

Response to Reviewer 3 Comments-

Round 2

Reviewer 3: I have reviewed the revised manuscript and the authors' responses and am satisfied that all major points raised in my previous review have been fully and appropriately addressed. The revisions have improved the conceptual clarity of the Discussion, strengthened the justification of methodological choices, and resulted in a more balanced and cautious biological interpretation. The manuscript is now clear, coherent, and technically sound.

I have no further major concerns and support acceptance of the revised version. I would like to offer three minor, optional comments that may further strengthen the manuscript:

Dear Reviewer,

Thank you very much for taking the time to review the manuscript again and for your valuable comments, which have helped to improve the quality of the article. In response to your suggestions, I have revised the relevant sections of the manuscript accordingly. Detailed responses to each comment are provided below, and all corresponding revisions and corrections have been highlighted in yellow in the revised manuscript.

Point-by-point response to Comments and Suggestions for Authors

Comments 1: *In a few places, interpretations of $Ka/Ks > 1$ could be phrased more cautiously (e.g., "signals consistent with positive selection"), given the plastome-based and pairwise nature of the analysis.*

Response 1: Thank a lot for this valuable suggestion. The Discussion has been revised to adopt more cautious and precise language when interpreting Ka/Ks values > 1 , consistent with the plastome-based and pairwise nature of the analysis. Statements implying positive selection have been softened to indicate putative or possible adaptive signals, using terms such as "suggesting possible adaptive evolution" and "may reflect," and interpretive claims have been framed in a hypothesis-driven context. These revisions reduce overinterpretation while preserving the biological relevance of the observed patterns. The revised text presented in L726-760 is as follows:

The Ka/Ks ratio (also referred to as dN/dS or ω) is a key metric used to infer the nature and strength of selective pressures on protein-coding genes. A Ka/Ks ratio < 1 indicates purifying selection, where deleterious amino acid changes are eliminated, maintaining functional conservation. A ratio of 1 suggests neutral evolution, while values > 1 indicate positive selection, where beneficial mutations are favored [171-173]. Analysis of 79 protein-

coding genes across 15 Ceropegieae plastomes revealed that most genes are under strong purifying selection, reflecting their essential roles in plastid function. Notably, no genes were found to be evolving neutrally. However, four genes—*accD*, *ndhE*, *ycf1*, and *ycf2*—exhibited Ka/Ks ratios >1, indicating signals consistent with positive selection. The potential functional and adaptive significance of these genes is discussed below in a hypothesis-driven context, in relation to their known roles in plastid metabolism and stress adaptation.

The *accD*, which encodes a subunit of the plastid acetyl-CoA carboxylase complex critical for fatty acid biosynthesis [174], showed evidence of accelerated evolution. This gene has also been reported to evolve rapidly in *Medicago ruthenica* [175], *Ficus* [176], and cupressophytes [174], often due to repetitive sequence insertions that contribute to both high substitution rates and genome rearrangements. In Ceropegieae, many of which are succulents inhabiting arid environments, such selection on *accD* may reflect adaptive modifications in lipid metabolism that enhance membrane stability, cuticular wax formation, or water retention.

Similarly, *ndhE*, part of the NADH dehydrogenase complex involved in cyclic electron flow and chlororespiration [82], exhibited Ka/Ks patterns consistent with positive selection. This may suggest potential fine-tuning of photosynthetic energy balance under stress conditions such as drought or fluctuating light, consistent with the ecological niches of Ceropegieae. Unlike its loss in some non-photosynthetic plants [77], selection on *ndhE* here may indicate adaptive maintenance associated with stress resilience.

ycf1, one of the largest and most variable chloroplast genes, although its precise function has been a subject of debate, it has been identified as Tic214, a vital component of the *Arabidopsis* TIC (Translocon at the Inner Chloroplast envelope) complex [140]. Despite its conserved function, *ycf1* displays high sequence variability and has been identified as putatively positively selected in *Ficus* [176], *Caragana* [177], and *Medicago ruthenica* [175]. In Ceropegieae, elevated Ka/Ks values for *ycf1* may reflect adaptive changes in plastid protein trafficking, potentially enhancing import specificity or efficiency under environmental stress.

