Revised molecular phylogenetic analysis of *Leucheria* Lag. sensu lato (Asteraceae; Nassauvieae) and implications for morphological and ecological evolution

[updated version 3]

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ABSTRACT

In a preceding work, I reanalyzed published ribosomal and plastome DNA sequence data for selected species of *Leucheria* Lag. and related Nassauvieae (Asteraceae). I reported that the genus *Polyachyrus* Lag. is phylogenetically nested within *Leucheria*, hence I transferred species of the former into the latter. I also demonstrated that the monotypic *Oxyphyllum* Phil. pertains to the *Leucheria* crown clade, and that the available data do not resolve its relation within this group. The present work analyzes all of the available sequence data for the same loci, viz. the nuclear ribosomal DNA internal transcribed spacer region (ITS) and plastome (cpDNA) sequences of the *rpl32-trnL*(UAA) intergenic spacer (*rpl32-trnL*) and the *trnL*(UAA) intron plus *trnL*(UAA)-*trnF*(GAA) intergenic spacer (*trnL-trnF*). I report here that the previous phylogenetic analysis of these data was based on an evidently unedited computer-generated multiple sequence alignment of unedited and untrimmed computer-generated chromatograph reads from low quality sequence chromatographs. This yielded a highly erroneous and poorly resolved phylogenetic tree topology whose branch lengths likewise were highly distorted. Nonetheless, in a subsequent phylogenetic comparative analysis of *Leucheria* morphology and ecology by the same author group, this poorly resolved and erroneous topology and its branch lengths were presumed to be *perfectly* accurate. Here, I discuss implications of the present results on the interpretation of morphological and ecological evolution and also on the accuracy and integrity of biodiversity research.

Key words: *Leucheria*, *Polyachyrus*, *Oxyphyllum*, Asteraceae, Nassauvieae, phylogenetics, molecular systematics.

[CITATION: Hershkovitz, M.A. 2024. Revised molecular phylogenetic analysis of *Leucheria* Lag. sensu lato (Asteraceae; Nassauvieae) and implications for morphological and ecological evolution. EcoEvoRxiv https://doi.org/10.32942/X2NC9Q]

Introduction

Leucheria Lag. Lag. (Asteraceae; Nassauvieae) until very recently had been conceived as a genus including ca. 46 species of southern South America (Crisci, 1976; cf. Rodríguez et al., 2018), though Katinas et al. (2022) consolidated these among 26 species and recognized three more. Jara-Arancio et al. (2017; including M.T.K. Arroyo; hereafter Jara2017) published a phylogeny of *Leucheria* based on DNA data and using Bayesian maximum likelihood (ML) phylogenetic estimation (henceforth BE). They published a cladogram representing BE results from concatenated DNA sequences of three loci, the nuclear ribosomal DNA internal transcribed spacer region (henceforth ITS) and plastome (cpDNA) sequences of the *rpl32-trnL*(UAA) intergenic spacer (henceforth *rpl32-trnL*) and the *trnL*(UAA) intron plus *trnL*(UAA)-*trnF*(GAA) intergenic spacer (henceforth *trnL-trnF*). They reported that *Leucheria* comprised two major clades, one (Clade A) comprising (sub)acaulescent cold-steppe/steppe species, and

the other (Clade B) comprising caulescent species polymorphic for habitat. Clade B, in turn, comprised three subclades. They also concluded tentatively that the caulescent form was ancestral in the genus.

Using the same loci, Lavandero et al. (2020; including M.F. Pérez; henceforth Lavandero2020) undertook another phylogenetic analysis, the results of which they did not report in detail, since they focused on interspecific relations of a new species pertinent to Clade A. They did, however, find support for the clades and subclades reported by Jara2017. But they also remarked, without elaboration, that their Clade A results differed "slight[ly]" from Jara2017 at the interspecific level.

Earlier, Pérez et al. (2020; including Jara-Arancio, Arroyo, and Lavandero; hereafter Pérez2020) undertook phylogenetic comparative statistical analysis of morphological and ecological evolution among 34 species of *Leucheria*. I refer to this as a molecular phylogenetic "derivative" study because it uses a molecular phylogeny as a scaffold for statistical analysis of the evolution of something *else*, such as phenotype, ecology, or biogeography. Henceforth, I use the acronym MPE to describe the discipline that includes molecular phylogenetics *and/or* its derivative *evolutionary* studies. In this case, Pérez2020 used the Jara2017 topology with nine terminals removed. The Jara2017 topology also was reproduced by Katinas & Forte (2020) to map evolution of inflorescence characteristics in the genus. While Lavandero2020 reported topological disagreements between their reanalyzed phylogeny and that used in Pérez2020 (including Lavandero), they did not comment (then or since) on the implications for Pérez2020's results, which would be highly sensitive to both topology and branch lengths.

The work of Pérez2020 called my attention because of the theoretical overlap with my own studies of morphological and ecological evolution of South American Montiaceae. In fact, I had read previously both Jara2017 and Pérez2020 (cf. Hershkovitz, 2021a: 3). I had considered the analyses in these to be based on somewhat uncritical results of downloaded analytical software, but this would not, per se, invalidate the results.

But returning to Pérez2020, I noticed something peculiar. Pérez2020 claimed that their phylogeny, copied (poorly) from Jara2017, was completely resolved except at one node. Comparison of the Jara2017 and Pérez2020 topologies revealed to me that 18 of the nodes qualified as resolved in the Pérez2020 topology were supported at unacceptably low BE posterior probabilities (PPs) in the Jara2017 topology (see later discussion). Pérez2020 did not mention this, even though Jara-Arancio and Arroyo were coauthors. Assuming these authors had even the most basic understanding of molecular phylogenetics, they ought to have appreciated that even a *random* tree topology is *fully* "resolved." But a random tree is hardly a robust phylogenetic estimate. In any case, these observations alone would seem to invalidate the Pérez2020 analysis, at least to the degree that phylogenetic comparative analysis indeed *depends* upon phylogeny. I discuss this work further below.

