponds to cells in mitosis (Fig. 12).

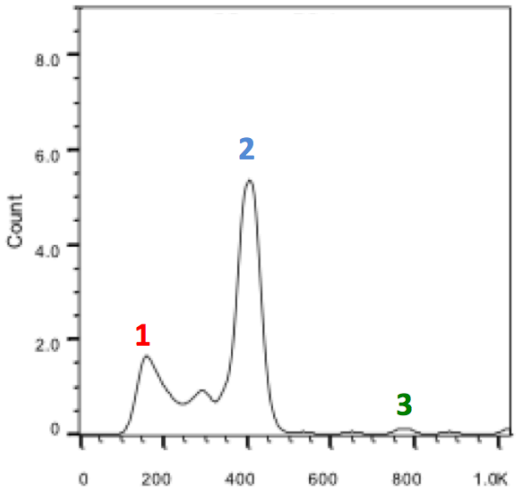


Figure 12. The peaks observed in the Flow cytometry experiments. The small first peak represents debris resulted from over chopping. The major peak in the middle (2) represent the most ploidy detected while the small peak (3) was the mitosis result. Y-axis representes the number of nuclear count and the X-axis represents the relative value of the ploidy level Notice that the value of peak # 3 on the X-axis is double of peak # 2.

Flow cytometry was run in duplicate or triplicate for each accession for accuracy in the measurement of the ploidy level. The value of the peak is the average of the measurements (minimal two) of each accession. The value for a species is the mean from all accessions for that particular species.

Flow cytometry is calibrated against an accession with known chromosome count. In this case, one set of chromosomes (haploid) gives a value of about 100 on the graph, therefore 200 refer to diploid and 400 would be tetraploid cytotypes. The rest of the Flow cytometry results were calibrated using this standard. Some

accessions had value not in an increment of 100 (*e.g.* 250). In this circumstance, the species would be considered diploid with a larger genomic content.

III. RESULTS AND DISCUSSION

Chromosome counting yielded in only one good result where *B. macra* 264 was determined to have *ca*. 40 chromosomes (tetraploid). Flow cytometry demonstrated that the majority of the accessions were either diploid (200) or tetraploid (400), with no hexaploid detected (Table 8). Some accessions had larger than normal values (250), such as *B. ischaemum*, *B. ewartiana*, *B. decipiens*, and *D. aristatum*. Since their values were between 200 and 300, these accessions were considered diploid but with a larger genome content since no species are known to be triploid. This is evidently detects aneuploidy or diploid with larger chromosome. Table 8 compares flow cytometry with chromosome counts from De Wet and Harlan (1970), Estep et al. (2014) and Rice et al. (2014).

The Flow cytometry results were congruent with most of the published chromosome data. By applying a flexible delimitation on genomic content, I deducted that, *B. decipiens* (250) was diploid, whereas *B. insculpta* (400), *Capillipedium spp.* (400), and *D. annulatum* (400) were tetraploid (Table 8). For several species, our results were in disagreement with previous work (De Wet and Harlan 1970, Rice et al. 2014, Estep et al. 2014). *Bothriochloa bladhii* diploid value (200) is a new finding since previous work (De Wet and Harlan 1970, Rice et al. 2014, Estep et al. 2014) noted tetraploid or hexaploid counts. Similarly, the *B. ewartiana* (250), *B. ischaemum* (250), and *B. insculpta* (400) accessions were diploid and tetraploid instead of the tetraploid, pentaploid or hexaploid as reported by De Wet and Harlan (1970) and Estep et al. (2014) (Table 7). These new findings maybe due to studying different accession, stressing the high degree of chromosome variation in these species and the potential of cross hybridization.

Table 8. Result of Flow cytometry, chromosome count database (sporophytic and gametophytic), and the sources of the data. Shaded area refers to differences in chromosome count. Symbol (–) refers to unavailable data.

Species	Flow cytometry values	Sporophytic **/Gametophytic** chromosome counts	Ploidy (De Wet and Harlan (1970) X value	Ploidy (Estep (2014)) ploidy format
B. bladhii	200	30, 34-44, 50, 60, c.70, 80	4x,6x	tetraploid, hexaploid
B. insculpta	400	50,60	5x, 6x	tetraploid
B. ischaemum	250	30, 31, 36, 40, 42, 45, 50, 60	4x,6x	tetraploid, diploid
B. ewartiana	250	-	5x, 6x	-
B. decipiens	250	-	-	diploid, tetraploid
B. macra	400	-	-	tetraploid
C. parviflorum	400	20, 40, 60	2x, 4x	tetraploid
C. spicigerum	400	40	-	tetraploid
D. aristatum	250	20, 40, 50, 60	2x, 4x	diploid
D. annulatum	400	20,40,60	2x, 4x	tetraploid
D. sericeum	400	**5**	-	

