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Reviewer's report on paper 19682:

A 250 plastome phylogeny of the grass family (Poaceae)

1.Basic reporting

The paper is an important contribution and a first step to obtaining a tree-of-life of grasses using plastome data analysis. The authors have used their own data and data generated by previous authors to build an updated plastome-based phylogeny of Poaceae. The background and literature provided is sufficient, but sometimes it is not discussed appropriately. The English style, figures and tables are correct and the authors share the raw data. The paper is extremely long (101 pages manuscript, excluding tables and figures) and basically descriptive. The authors describe, too profusely and sometimes redundantly, the phylogenetic relationships among taxa within each subfamily, tribe and other taxonomic ranks in the Results and Discussion sections; these two sections could probably be re-organized and reduced to make a shorter paper keeping only essential novel information. Some parts of the paper and even subsections deal with aspects/taxa that have not been analysed in this paper and are therefore superfluous.

2.Experimental design

The research is primarily original as the authors have used methodological approaches (e. g., phylogenetic searches using 14 alternative data partitions and a basic test for detection of purifying/non-purifying selection within coding regions) not assayed before in grass plastomes. The methods have been described in detail and the performed analyses are overall correct. However, some procedures could be questionable. For example, 1) the alignments were not curated manually, at the risk of leaving some microstructural mutations/indels to be misaligned or deleting valuable information; it would, indeed, have a minimum effect at deep-level phylogenies, but perhaps not at shallow-level phylogenies, especially for very closely related taxa, though the authors solved partially the problem by discarding the gaps in some partition analyses; 2) the authors replaced stop codons with gaps in codon data if they were present; it could leave putative non-functional copies (e. g., pseudogenes) in the CDS data set. Despite the number of pseudogenes is low in the grass plastomes, it would be desirable to discard them from the CDS-based analysis. The authors have not commented the presence of potential pseudogenes in the data set (nor if some of them were present in one of the discarded IR); 3) the authors used a codon-based Z test to test for purifying vs positive selection in coding regions across the 250 grass plastomes using the default options in MEGA and Positive selection options in HyPhy (using MEME, mixed effects model of evolution to search for episodic selection at individual sites), and provide a Fig. 1 with number and proportion of codons under purifying/positive selection for each CDS and a Suppl. Table 1 with information on dN, dS, omega and p and test values for purifying/positive selection for each CDS; it is not clear, however, which codons of the genes show positive selection (those located in the more conserved 5'-end or those located in the more variable 3'-end of the genes?) and which values of the tests support one hypothesis over the other (in Table S1). Also, and most importantly, the authors have not tested if episodic positive selection is distributed across all grass lineages or, more likely, only across a subset of them (or even a few of them) in each case. To do it

the authors should run a complementary approach to find selected branches (under positive selection) by pooling information over sites as indicated by Kosakovsky Pond et al. (2011) Mol Biol Evol 28: 3033–3043 (branch-site REL model of evolution).
The hypothesis testing is mainly based in a classification of grasses proposed by several of the current authors (Soreng et al. 2017) which, in turn, compiles molecular and morphological data from different researchers (see General comments to author).

3.Validity of the findings
The data is robust and statistically sound, though most of the novelty resides in the re-analysis of an enlarged data matrix using different data partitions (coding and non-coding regions, full plastome, selected genes) with and without gaps and positively selected codons, and the comparisons of the topologies and support of clades between partitions. Discussions with respect to previous phylogenetic works and classifications are exhaustively, but some of them are misleading or are not limited to supporting results (see General comments to author). The interpretation of the potential negative impact of positively selected sites should be re-evaluated with more precise data on distribution of sites across lineages, as well as that of the informative value of coding and non-coding regions with respect to the evolutionary depth of the groups under study.

General comments for the author
The paper is an important contribution to the tree-of-life of grasses using plastome data analysis. It deals with an evolutionary systematic study of the grass family based on phylogenomic analysis of a large collection of plastome data (250 taxa, representing 180 genera and 44 tribes of Poaceae) generated by the authors and by previous researchers that have been jointly analysed for the first time using 14 different data partitions. The data, methodology and results are overall sound; however, the paper presents some flaws and misinterpretations that should be corrected and results that should be properly addressed. Additionally, the paper is extremely long, and parts of the Results and Discussion sections could probably be deleted or summarized to make a more readable paper.