But, this aside, my reexamination of the Jara2017 topology itself then piqued my curiosity for several reasons. First, they illustrated only a cladogram and not a phylogram that would show divergence. This would have helped in interpreting the results, e.g., whether poorly resolved nodes involved shorter or longer branches. Second, they illustrated and described results only for *combined* nuclear and plastome sequences. More often than not in sizeable genera, nuclear and plastome sequence phylogenies manifest some degree of incongruency owing to, e.g., hybridization and lineage sorting. This has been known since the dawn of modern molecular phylogenetics in the early 1990s. Jara2017 briefly acknowledged the existence of such incongruencies in their data, but they did not describe any specific examples, and they dismissed the possibility that these significantly affected their final result.

But they did include in the supplemental data files the alignment used in the analysis and cladograms (not phylograms) of the results for each locus analyzed separately (Jara2017: Online Resources 1–4). Studying these, I found that the individual locus trees, indeed, manifested multiple

"significantly supported" topological incongruencies, viz. with ≥ 0.95 PP. Of course, this further undermines the validity of the Pérez2020 analysis. But perhaps this validity required no further undermining, since the combined data topology was poorly resolved statistically in any case. Third, the analysis relied *exclusively* on BE, which, however "popular," remains theoretically poorly understood and also is prone to artifacts that have been reported repeatedly since the late 1990s (see Appendix 1 and Hershkovitz, 2021b).

Since gene tree incongruence is another theme of interest to me (e.g., Hershkovitz, 2021b), I decided to download and study the alignment included in the supplementary data of Jara2017 (Online Resource 1). I was so dumbstruck that I thought they must have uploaded the wrong file, because no *minimally* competent molecular phylogeneticist would have used *this* alignment for phylogenetic analysis. In particular, *simple* inspection that revealed most sequences for all three loci included varying lengths of extra-locus 5' and 3' sequence, often 50 but up to more than 200 bases long. These extra-locus regions were "noisy" and very poorly aligned. While alignment was much better within each locus, there were a substantial number of obvious misalignments, especially involving plastome sequences with large deletions in some species. Moreover, I found several cases of *infralocus* incongruency, i.e., where different portions of the *same* locus or sequence mapped to different clades. This is something I had never encountered in the plant molecular systematics literature dating to its inception. Wow. I also found many of the sequences to be poor quality as evidenced by regions of random sequence, sequence "smear," and numerous evidently spurious insertions or deletions.

Although Jara2017 reported that they had edited their alignment using an appropriate tool, the alignment and individual sequences suggest otherwise. The data suggest that unedited, untrimmed data from computer-read low-quality sequence chromatographs were loaded into an unspecified sequence alignment program, and that this unedited alignment was loaded into a BE phylogenetics program. Furthermore, some of the sequences suggest that either the sequencing samples were contaminated or their sequence files mixed. This may be consequent to the high proportion of DNA samples (30/44) that were derived from herbarium (viz. museum) specimens.[1] Compared to properly preserved fresh specimens, museum specimens often yield low DNA quality and quantity. Jara2017 described no protocols for special handling and processing of museum specimens. Indeed, the PCR protocol evidently failed to amplify 1–2 loci in many of the specimens, as these sequences are missing from the alignment.

In Hershkovitz (2024a), I published a partial molecular phylogeny of *Leucheria* using mainly the sequences published by Jara2017 but edited and realigned. For each locus, that alignment trimmed the 5'- and 3'-ends of the Jara2017 alignment to the limits for which clean sequence was available for almost all sequences and appeared to be unequivocally alignable at that limit. The purpose of that work was to demonstrate that the genus *Polyachyrus* Lag.[2] was nested within *Leucheria* and that the genus

[1] This, itself, is unusual. Herbarium specimens might yield satisfactory DNA samples, but often they do not, as evident in Jara2017. To obtain high quality and uncontaminated DNA, researchers prefer fresh samples that have been thoroughly washed and lyophilized. Herbarium specimens usually are a last resort for specimens that would otherwise be too difficult or expensive to obtain in the field. Jara2017 used herbarium specimens for some taxa not especially difficult to obtain from the authors' collective locations.

[2] Hershkovitz (2024a, b), per current conventions of phylogenetically-correct taxonomy, recombined in *Leucheria* the names of accepted (sub-)species of *Polyachyrus*. This was to minimize the number of disadvantageous and/or unnecessary nomenclatural changes, hence maximize taxonomic stability. In particular, the generic names had equal priority, but *Polyachyrus* was a much smaller genus with a much more restricted distribution. I recognize the desirability of continued recognition of *Polyachyrus* given its obvious morphological distinctions from *Leucheria*. However, the phylogenetic evidence yields no satisfactory taxonomic alternative. For example, retention of the circumscription of *Polyachyrus* would require splitting *Leucheria* into four genera (viz. Clade A and Subclades I, II, & III of Jara2017). This would require changing a large number of names that have been applied stably for more than a century (cf. Katinas et al., 2022). Moreover, there are no obvious morphological distinctions between the

Oxyphyllum Phil. pertained to the *Leucheria* crown group but that its relation to the *Leucheria* clades and subclades remained unresolved. Jara2017 reported that they were unable to "extract quality DNA from *Polyachyrus*," which itself I found very peculiar, unless this *also* was extracted from a herbarium specimen. Populations of plants of this suffrutescent genus grow on the beach less than 2 hours from Santiago and also in La Serena < 10 km from where one of the Jara2017 coauthors lives and works. But no matter, Jara2017 overlooked DNA sequences available in GenBank for all three loci and reported in a paper they *cited*, Katinas et al. (2008). They also overlooked available sequences from *Oxyphyllum*, reported in a paper they overlooked, Luebert et al. (2009), which reported an intimate relation between this genus and *Leucheria*, confirmed in Hershkovitz (2024a).

The purpose of the present work is to expand upon the analysis of Hershkovitz (2024a) to include all of the samples. A subsequent work will offer consequent insights on the systematics and evolution of *Leucheria*. I also document some of the characteristics of the Jara2017 sequences and alignment that undermine the reliability of its phylogenetic conclusions. Finally, I comment briefly on the significance of the present results in terms of the formal scientific institution in an age ever more dependent upon the reliability of knowledge purported to be "scientific."