Superimposing the Flow-cytometry results on the phylogenetic tree based on the Region–Focus approach using concatenated *trnT-F, rps16 intron*, and *3'trnK* dataset (Fig. 13) revealed new information. Clade-based perspective shows a consistent ploidy level for *Bothriochloa* species. All diploid *Bothriochloa* were in clade 1 (*B. decipiens*) and clade 2 (*B. bladhii, B. ischaemum, B. ewartiana*). Species of *Capillipedium* and *Dichanthium,* however, were tetraploid, but *D. aristatum* was diploid. These results (Table 8) are in partial agreement with De Wet and Harlan (1970) and Estep et al. (2014). De Wet (1968) pointed out the extreme rarity of diploids in *D. annulatum* and *D. aristatum* since their collection of hundreds of accessions represent only about 2% of the populations. There were no data for *D. sericeum* from previous literature (De Wet and Harlan 1970, Estep et

al. 2014). The Chromosome Count Database gives a gametophyte count for this species as x=5, but further information is needed to discuss this species.

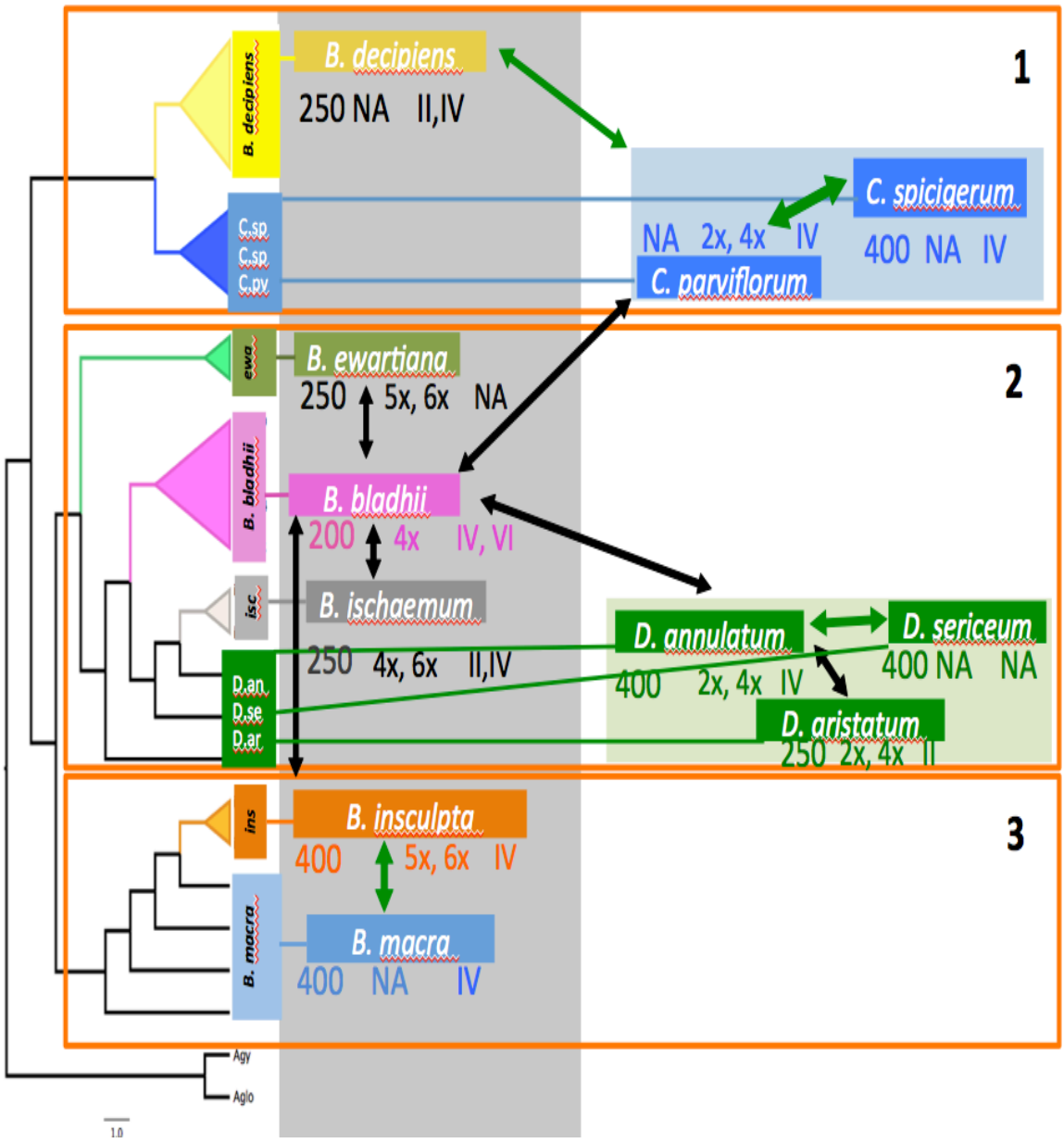


Figure 13. Phylogenetic tree of the BCD complex based on the Region-focus approach with ploidy status reported below each taxon. Number reported below each species correspond to from left to right: Flow Cytometry result, ploidy level following De Wet and Harlan (1970), and the ploidy level reported by Estep et al (2014) in Roman numbers. NA refers to Not Available. Legend: ***Aglom***: *A. glomeratus*, ***Agy***: *A. gyrans*, ***isc***: *B. ischaemum*, ***ewa***: *B. ewartiana*, ***ins***: *B. insculpta*, ***C.pv***: *C. parviflorum*, ***C.sp***: *C. spicigerum*, ***D.an***: *D. annulatum*, ***D.ar***: *D. aristatum*, ***D.se***: *D. sericeum.*