I acknowledge the valuable efforts made by the authors to compile and analyse a large amount of current plastome data and to use it to help to resolve the phylogeny and systematics of Poaceae. Nonetheless, I am concerned about several issues that require throughout revision:

1.The paper is extremely long (101 pages manuscript, excluding tables and figures) and basically descriptive. The authors describe, too profusely and sometimes redundantly, the phylogenetic relationships among taxa within each subfamily, tribe and other taxonomic ranks of Poaceae in the Results and Discussion sections. These two sections could probably be re-organized and reduced to make a shorter paper keeping only essential novel information. Some parts of the paper deal with aspects/taxa that have not been analysed in this paper. For example, “Rooting the grass phylogenetic tree” subsection (of Discussion) is out of place here as the authors did not root the Poaceae tree with any close outgroup (but with basal Anomochlooideae lineages). In several instances, the authors comment grass subtribes for which plastomes have not been published yet. It is superfluous, as the current work is not intended to be a full revision.
2. The hypothesis testing is mainly based in a classification of grasses proposed by several of the current authors (Soreng et al. 2017) which, in turn, compiles molecular

and morphological data from different researchers. It is appropriate to use it as a baseline hypothesis and to compare the new results to it; however, the authors should give the credits to the researchers that contributed most to the phylogeny/classification of each group, trying to avoid undermining or skewed statements. For example, the authors indicate that "Danthonioideae includes a single tribe comprising 18 genera …. (Soreng et al., 2017)" and that "the plastome trees" (-based on 7 species representing 6 genera) "are better supported than trees based on a few plastid regions (Linder et al. 2010)". However, systematic circumscription of Danthonioideae is mostly based in the work of Linder et al. (2010), who recognized 17 genera after their comprehensive molecular and morphological study of 281 danthonioid species, and the additional contribution of Teisher et al. (2017) with 1 genus. It is not surprising to know that the 7 species plastome trees of Danthonioideae are better supported than the >200 species plastid trees of Linder et al. (2010) but the two studies and their respective sampling sizes are not comparable.

3. The authors used a codon-based Z test to test for purifying vs positive selection in coding regions across the 250 grass plastomes using the default options in MEGA and Positive selection options in HyPhy (using MEME, mixed effects model of evolution to search for episodic selection at individual sites), and provide a Fig. 1 with number and proportion of codons under purifying/positive selection for each CDS and a Suppl. Table 1 with information on dN, dS, omega and p and test values for purifying/positive selection for each CDS. It is an interesting approach though it is not clear, however, which codons of the genes show positive selection (those located in the more conserved 5'-end or those located in the more variable 3'-end of the genes?) and which values of the tests support one hypothesis over the other (in Table S1). Also, and most importantly, the authors have not tested if episodic positive selection is distributed across all grass lineages or, more likely, only across a subset of them in each case. It would be advisable to conduct a complementary approach in order to find selected branches (under positive selection) by pooling information over sites as indicated by Kosakovsky Pond et al. (2011) Mol Biol Evol 28: 3033–3043 (branch-site REL model of evolution). It is highly relevant because some of the conclusions drawn by the authors about the potential effect that genes containing positively selected codons can have on phylogenetic disturbance (or "systematic error") should be carefully checked.

4. The authors have built a grass phylogeny showing different evolutionary depths (from subfamily (deep phylogeny) to species/variety (shallow phylogeny) levels and have compared the levels of resolution and support of each of the 14 data partitions across the main clades. However, it would be worth to know which data partition (or even subpartition) is most adequate for solid reconstruction of the deep and shallow branches of the tree clades. Additionally, they indicate that widely used loci in grass systematics, such as ndhF, matK, rpoC2 and rbcL, particularly prone to positive selection in some codons, could cause phylogenetic artifacts. I am not convinced about this conclusion. These genes (or parts of them) are more variable than other plastid genes, and therefore more useful to resolve deep vs shallow phylogenies, as demonstrated in early studies of plastid phylogenies of angiosperms. All of them encode functional proteins, and episodic positive selection in some codons may occur only in some lineages. At least two of them (rbcL, matK) have been selected as barcoding molecules for plants (ToL project). The authors should make more solid arguments about the phylogenetic value/failure of these genes before recommending caution about them. Moreover, the suggestion of using the highly conserved psaA and psaB genes for phylogenetic reconstruction is not very useful for shallow phylogenies of grasses; they