Materials and Methods

This work analyzes reported sequences for ITS, *rpl32-trnL*, and *trnL-trnF*. Hershkovitz (2024a) reported that DNA sequences of species of the relevant taxa were downloaded from GenBank. More accurately, most of the sequences were obtained from the alignment in the supplemental data of Jara2017. Comparison of selected sequences with the corresponding documents in GenBank confirmed their congruence, hence the congruence of all of them is presumed. The current analysis incorporated sequences from additional taxa as documented in Hershkovitz (2024a). I excluded sequences of additional outgroups analyzed by Jara2017. Because of their relatively high divergence at these relatively rapidly evolving loci, these sequences were less well alignable with each other and with the alignment used here.

Among the outgroups excluded here and in Hershkovitz (2024a) is *Macrachaenium* Hook.f., which includes a single morphologically peculiar acaulescent perennial herb endemic to cool temperate and subpolar Patagonian forest. Jara2017 reported this genus to be sister to *Spinoliva*, a semiarid to arid zone shrub. I did attempt to include *Macrachaenium* in the alignment, but this proved to be problematic. The *Spinoliva* sequences, although highly diverged from all other sequences in Hershkovitz (2024a), nonetheless aligned with the other sequences very easily and introduced only a few and very minor alignment insertions. Insertion of the *Macrachaenium* sequences, however, introduced numerous and often larger insertions, and a few sequence regions were unalignable. Thus, I excluded it. I will discuss its relations in the forthcoming work.

The sequences were collated and (re-)aligned[3] manually using the BioEdit alignment editor (Hall, 2004), and the alignment trimmed to reduce 5' and 3' ambiguity in some of the sequences. Problematic sequences were either deleted or their problematic regions trimmed, as described in the results. The

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three subclades (Jara2017; cf. Katinas et al., 2022), such that identifying the new genus would require *first* identifying the species according to the *replaced* taxonomy of *Leucheria*. The other problem is that different individuals of one morphological species per Katinas et al. (2022) pertain to two divergent subclades per Jara2017 and the present analysis. Of course, there is no "law" compelling cladistic taxonomy, hence neither the inclusion of *Polyachyrus* in *Leucheria*. But this renders scientific taxonomy rather capricious, since generally molecular phylogenetically-based cladistic taxonomic revision has been accepted and applied in the taxonomy of plants in general and South American Nassauvieae in particular.

[3] https://osf.io/xmnhf/files/osfstorage/663e243c4664da60d3ed6b4a/?pid=xmnhf

manual alignment procedure itself was an intuitive emulation of that codified in the CLUSTAL alignment tool (Thompson et al., 2002).[4] This first groups very similar sequences and then aligns the groups according to phylogenetic relations approximated using a distance tree derived from pairwise sequence similarity, viz. an alignment "guide tree." However, both the local and global alignment procedures are "fixed" at each stage according to preset "penalty" values. The program does not explore alternatives and is not capable of finding the best global alignment. Thus, it provides merely a heuristic first approximation that must be studied and adjusted manually. Jara2017 evidently failed to do this. To illustrate artifacts of the Jara2017 alignment, I juxtaposed critical regions side-by-side and documented the artifacts using the screen capture function. I also examined the alignment and sequences for additional artifacts, as described in the results.

Hershkovitz (2024a) added to this alignment a matrix of phylogenetically informative length-variable regions consequent to phylogenetic sequence insertion or deletion (indels). The present analysis excludes this indel data, because I decided that phylogenetic artifacts consequent to homoplasy at deeper phylogenetic nodes outweighed the value of their phylogenetic signal at shallower nodes.

As in Hershkovitz (2024a), this work applies the taxonomic identifications given in the sequence documents. Most of these are from Jara2017. Katinas et al. (2022) radically modified the species concepts in *Leucheria* such that several of the names applied here are reduced to synonymy. However, DNA sequences corresponding to Katinas et al.'s species are divergent and, more importantly, appear polyphyletic in Jara2017 and the present work. Mainly for this reason, the names used here, corresponding to earlier taxonomies, are retained. However, the taxonomic implications of the present results will be discussed in a follow-up work.

Phylogenetic analysis applied maximum parsimony (MP) and ML methods (see Appendix 1 and Hershkovitz, 2021b) as implemented in PAUP version 4 (Swofford, 2003). MP tree construction and bootstrap analysis (1000 replicates) were undertaken using the default algorithm and parameters, except that the bootstrap analysis was performed using random addition sequence (10 replicates), holding 10 trees at each addition step, with maxtrees fixed at 100. ML analysis used the 6-parameter plus gamma general time-reversible substitution model with correction for estimated base frequencies and among-site rate variation. All parameters were estimated using an MP tree.

In addition to analyzing the corrected alignment, I also performed briefer analyses of the original Jara2017 aligned phylogenetic data set. This was in order to verify whether or not the phylogenetic results of Jara2017 emerged from analysis of the complete alignment data set they made available or only portions thereof. I did not attempt to duplicate their Bayesian analysis, because MP analysis sufficiently answered my question.

Hershkovitz (2024a) analyzed the ITS and cpDNA (*trnL-trnF* and *rpl32-trnL*) data separately and in combination for all taxa represented by sequences for all three loci, excluding certain "problematic" sequences. The present work analyzes a more complete taxonomic sampling, but only presents results for separate ITS and cpDNA. This is in part because of phylogenetic incongruencies between these, which decreases the accuracy, validity, and utility of their combined analysis, as discussed later. But it is also because the separate analyses reincorporates all taxa represented by sequences (again, excluding "problematic" sequences) for one or the other genome but not both. This, again, can introduce artifacts in combined data analysis. Combination of the phylogenetic information from each incomplete data set thus requires a "supertree" approach.

[4] Actually, quite to the contrary, CLUSTAL codifies an intuitive alignment procedure identical to the one I use here. Like all computer programs, CLUSTAL emulates the mind, not vice versa. But, unlike computer programs, the mind is self-correcting.

At the same time, I note that the ITS phylogenetic data includes three species for which only ITS2 sequence is available, even though ITS1 is more variable than ITS2. In two cases, the ITS1 sequence clearly did not pertain to that species; in the third case, sequence upstream of the ITS2 was random noise. But I decided that ITS2 for these taxa was *sufficiently* variable for the present purposes, hence some ITS data was better than none. The cpDNA data set includes some taxa for which only *rpl32-trnL* sequences are available. This is because *rpl32-trnL* is more than twice as variable as *trnL-trnF*, hence adequately represents the cpDNA data. Again, the outgroup *Moscharia* was excluded from the cpDNA analysis because only the less variable *trnL-trnF* sequence was available. But I reported in Hershkovitz (2024a) that separate analysis of the *trnL-trnF* data demonstrates that *Moscharia* is sister to *Marticorenia*, the same as the ITS data shows.