ycf2, the largest plastid gene in angiosperms and it is unequivocally identified as a functional gene whose products are essential for cell survival and plant viability [178], also showed signatures consistent with adaptive evolution. Similar selection has been documented in *Cerasus* [179] and Zingiberaceae [180], where it is associated with adaptations to varying light conditions. In Ceropegieae, elevated Ka/Ks values for *ycf2* may be associated with optimization of ATP-driven protein transport in response to the energy demands of plastid function under arid or fluctuating light conditions. Collectively, while most plastid genes remain under purifying selection, *accD*, *ndhE*, *ycf1*, and *ycf2* may contribute to evolutionary flexibility enabling persistence in diverse and challenging habitats.

Comments 2: *The discussion of possible plastome heteroplasmy in Orbea wissmannii var.*

eremastrum is interesting but could more explicitly emphasize its speculative nature and the need for further validation.

Response 2: Thank you for this helpful comment. The Discussion has been revised to more explicitly emphasize the speculative nature of the proposed plastome heteroplasmy in *Orbea wissmannii* var. *eremastrum*. Interpretations have been softened, and I now clearly state that the biological significance of the detected inversion remains uncertain and requires validation. The revised text presented in L580-594 is as follows:

In *O. wissmannii* var. *eremastrum*, the 8.4 kb inversion disrupts the canonical *ndhH–D* operon (Figure 2), which may potentially influence transcriptional regulation and co-expression. Loss or pseudogenization of plastid *ndh* genes has occurred repeatedly and independently across diverse plant lineages—most notably in heterotrophic taxa such as *Orobanchae* and many *Orchidaceae* [87,88], but also in fully photosynthetic species including *Pinus thunbergii* [89], members of *Ericaceae* [90,91], *Gentiana* sect. *Kudoa* [92], some *Cactaceae* [93], *Corydalis* [94] and *Capparis* [95]. At present, the biological significance of the inversion observed in *O. wissmannii* var. *eremastrum* remains speculative, and it is unclear whether it represents a fixed structural feature or a rare plastome configuration. If validated, this inversion would represent the first documented case within *Apocynaceae* and could provide a useful system for investigating operon stability, transcriptional flexibility, and plastome evolution within the family. Targeted population-level sampling combined with long-read sequencing will be essential to determine whether this inversion reflects a stable plastome architecture, an isomeric variant, or possible plastome heterogeneity.

Comments 3: Some statements could be slightly refined to stress that conclusions mainly apply to the Arabian *Orbea* taxa analyzed here, rather than to the genus as a whole.

Response 3: Thank you very much for this helpful comment. The Conclusions section has been revised to more clearly emphasize that the interpretations and implications of this study primarily apply to the Arabian *Orbea* taxa analyzed here, rather than to the genus as a whole. The revised text presented in L803-819 is as follows:

5. Conclusions

This study provides the first complete chloroplast genomes of two Arabian *Orbea* taxa—*O. sprengeri* subsp. *commutata* and *O. wissmannii* var. *eremastrum*—and offers new insights into plastome structure, variation, and evolutionary placement of these taxa within the tribe *Ceropegieae*. Despite the limited taxon sampling within *Orbea* (only three plastomes out of over 60 described species), the findings reveal unique structural features specifically in the Arabian taxon *O. wissmannii* var. *eremastrum*, including a flip-flop inversion spanning *ndhG* to *ndhF* and extreme IR expansion, which contribute to plastome size variation, gene rearrangements and gene duplication. The observed structural divergence in this taxon may be a sign of plastome heteroplasmy, prompting further investigation through broader population-level sampling. Although the current data set does not

allow for broad generalizations across the genus *Orbea*, the results highlight the genomic distinctiveness of Arabian *Orbea* taxa analyzed here relative to the only previously sequenced African representative (*O. variegata*). Expanding plastome samples across *Orbea* is essential to capture its full genomic diversity and to clarify evolutionary trends within Ceropegieae. Within this context, the present study lays a foundation for future plastid phylogenomic, DNA barcoding, and conservation research of arid-adapted Arabian *Orbea* taxa.