may content very few positively selected codons but do not contain enough phylogenetic signal for recently evolved lineages.
5. The core Pooideae clade should not include Brachypodieae. Several evidences support it. The sister but non-inclusive relationship of *Brachypodium* to the core pooid clade [Triticodae (Triticeae+Bromeae)/Poodae (former Poeae+Aveneae; now Poeae s. l.)], originally proposed by Davis and Soreng (1993), was abandoned in favor of the inclusion of *Brachypodium* within the 'core pooids', a non-taxonomic but independently evolved natural group, in some recent analyses (Davis & Soreng, 2007; Saarela *et al.*, 2015; Soreng *et al.*, 2015, 2017). However, recent studies (Minaya et al. 2015; pooids, b-amylase; Sancho et al., 2017, New Phytol. (accepted paper, phylogenomics of Brachypodium and grass plastomes) support the sister relationship proposed by Davis and Soreng as well as divergence times intermediate between those of the basal ancestral pooids and the recently evolved core pooids (Sancho et al. 2017). Additionally, pairwise plastome genetic and patristic distances have further confirmed that *Brachypodium* is closer to some basal pooid lineages than to the core pooid lineages (Sancho et al., 2017), corroborating similar results based on nuclear single copy genes (Minaya *et al.*, 2015) and functional genomic studies of regulation of vernalization and flowering time genes (Fjellheim et al. 2014; Front. Plant Sci. 5: 431; Woods et al. 2016, Plant Physiol. 170:2124-2135). *Brachypodium* is a highly isolated lineage, and the large length of its stem branch (compared to the short lengths of its crown branches) has been recovered in all phylogenetic studies conducted with representative species of this genus and other Pooideae lineages (see, for example, Minaya *et al.* 2015; Catalan et al. 2016 (not 2015); Sancho et al. 2017). It would be advisable to recognize that Brachypodieae is not part of the core Pooideae from the very beginning; the authors could explain better their current results based on this hypothesis.
6. The authors have misinterpreted the results of Christin et al. (2007, 2008) about convergent evolution of C4 photosynthetic pathway genes. The sentence (ls. 758-760) "For example, in grasses in which photosynthetic genes, such as *rbcL* or *PEPC*, converge under selection for C4 photosynthesis, misleading phylogenies can result (Christin et al., 2007, 2008)" is incorrect. Convergent evolution caused by selection resulting in (false) monophyly of C4 grasses was found in analysis of some coding (non-synonymous) positions of the PEPC gene (Christin et al. 2007), whereas polyphyletic origin of C4 grass lineages was recovered in analyses of synonymous and non-coding positions of the same PEPC gene (Christin et al. 2007) as well as in plastid rbcL and ndhF genes (Christin et al. 2008). Christin et al. never indicated that the plastid rbcL gene was under convergent selection; by contrast, they used this gene and the plastid ndhF gene to construct a reliable phylogeny to estimate the divergence times of the polyphyletic C4 grass lineages.

Additional points:

7. The alignments were not curated manually, at the risk of leaving some microstructural mutations/indels to be misaligned or deleting valuable information; it would, indeed, have a minimum effect at deep-level phylogenies, but perhaps not at shallow-level phylogenies, especially for very closely related taxa, though the authors solved partially the problem by discarding the gaps in some partition analyses.
8. The authors replaced stop codons with gaps in codon data if they were present; it could leave putative non-functional copies (e. g., pseudogenes) in the CDS data set. Despite the number of pseudogenes is low in the grass plastomes, it would be desirable to discard them from the CDS-based analysis. The authors have not commented the