Results

1. Alignment and sequence characteristics

Figures 1–8 compare fragments of the alignment used in the phylogenetic analysis of Jara2017 and that used here. Figures 1–3, 5, and 7 illustrate 5'- and 3'-ends of the alignment of the three loci. The Jara2017 alignment includes variable lengths of poorly and inaccurately resolved and aligned sequences in these regions, whereas in most cases, the sequence in these regions should be highly conserved, hence perfectly aligned and with few site-wise polymorphisms. These figures also show examples of misalignment in the targeted loci regions. But even the corrected alignments show likely chromatographic artifacts in the targeted regions, especially single-based insertions and deletions (indels) that, even if correct, require confirmation.

Figures 3 and 6 highlight major misalignments in the Jara2017 alignment associated with large deletions in some sequences in the cpDNA loci. Such large deletions are fairly common in non-coding cpDNA regions. Computer alignment programs in the CLUSTAL family habitually misalign such regions, because the penalties for gap insertion and gap length are fixed and usually optimized for small indels.

Figure 8 shows an example of a chromatographic artifact present to a greater or lesser degree in several of the sequences. Such artifacts owe to different possible causes. One is disproportions among fluorescent-labeled fragments produced during the Sanger sequencing reaction.[5] Another is chemical interactions within and among DNA fragments, such that, during electrophoresis, some fail to migrate according to their canonical length and thus appear to be underrepresented or even missing from the sequence. The consequence in either case is that the signal of DNA fragments one base longer and/or

[5] For those unfamiliar with the chemistry and physics of Sanger sequencing, e.g., evidently the authors of Jara2017, sequence data are generated from a DNA fragment by a DNA polymerase in a reaction containing a mixture of deoxynucleotides (dNTPs). But the mixture contains also labeled dideoxynucleotides (ddNTPs) that terminate continued *extension* of polymerization. The reaction thus produces a *population* of labeled single-stranded DNA sequence fragments of variable length that represent every possible length of the original sequence from its origin up to the point that the labeled ddNTP was incorporated (viz. N+n-ddNTP, N+n+1-ddNTP…). This population of length-variable fragments is then subjected to electrophoresis, which sorts them according to their specific length. Because the length of each labeled fragment corresponds to the number of bases between the sequence origin and every possible termination point, the original sequence can be determined according to the *order* in which each fragment migrates. But sequence is poorly resolved at the beginning of the sequence and beyond the sequence length for which fragments can be distinguished electrophoretically. This poorly resolved sequence at each end must be manually deleted from chromatographic output. Possibly Arroyo neglected to explain this to her PhD student Jara-Arancio when she trained her in molecular systematics theory and methods.

shorter superimposes over the weak signal in between, such that the computational base reader reads the adjacent base twice. Other artifacts cause the reader to either miss a base or insert an extra base in the sequence. These appear in the alignment as spurious single-base insertions and deletions.

While I was greatly able to improve the alignment of Jara2017, one problem that I could not overcome was poor sequence quality and other artifacts, and these affected downstream phylogenetic analyses. Some problems were more serious than others. The numerous likely spurious single-base indels concentrating especially towards both ends of the sequences were not so problematic, because they only aligned with themselves, and thus amounted to "missing data" in the phylogenetic analyses. However, many sequences also manifested unexpected substitutions in these regions, and these require chromatograph analysis to verify. The smeary *rpl32-trnL* sequence of *L. polyclados* Reiche however, proved to introduce severe branch attraction in the phylogenetic analysis, so I had to simply discard it.

Some sequences manifested indications of PCR contamination. The ITS sequences of *L. congesta* D.Don and *L. senecioides* Hook. & Arn. were "hybrid," though not in a biological sense. I found that the ITS1 portion of both sequences of these Subclade III species had high identity with a certain Clade A sequence, while the ITS2 portion pertained as expected to Subclade III. I also found a similar case in the cpDNA data. The *trnL-trnF* sequence of the Subclade III species *L. gilliesii* Hook. & Arn. corresponds to Subclade III. But the *rpl32-trnL* sequence has high identity with Clade A species. This also represents an infralocus difference, because cpDNA is inherited as a single locus.

While a truly recombinant infralocus ITS or cpDNA recombination is not out of the question theoretically, and such can be biotechnologically created, I am not aware that any such recombinants ever have been found in nature. My impression is confirmed by world authority on plant hybridization Loren Rieseberg (written comm., 2 April 2024). But given the poor quality of the Jara2017 sequences, alignment, and phylogenetic analysis, most likely these represent researcher error.

The "recombinant" ITS sequences possibly represent PCR contamination combined with poor quality/quantity of sequencing template. But another possibility is errant merging of chromatograph files of low-quality forward and reverse sequencing reactions, neither of which succeeded in spanning the entire region. Evidence for this includes the ITS sequence of *L. suaveolens* Druce, which is much shorter than the other sequences. But the 5' portion of this sequence is random noise that, per a BLAST search (Camacho et al., 2009), corresponds to no known sequence. The 3' portion includes all of the ITS2 sequence, and this maps to the proper phylogenetic position. This thus probably represents a low quality reverse reaction (i.e., extended using the 3' primer) that failed to extend upstream of ITS2. And probably the forward sequencing reaction did not work at all, explaining the shortness of the published sequence. In the case of the "recombinant" cpDNA locus in *L. gilliesii*, this owes either to contamination or mixing of samples or files.

As noted above, while I corrected the Jara2017 alignment to the degree possible without studying the original chromatographs, the corrected alignment probably *still* includes some spurious base calls. Evidence for this emerged in the phylogenetic analysis, as discussed below.

2. Phylogenetic analytical results

a. Phylogenetic reevaluation of the Jara2017 DNA sequence alignment. To verify whether the Jara2017 trees reflected analysis of the complete alignment they made available, I performed MP bootstrap analysis of the Jara2017 ITS and *rpl32-trnL* loci separately. This is because these regions are the most variable, and because the corresponding Jara2017 trees included, besides their topology, several branches supported by high PPs. For each locus, I bootstrapped the Jara2017 alignment (200 replicates)

and, separately, each *original* alignment but "trimmed" to the same 5' and 3' limits of the corrected alignment used here and in Hershkovitz (2024a).