Clade 3 **(**Fig. 13**)** consisted exclusively of tetraploid estimates for *B. macra* and *B. insculpta* based on the flow cytometry data. This is congruent with Estep et al. (2014) findings, though De Wet and Harlan (1970) considered *B. insculpta* as pentaploid and hexaploid, but gave no information for *B. macra*. This again underscores the potential of excessive variation in chromosome numbers existing in the compilospecies complex yet to be fully reported.

Superimposing flow-cytometry on the nucleotide substitution (TCS) map of Region-Focus (Fig. 13) confirmed the ploidy status of some species and provides a different perspective. *Bothriochloa decipiens* (250) was positioned in the middle as the diploid ancestor with larger genomic content. All of the diploid species were linked directly to *B. decipiens.* These diploid species include normal diploid *B. bladhii* (200) and diploids with larger genome (250), *B. ischaemum*, *B. ewartiana* and *D. sericeum.*

Tetraploid taxa C. *spicigerum* (400) and *B. macra* (400) were also linked directly with *B. decipiens* (Fig. 14), indicating direct evolution from diploidy to tetraploidy. Other instances represent indirect evolution of tetraploidization, such as the emergence of tetraploids *D. sericeum* (400). *Bothriochloa insculpta* (400) represent a case of speciation at a tetraploid level since it is directly link to tetraploid *B. macra* (Fig. 14).

Some of the conflicting result in the ploidy level could be explained by the use of different accessions in the studies focusing on the compilospecies. This study and Estep et al. (2014) only have a single accession in common (*B. decipiens*, PI 153). This accession is consistently shown as diploid in both studies. Although inconsistancies in flow cytometry data are not unexpected, this study is based on duplicate or triplicate sampling with the result are highly consistent for the ploidy level.