presence of potential pseudogenes in the data set (nor if some of them were present in one of the discarded IR).
9. Despite their critics about genes showing positively selected codons the authors chose as their reference tree, tree X, based on plastome data including positively selected codons.
10. l. 250. 'Unsupported' should not be used for clade support < 50%, you could use very low or very weak support instead.
11. ls. 304-306. What is the sentence for?
12. l. 663. Please give details on number of clades and percentages.
13. ls. 667-668. Please identify the 30 clades. Do they correspond to shallow clades?
14. ls. 674-675. The argument is not convincing; character conflict does not necessarily to be connected with selection effect. The authors should test this hypothesis.
15. l. 821, 836-841. Bambusoideae. Most of the Discussion on bamboos is based on previous plastid-based phylogenetic studies, despite the fact that many bamboos are polyploids. What is the conclusion here? Can the authors identify hybrid/allopolyploid clades or introgression events that could explain the conflicting topologies obtained from plastomes and from nuclear data?
16. ls. 964-966. Confusing sentence, the authors have not described properly the relationships among the 5 Phyllostachys species.
17. ls. 1003-1008. Unnecessary paragraph. The current study could not resolve it as Neohouzeaua has not been included in the study.
18. ls. 1053-1054. The pivotal paper that proposed Brachypodium distachyon as model system for grasses was that of IBI (International Brachypodium Initiative) or Vogel et al. 2010. Nature 463: 763-768.
19. 1066-1069. A sister relationship of Diarrhena and Brachypodium was recovered in the beta-amylase tree of Minaya et al. (2015) though some sequences (Diarrhena, B. distachyon clone 2-3) could be recombinant. The authors cite this reference in the manuscript but do not comment these results.
20. ls. 1085-1098. See comments above about phylogenetic studies of Brachypodium (Minaya et al. 2015; Catalan et al. 2016 (book chapter), and the accepted work by Sancho et al. 2017, that could be discussed by the authors.
21. ls. 1102-1109. I recommend the authors to read the recent work by Sancho R, Cantalapiedra CP, López-Álvarez D, Gordon SP, Vogel JP, Catalan P, Contreras-Moreira B. 2017. Comparative plastome genomics and phylogenomics of *Brachypodium*: flowering time signatures, introgression and recombination in recently diverged ecotypes. *New Phytologist* (in press) for a robust dating analysis of the Brachypodium lineages within the grass phylogeny framework based on plastome analysis and nesting dated approaches. The paper will be published as early view soon.
22. ls. 1197-1198 and more. Please indicate if the work by Saarela et al. (in review) is already published.
23. ls. 1205-1207. Pimentel et al. (2017) found a sister relationship of Lagurus to Aveninae-Koeleriinae in their 5-genes plastid tree. The authors should comment it. This paper [Pimentel M, Escudero M, Sahuquillo E, Minaya MA, Catalán P. 2017. Diversification rates and chromosome evolution in the temperate grasses (Pooideae) are associated with major environmental changes in the Oligocene-Miocene. *PeerJ*] has been accepted for publication and will be published soon.
24. ls. 1236-1243. Ammophila has not been included in this study. These sentences are unnecesary here.
25. 1245-1247. Pimentel et al. (2017) in their 5-genes plastid phylogeny do not recover a sister relationship of Anthoxanthiinae to Agrostidinae+Brizinae but that of