The results (not shown) rendered very clear that the Jara2017 trees were derived using the complete alignment of Jara2017 (Online Resource 1), i.e., included the expansive poorly aligned and otherwise "noisy" flanking regions. In particular, the bootstrap consensuses using the complete alignments were highly compatible with the Jara2017 trees and shared the same spurious relationships. Many, but not all, of these spurious relationships were absent in the bootstrap consensuses of the trimmed alignments. As expected (see discussion below), bootstrap values were not especially high, but nonetheless sufficiently high as to anticipate that, in BE analysis, the corresponding PPs would approximate those reported in the corresponding Jara2017 trees. More importantly, the consensuses were strikingly different, with the *original* alignment consensuses highly compatible with the corresponding Jara2017 topologies, and the *trimmed* alignment consensuses incompatible with Jara2017 and more similar to trees derived with the maximally corrected alignment used here. Some incompatibilities persisted between the Jara2017 trimmed alignment trees and the maximally corrected alignment trees presented here. This is because trimming the noisy ends of the Jara2017 alignment corrected some but not all of the alignment errors and artifacts.

To appreciate the impact of the contribution of error to the Jara2017 trees, I computed the number of phylogenetically informative sites for each of the three alignments. For the *original* Jara2017 alignment, the total was 646, for the *trimmed* alignment, 363, and for the maximally corrected alignment used here, 257. The differences, of course, are apportioned among the three loci. These differences have considerable significance. Phylogenetically informative sites are alignment positions where two or more sequences share bases distinct from those of the rest of the sequences. These influence directly the estimation of phylogenetic relations using *any* method. Uninformative sites are either constant in all sequences or variable in only one of them. The former do not influence estimated relations, while the latter might have an indirect influence by affecting branch lengths calculations in ML and distance methods. Axiomatically, of course, the number of informative sites correlates with the number of variable but uninformative sites, so the latter statistic can be ignored for purposes of the present comparison.

In summary, MP strict and bootstrap analysis of the separate and combined loci in the aligned data set made available in Jara2017 (Online Resource 1) generally agreed with their Bayesian topologies. More importantly, the MP analyses reproduced the topological incongruencies between their topologies and those presented here. This reanalysis thus demonstrates that: **(1)** the Jara2017 alignment included, besides other artifacts, considerable lengths of 5' and 3' poorly or unaligned sequence and chromatographic artifacts (Figures 1–3, 5, 7); **(2)** the phylogenetic trees in Jara2017 were generated using these termini; **(3)** these termini include 44% of the phylogenetically informative sites in the Jara2017 analysis; and **(4)** the phylogenetic "misinformation" in these termini yielded spuriously high PP branches in the Jara2017 trees.

b. Results of the present phylogenetic analysis.[6]

Results of the phylogenetic analyses obtained here are illustrated in Figures 9–12: Fig. 9 shows the ITS MP bootstrap consensus; Fig. 10 shows one of 13 ITS ML trees with equal likelihood; Fig. 11 shows the cpDNA MP bootstrap consensus; and Fig. 12 shows the single cpDNA ML tree.

[6] I refer here again to Appendix 1 and Hershkovitz (2021b) as the basis for my interpretations. Accordingly, in the discussion below, I use the term "significant" in the generic and not the statistical sense. For bootstrap analyses, I regard ≥ 70% bootstrap support as significant. For BE, I consider the cutoff to be 0.95 PP (see Appendix 1). Jara2017 illustrated values ≥ 0.90 but did not explain why they chose this value.

Beginning with the ITS results, the MP bootstrap consensus (Fig. 9) supports *Leucheria* Clade A of Jara2017, but not Clade B. It does, however, separately support Clade B Subclades I–III. Also, the consensus does not confirm the inclusion of *Polyachyrus* within *Leucheria*. It also shows *Marticorenia* and *Moscharia* as outgroups of a clade comprising *Leucheria*, *Polyachyrus*, *Oxyphyllum*, and *Spinoliva*. These results are the same as Hershkovitz (2024a) found for ITS. Hershkovitz (2024a) explained this as a consequence of variable GC frequencies among the clades, such that in the MP strict consensus and many bootstrap trees, the high GC taxa *Oxyphyllum* and *Spinoliva* were, along with *Polyachyrus*, attracted to the high GC Subclade I.

In fact, monophyly of Clade B in Jara2017 was supported with 0.91 PP, which I consider to be insignificant, and relations among the subclades were supported at an unspecified PP < 0.90. However, Lavandero2020, based on a *realigned* combined data set, later reported monophyly of Clade B supported with 1.0 PP and 100% ML bootstrap. This data set is not available. They also showed Clade B as comprising *two* subclades, not three, each well supported by PP and ML bootstrap %. I presume that that one of these two subclades comprises Subclades I and II of Jara2017, because this is consistent with the present results, though not well supported.

Meanwhile, the ML consensus shows the relation in Fig. 2, with *Oxyphyllum* and *Spinoliva* as outgroups of *Leucheria*, while *Polyachyrus* is included, though as sister to Subclade I. But Hershkovitz (2024a) suggested that the short branch length supporting the last relation would not withstand an ML bootstrap analysis, such that the relations between Subclades I and II and *Polyachyrus* would remain poorly resolved. However, since Bayesian analysis estimates not per se the probability of the tree, but rather the probability that the tree is the ML tree per the (inadequate) model (Hershkovitz, 2021b), the resolution shown in Fig. 10 might well be supported by significant PP values following Bayesian analysis. But this would not make it "true."

Aside from supporting Clade A and the Subclades, the results otherwise correspond poorly to the ITS results of Jara2017 (Online Resource 2). The latter I consider to be poorly resolved, with only five branches supported by PP ≥ 0.95, three of which conflict significantly with the present MP bootstrap consensus. In fact, this *overall* low degree of PP support itself is unusual, because BE trees usually show a higher proportion of ≥ 0.95 PP branches than bootstrap trees do ≥ 70% branches. This, again, is because the PPs represent the probability that the BE tree is the ML tree, whereas the bootstrap approximates uncertainty in the underlying data (Appendix 1).