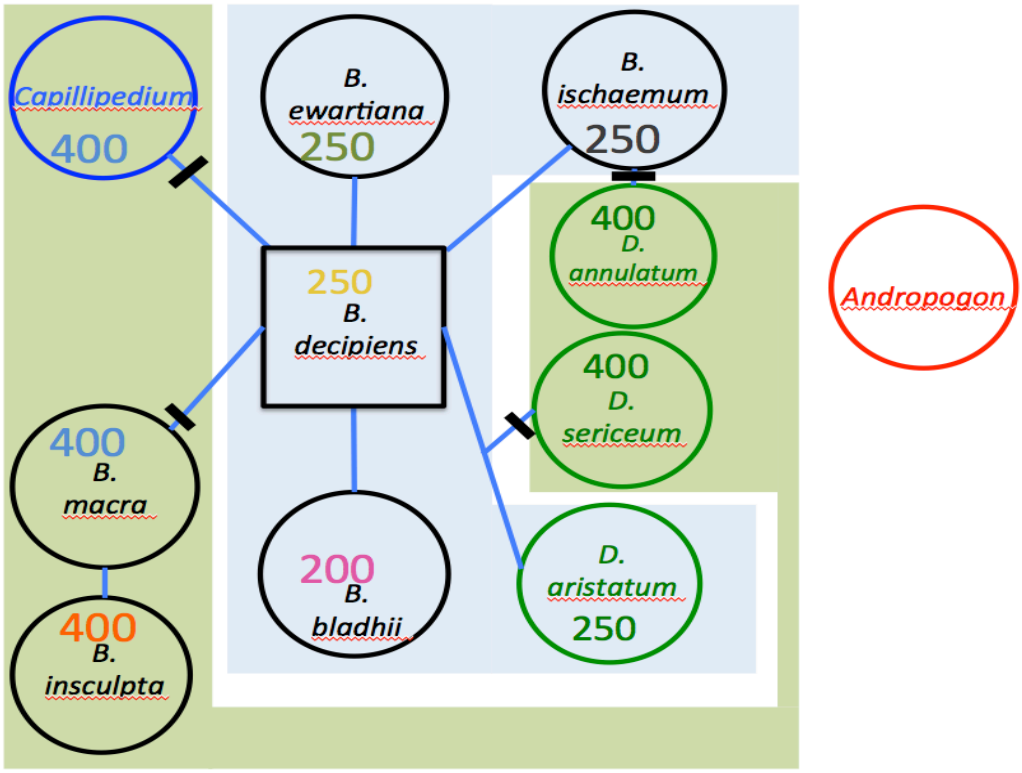


Figure 14. Superimposing average flow cytometry result (number inside circle) on TCS mapping of Region Focus (95% parsimony probability, gap coded as missing data). Tetraploids and diploids are highlighted in green and blue shades. Four black bars refer to tetraploidization events. Square box refer to ancestor species. The outgroup *Andropogon* is isolated from the rest of the network. The letters *B.* refers to *Bothriochloa*, *C.* refers to *Capillipedium*, and *D.* refers to *Dichanthium.*

V. SUMMARY AND CONCLUSIONS

Flow-cytometry delivers the fastest result for assessing the genomic content. It detected diploids and tetraploids but not hexaploids. Flow cytometry points to diploid values for *B. decipiens*, and a clade consisting of *B. bladhii, B. ewartiana,* and *B. ischaemum. Bothriochloa insculpta* and *B. macra* formed an exclusively tetraploid clade. *Capillipedium spicigerum* and *Dichanthium sericeum* were tetraploid. However, no hexaploids were detected during the measurement. The TCS result showed that speciation has taken place in different paths. The ancestral diploid species *B. decipiens* was linked directly to diploid species,

tetraploid species, and indirectly to tetraploid species.

Unfortunately, polyploidy and hybridization remained largely unresolved in explaining the adaptation to variable environmental conditions and potential invasiveness (Mable 2013). Evaluation of the whole compilospecies would be a huge effort. Multidimensional studies using aspects such as geography, dispersal, and various cytotypes may provide more insight into the evolution of the compilospecies. Until that moment, no comprehensive taxonomic revision of individual genera can be finished with great confidence, nor can the hybridization issue in BCD be resolved.

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FINAL SYNTHESIS

As the first empirical study on the compilospecies, the phylogenetic analyses based on morphological characters using MP and BioNJ showed that all three genera *Bothriochloa*, *Capillipedium* and *Dichanthium* (BCD) are individual monophyletic units. This was resolved despite reported intergeneric hybridization among them. The study also revealed the close relationship between *Capillipedium* and *Dichanthium* since they were recovered as sister clades in one well-supported lineage. Their relationship with each other and with *Bothriochloa* has been disputed. Tree topology based on BioNJ and MP analyses are highly congruent. *Bothriochloa* consisted of two subclades.

In the chloroplast-based phylogeny, *Bothriochloa* is not monophyletic since *Capillipedium* and *Dichanthium* are nested within it. *Capillipedium* appeared monophyletic, whereas *Dichanthium* is paraphyletic with *B. ischaemum* and *B. bladhii* consistently nested inside it in trees based on individual data sets (*trnT-F, rps16 intron* and *3'trnK*) and concatenated data set of Region-Focus and Taxa-Focus. This is a sharp contrast with the results of the morphological analyses where all three genera were recovered as monophyletic units. Composition of the clade remains stable and addition of two new species of *B. bunyensis* and *B. erianthoides* only shifted the placement of *B. ewartiana*. Concatenated data sets showed the greatest informativeness compared with individual genomic regions. Of the individual genomic regions, the *trnT-F* is the most informative, followed by *rps16 intron* and *3'trnK;* the latter region was considerably less informative. A high degree of congruency was evident when comparing trees based on concatenated data set of Region-Focus with the TCS network constructed here and the De Wet and Harlan (1970) network of the compilospecies. The addition of species in our study that were missing from the De Wet and Harlan (1970) work provided new understanding of the compilospecies complex in the form of hypothesis that can be tested by hybridization.

Flow-cytometry study recorded diploids and tetraploids but no hexaploids. In

combination with the TCS result, new patterns of evolution have been detected. The ancestral species appeared to be diploid *B. decipiens* not *B. bladhii*. *Bothriochloa decipiens* was linked directly to diploid species, tetraploid species, and indirectly to tetraploid species of the BCD complex.

The evaluation the whole compilospecies will be a massive undertaking. This Australia-focused study provided a significant new insight into the relationship among species of the compilospecies complex in general and on the taxa of this continent in particular. Thus it represents a valuable starting point for understanding the details of the compilospecies complex. Incorporating other species of *Bothriochloa* beyond Australia and using sequence information from nuclear genomic regions will refine the overall picture of the classification and evolution of the compilospecies.