Anthoxanthinae to Aveninae-Koeleriinae-Lagurus. These authors have a larger sampling of Agrostidinae, and Aveninae-Koeleriinae than the one presented here. The authors should discuss it and be cautious about their conclusion.
26. ls. 1275-1278. Minaya et al. (2015) included Avenula bromoides (Gouan) H. Scholz in their study (not A. hookeri). It is not surprising to know that the plastome tree does not agree with the ITS (nuclear)+plastid tree of Minaya et al. (2015) for these particular taxa, considering the high reticulation of the groups involved.
27. 1278-1279. The authors do not explain the conflicts of their plastome tree with those of the b-amylase tree of Minaya et al. (2015). They should comment them. Again, it would not be surprising to find differences between the nuclear beta-amylase tree and a plastome tree in highly reticulate groups. The authors do not extract conclusions about the observed differences. Moreover, Minaya et al. (2015) detected incongruent resolutions for some beta-amylase sequences, caused by recombination or selection, and more specifically they explained the cases of Avenula bromoides, Desmazeria rigida, Deschampsia antarctica, Alopecuros arundinaceus, Colpodium drakensbergense, Ammophila arenaria, Vulpia alopecuros, and Festuca ovina. It looks as if the authors have not paid attention to the paper by Minaya et al. (2015), in which they throughly investigated the potential origins of phylogenetic incongruence in cloned copies of the single copy gene beta-amylase.
28. 1279-1282. The sentence is superfluous; it is not currently supported by the data.
29. ls. 1288-1289. This relationship is only supported by 8-9 trees and, as stated by the authors, they haven't sampled other representatives of Airinae (apart from H. hookeri), Holcineae (apart from H. lanatus) and Deschampsia s. s. (apart from D. antarctica). In the 5-genes plastid phylogeny of Pimentel et al. (2017) those relationships are not well supported.
30. ls. 1299-1305. The 5-genes plastid phylogeny of Pimentel et al. (2017) (including matK) does not recover a strong support for the Cynosurinae+Dactylidinae+Parapholiinae+Loliinae clade. A tree based solely in matK might not be an improvement to the resolution of the phylogeny of the group.
31. ls. 1306-1307. 98% in tree X (Fig. 4) for the branch showing the sister relationship of Dactylidinae/Cynorusinae+Parapholiinae to Loliinae. Taxon sampling has increased in Dactylidinae, Cynosurinae and Parapholiinae but not much in other close groups (Airinae, Holcinae, Deschampsia s.s.).
32. ls. 1318-1319. Incomplete sentence.
33. ls. 1467-1469. The authors recognize that their plastome gapped regions could be ambiguously aligned. It could be avoided in part through manual alignment curation.
34. ls. 1478-1479. “lack of support”. Replace with very low support.
35. l. 1511. The authors recognize here the potential influence of nuclear genes in combined nuclear+plastid topologies and their contrasting resolution with respect to plastid or plastome topologies alone, but they haven't done it with respect to Minaya et al. (2017) analyses (see comments above).
36. ls. 1634-1638. Please compare levels of support with sampling sizes.
37. ls. 1653-1656. Confusing sentence. It is not clear which studies show stronger support than others and which studies were considerably less sampled than others.
38. ls. 1656-1661. Please indicate if the Washburn et al. (2015) tree is their only-plastid tree, -which would be congruent with the plastome tree-, and if conflict with the Vicentini et al. (2008) tree is because the latter is a nuclear-based tree (as indicated by Washburn et al. 2015).
39. ls. 1683-1687. Please indicate the ploidy level of W. capillipes. Bidirectional crosses are common in grass allopolyploids (e. g. Brachypodium hybridum, Lopez-Alvarez et

al. 2012, 2017; Catalan et al. 2016) and allopolyploidy and bidirectional origin could explain the coexistence of two plastid types within the same taxon (see discussions in Catalan et al. 2016 (Phylogeny and evolution of the genus *Brachypodium*, book chapter); Lopez-Alvarez et al. 2017, Annals of Botany 119: 545-561.).
40. ls. 1694-1698. The authors should discuss their results regarding Setaria and Paspalidium with respecto to the broadly sampled study of both genera and close allies conducted by Kellogg et al. (2009) using plastid ndhF data. Please specify the two plastome trees that support the monophyly of Setaria.
41. ls. 1816-1817. Speculative assessment. Denser plastome sampling could also reduce the resolution or the support of some evolutionary relationships.
42. ls. 1836-1839. The analysis of perennial Brachypodium spp plastomes is currently underway (Sancho et al. unpub. data). Nonetheless, resolution of Brachypodieae with respect to Diarrheneae and the core Pooideae (Poeae+Bromeae+Triticeae) do not depend on it. Brachypodium is a strongly supported monophyletic lineage in all evolutionary analyses.
43. ls. 2023-2025. The correct reference is as follows: Catalán P, López-Alvarez D, Díaz-Pérez A, Sancho R, López-Herranz ML. 2016. Phylogeny and evolution of the genus *Brachypodium. In* Vogel J (ed.). Genetics and genomics of *Brachypodium.* pp. 9-38. Series Plant Genetics and Genomics: Crops Models. Springer. New York.