In contrast, the MP bootstrap shows 14 branches supported by ≥ 70%. Three of these support Clade A and Subclades II and III (the two Subclade I sequences are identical), and one supports an outgroup clade. The remaining 10 strong bootstrap values support relations within the *Leucheria* clades. I add that possible artifacts owing to GC base bias that I reported in Hershkovitz (2024a) are not an issue for these last 10 bootstrap values, because the biases are among clades, not within them.

The cpDNA results (Figures 11 & 12) likewise are in agreement with Hershkovitz (2024a). These trees represent results of the combined cpDNA analysis, with values indicated for analyses using *rpl32-trnL* and *trnL-trnF* alone. The combined and separate bootstraps are largely compatible, with a notable exception described below.

In Fig. 11, Clade A and each of the Subclades is strongly supported, with *Polyachyrus* strongly supported as sister to Subclade II. However, as in Hershkovitz (2024a), neither the MP bootstrap nor the ML tree support Clade B and, moreover, do not support exclusion of *Oxyphyllum* from *Leucheria*. These data suggest that the *Leucheria* crown group consists of four lineages whose interrelationships are not resolved, viz. Clade A, each of the Subclades, and *Oxyphyllum*.

Meanwhile, the Jara2017 *rpl32-trnL* tree (Online Resource 4) shows Clade A as polyphyletic, with its members separated by three significantly supported branches. Their *trnL-trnF* tree (Online Resource 3) does not show significant support for Clade A. Within Clade A, their *rpl32-trnL* tree shows two significantly supported branches, one supported in the cpDNA MP bootstrap and one lacking support. Their *trnL-trnF* tree shows four significantly supported branches, *all* in significant conflict with the cpDNA MP bootstrap.

The Jara2017 *rpl32-trnL* tree shows Subclade I as polyphyletic, the two species separated by two significantly supported branches. Their *trnL-trnF* tree shows Clade I as diphyletic but without significant support. It also shows Subclade II as triphyletic with significant support. The remaining monophyletic Subclade II includes five branches supported significantly, though one is trivial in my analysis because the sequences are identical. Of the remaining four branches, two agree and two conflict with the cpDNA MP bootstrap. The Jara2017 *trnL-trnF* tree also shows no significant support for Subclade II, and the single significantly supported branch associated with these species conflicts with the cpDNA MP bootstrap.

The Jara2017 *rpl32-trnL* tree shows Subclade III as diphyletic with significant support. The two components are separated by two significantly supported branches. Otherwise, the Subclade III relations are supported by (effectively) four significant branches. One of these agrees and one conflicts with significant branches in the cpDNA MP bootstrap, while the other two are not supported. The Jara2017 *trnL-trnF* tree does not significantly support Subclade III. The single significantly supported branch also is significantly supported in the cpDNA MP bootstrap.

Subclade III provides the only case in Fig. 11 where the results for combined and separated cpDNA data seem to differ significantly. This Subclade comprises two further divisions. “Division I” comprises three species that Katinas et al. (2022) includes in *L. tomentosa* (Less.) Crisci of Subclade II, thus rendering the latter species grossly polyphyletic. “Division II” includes six species shown in Fig. 11, which Katinas et al. (2022) reduced to five. The combined cpDNA bootstrap strongly supports monophyly of both Subclade III divisions and somewhat less, though still significant, support for monophyly of *all* of Subclade III. The MP bootstrap of the more variable *rpl32-trnL* data strongly support monophyly of “Division I,” < 50% support for “Division II,” and much stronger support for Subclade III than in the combined cpDNA bootstrap. Meanwhile, the much less variable *trnL-trnF* data trivially support “Division I,” because the three sequences are identical, fairly strong support for “Division II,” but < 50% support for Subclade III. In fact, the *trnL-trnF* ML tree and MP strict consensus show “Division I” as the *outgroup* of the remaining *Leucheria* crown, and this relation has 53% support in the MP bootstrap!

To explain these paradoxically contradictory results, I studied the alignment to locate sequence sites that were informative with respect to these taxa. The results are revealing. In the case of *rpl32-trnL*, several sites supported monophyly of Subclade III and “Division I,” but not “Division II.” However, this seemed consequent to the fact that “Division I” sequences share two deletions totaling 263 bases, or slightly more than a quarter of the total alignment. In the deleted regions, “Division II” sequences shared unique substitutions, but since “Division I” sequence was “missing” (viz. deleted), there was no possibility that these sites could support “Division II” monophyly relative to “Division I.” However, they did effectively support overall monophyly of Subclade III, and that is precisely what the results show.

In the less variable *trnL-trnF* data, in contrast, “Division II” monophyly *was* supported at some sites, but these sites were uninformative vis-à-vis the relations of “Division I,” except at *one* site that supported a relation of the latter with Clade A. Otherwise, informative “Division I” sites were shared not specifically or even at all with “Division II.” They were shared with Subclades I and/or II or, alternatively, with *Clade A and the outgroups*. Again, that is precisely what the results show: ambiguous

relations of "Division I." So this is a question of "half-full" versus "half-empty." Yes, adding the *trnL-trnF* to the *rpl32-trnL* data *reduces* the significance of the support for Subclade III relative to support from *rpl32-trnL* alone. Alternatively, adding the *rpl32-trnL* to the *trnL-trnF* data *creates* significant support for Subclade III relative to that from *rpl32-trnL* alone. But there is no real *conflict*. In view of independent strong ITS support for Subclade III, the latter interpretation seems more reasonable.

Two further remarks regarding the cpDNA data. One is the observation that the branches for *C. tomentosa* and *C. viscida* (Bertero ex Colla) Grau & Zinnecker in the ML tree are extremely long. Indeed, high divergence of these sequences is evident upon inspection of the alignment. But, given artifacts evident in so many other Jara2017 sequences, the accuracy of these sequences should be questioned. For example, I excluded the *C. polyclados* sequence from the cpDNA analysis because the *rpl32-trnL* sequence was "smeared" (see above). In preliminary analyses that included this sequence, the consequently long branch substantially reduced bootstrap support at all deeper nodes in the tree. This is precisely the effect of adding, e.g., a *random* sequence or, alternatively, an "*empty*" sequence, to a phylogenetic data alignment. These branches fit anywhere, so support for the *entire* tree is reduced.

The other observation regards the position of *L. rosea* (Less.) Reiche, whose inclusion in Subclade III is strongly supported by the ITS, whereas inclusion in Subclade II is strongly supported by the ITS data. This suggests hybridization. But as it happens, independently obtained ITS and *trnL-trnF* sequences for *L. rosea* (GenBank EF530254.1 and EF530300.1, respectively) *both* are identical to other Subclade III sequences. This does not rule out the possibility that one individual is a hybrid and the other not. But, again, given the overall low quality of the Jara2017 data and analysis, sample mix-up or contamination are highly plausible.

Since Jara2017 did not include a combined cpDNA analysis, I cannot compare the results directly with the cpDNA analyses here. But it is worth noting that: **(1)** neither locus tree in Jara2017 is well-resolved in terms of the proportion of branches supported significantly; **(2)** neither locus tree supports monophyly of Clade A or Subclades I, II, or III; **(3)** *both* trees show one or more of these clades as polyphyletic with significant support; and **(4)** the trees conflict significantly with each other, e.g. the relations of *L. tomentosa* and *L. floribunda* DC.

Hershkovitz (2024a) presented results for combined ITS and cpDNA analysis of a reduced taxon dataset. I do not present such results for the present dataset. The ITS and cpDNA trees are largely but not completely compatible with the smaller combined data tree in Hershkovitz (2024a), viz. many branches are mutually supported or at least are not significantly incongruent. The exception is the clade comprising the species *L. cantillanensis* Lavandero and *L. salina* (R.Rémy) Dusén subsp. *salina*. The relations in the ITS and cpDNA trees are significantly incongruent. Notably, neither MP bootstrap tree shows the relations significantly supported by both BE and ML bootstrap in the combined ITS + cpDNA analysis of Lavandero2020. The latter, however, shows the same topology as the present ITS ML consensus. This is not surprising, since ITS accounts for ca. 70% of the informative variation in the combined data (Hershkovitz, 2024a). Clearly the ITS and cpDNA trees are incongruent for these species. In this sense, the results of Lavandero2020 are positively misleading, because they yield no hint of this incongruency. But given the considerable noise and missing data remaining in dataset, there is no point in dwelling on these points or performing and agonizing over an additional combined data analysis.

Notably, aside from support for the major clades, the results of Lavandero2020 depart *significantly* from those of Jara2017, and, as expected, much more closely resemble the ITS results presented here (see above). But this is *contrary* to what Lavandero2020 reported. Lavandero2020 illustrated their interspecific topology *only* for Clade A. For this case, they described the differences between theirs and Jara2017's topology as being "slight." This rather obviously is not correct. The Clade A tree comprises 12 branches, only *three* of which are shared between Jara2017 and Lavandero2020. The

other nine *conflict*, most with high PP. These observations are peculiar, and they will be discussed further below.

Discussion

1. Molecular phylogenetics of *Leucheria*

The present work adds to the previous analysis of the *Leucheria* crown (Hershkovitz, 2024a). At least it demonstrates that the additional sequences do not perturb the earlier conclusions. In particular, it confirms monophyly of four clades recognized by Jara2017, Clade A and the three subclades of their Clade B. However, evidence for monophyly of Clade B is evident only in the ITS MP and ML strict consensuses. It is not supported in the ITS MP bootstrap. The relations among Clade A and the Clade B subclades in the cpDNA MP bootstrap are completely unresolved, although a clade comprising Subclades I and II plus *Polyachyrus* is supported in the cpDNA strict MP consensus and ML tree. For *Polyachyrus* Lag. and *Oxyphyllum* Phil., the present results for cpDNA and ITS are the same as reported in Hershkovitz (2024a). In fact, Clade B was supported in Jara2017 with only 0.91 PP, a value I consider to be insignificant. Monophyly of the subclades was supported with 1.0 PPs. Monophyly Clade B was well-supported by both BE and ML bootstrap in Lavandero2020, but this data set is not available.

In Hershkovitz (2024a), I pointed out that *Polyachyrus* was segregated from *Leucheria* principally because of inflorescence and floral characters, but also its habit, which is more suffrutescent versus the more herbaceous *Leucheria* habit. The leaves in these species also are more succulent and rather more "robust" than leaves of lowland caulescent *Leucheria* species. However, I neglected to mention *L. annua* (I.M.Johnst) Hershk., a smallish annual herb endemic to northern Chile's distinctive coastal fog zone. Its leaves are thin, lax, and subglabrous and thus superficially resemble those of some caulescent *Leucheria* species. But this does not mean that this is the ancestral habit. For example, that zone also hosts the endemic *Alstroemeria graminea* Phil., a diminutive annual species derived within the otherwise perennial *Alstroemeria* lineage (Rougier, 2005), this lineage itself nested among other perennial lineages of Liliales. I suspect, therefore, that the habit and morphology of *L. annua* also are derived, most likely via paedomorphosis. There are not at present DNA sequences of *L. annua* for the loci analyzed here.

As for interspecific *Leucheria* relations, the present results for ITS and cpDNA depart radically from those reported by Jara2017 for the same data. Likewise, relations among Clade A species reported by Lavandero2020, the authors' contrary assertion characterization notwithstanding, also depart radically from those reported by Jara2017 and are compatible with the present results for ITS. Conflicts with Jara2017 owe, in part, to significant incongruencies between the combined and separated data trees in Jara2017. Their combined data analysis significantly supported monophyly of Clade A and the three Clade B subclades, but their individual locus trees variously showed one or more of these clades as either polyphyletic with significant support or at least without significant support for monophyly.

In the present work, the ITS, combined cpDNA, and *rpl32-trnL* analysis showed significant support for monophyly of all four clades. Not entirely unexpectedly, the much less variable *trnL-trnF* data did not, but it did not show conflicting relations either. In any case, the Jara2017 locus trees effectively show very different relations for species *within* the four clades, and this probably explains differences between interspecific relations supported in their combined data tree versus those in the present work. For example, both the ITS and cpDNA trees show a significantly supported clade comprising the samples of *L. menana* J.Rémy, *L. cerberoana* J.Rémy, and *L. cumingii* Hook. & Arn. The Jara2017 combined data tree significantly supports polyphyly of this clade. The present finding is significant taxonomically. Katinas et al. (2022) considers all three of these species to be synonyms of *L. tomentosa*. *Leucheria tomentosa* sensu Katinas is polyphyletic in both Jara2017 and the present work, with a clade of three

included species pertaining to Subclade III and the remainder to Subclade II. But the present analysis suggests that the Subclade II species might be monophyletic rather than polyphyletic, as indicated in Jara2017.

As noted, Jara2017 did briefly mention disagreements between their locus trees, and they parroted a list of well-known possible causes, e.g., hybridization. They then asserted that high PPs in the combined data tree for branches less well supported, absent, or significantly conflicting in the locus trees were consequent to expected amplification of phylogenetic signal when the data sets were combined. But they did not demonstrate this analytically, and evidently they did not consider the possibility of *error*. One thus is left with the impression that the combined tree itself is accurate.

But their argument is specious for two reasons. First, it seems to me well-known for decades that combination of phylogenetic data sets yield *four* different possible outcomes. One is that, indeed, the "true" underlying signal might emerge from the combined data. Alternatively, data combination might support one or the other relation in the separated data. This is evident in Lavandero2020, as described above. Here, the results of the combined data set support the relations derived from the ITS data alone. They are incongruent with relations strongly supported by the less informative cpDNA data alone. However, the combined data analysis of Hershkovitz (2024a) showed the relations supported by the cpDNA data alone. The difference might owe to the different methodology, perhaps a different sequence alignment, or the different complement of sampled taxa. It does not matter. The results demonstrate that the phenomenon occurs.

The other possible result is a taxon whose *incongruent* relations are strongly supported in separate data, but a *different* relation appears in the combined data analysis. An example is the relations of *L. rosea* in Jara2017. As I noted above, Jara2017 failed to notice that the data were "hybrid," whether the result of real hybridization or error. In any case, in the combined data analysis, the taxon is placed in a position rather different than *either* the ITS or cpDNA trees (as articulated further below).

Another source of spurious support for clades in Jara2017 arises from misalignment. For example, Fig. 6 shows three sequences that share a common long deletion. Not surprisingly, the three taxa are closely related. But in Jara2017, support for this partition is reinforced by spurious "substitutions" in the misaligned portion.

In summary, Jara2017 recovery of *some* clades in their combined data but not separate data analysis may indeed reflect primarily phylogenetic signal amplification. But this does *not* reflect, as the authors suggested, amplification of weak but otherwise compatible signal in the separate data sets. Rather it is consequent to the *de*-amplification of *error* that the authors themselves *created*. But, even so, this is a red herring, for *two* reasons. First, *other* clades supported in Jara2017, and the overall topology itself, *remain* erroneous, and this clearly is *not* consequent to amplification of weak but otherwise compatible signal in the separate data sets. So their explanation is not merely incorrect, it is *post hoc*. It cannot be used as evidence for clades that turn out to be true while discarding it as evidence for similarly supported clades that turn out to be *false*.

2. Implications for morphological and ecological evolution of *Leucheria*

For purposes of a phylogenetic comparative analysis, Pérez2020 (including Jara2017 authors Jara-Arancio and Arroyo) used an unpublished/undocumented phylogram of the inaccurate and poorly resolved Jara2017 combined data topology as though it were both perfectly accurate and resolved. Well, it was not *exactly* the Jara2017 topology, and, while it was "resolved," it was not *well-supported* (see above). Relative to Jara2017, the tree shows a rearrangement of a terminal triplet of species that vary in

the very phenotypic characteristics that Pérez2020 analyzed. These include two species that Pérez2020 classified as warm-lowland (*L. amoena* Phil. and *L. hieracioides* Cass.) and one as cold-steppe [*L. gayana* (J.Rémy) Reiche]. The Pérez2020 tree shows the former as sister within a primarily cold-steppe clade, hence representing a single habitat transition. But Jara2017 shows the cold-steppe species as sister to only *one* of the warm-lowland species. This would require *two* habitat transitions.

The Pérez2020 tree includes 34 of the 45 taxa in the Jara2017 tree. They pruned from the latter branches of species they did not sample for the analyzed morphometrics characteristics. They reported that one node in their 34-taxon tree was unresolved, and they resolved it arbitrarily for analytical purposes. It is not clear which node this was, because the Jara2017 tree for the pruned taxa is completely resolved. The Jara2017 tree includes one unresolved tritomy, but one of the taxa was not included in the Pérez2020 tree, such that the original node became bifurcate.

Pérez2020 reconstructed habitat evolution among species in their tree. It is not clear why they used the pruned tree for this reconstruction, since this involves only geography and not the morphometrics characteristics. Inclusion of most of the excluded taxa would not have affected their reconstruction, but two of the excluded taxa would have included two additional transitions from cold-steppe to warm-arid in Subclade II. This in turn would have rendered more ambiguous the ancestral habitat of Subclade II, with additional reconstructive repercussions at deeper nodes.

Hershkovitz also pointed out that Jara2017's and Pérez2020's conclusions could not have been based upon the Jara2017 phylogeny, because the relevant relations of both ingroup and outgroup taxa were not resolved. Also, Pérez2020 does not seem to have even evaluated alternative scenarios by performing reconstructions with an outgroup included, in particular each of the Jara2017 outgroup taxa (*Moscharia* and *Marticorenia*) separately, one of which is cold-steppe and the other warm-lowland. But, notably, *neither* of these outgroups is acaulescent.

Thus, while Pérez2020, like Jara2017, concluded that the ancestral *Leucheria* habitat was cold-steppe and the ancestral life form acaulescent rather than caulescent. Hershkovitz (2024a) concluded that the caulescent habit and warm-lowland habitat were ancestral in *Leucheria*. This conclusion was reinforced by the incorporation of *Polyachyrus* and *Oxyphyllum* into the tree.

It must be emphasized that, for purposes of statistical comparative analyses such as Pérez2020, phylogenetic accuracy and precision is much more critical than it is per se in phylogenetic analysis. The latter is merely an approximation for its own sake. Phylogenetic comparative analysis bases upon not only the genealogy of the species, but also the "amount" of evolutionary history shared with their relatives during the course of their supposed cladogenesis, as well as the "amount" of their independent evolution. These quantities are estimated conventionally using standardized branch lengths of phylograms estimated using gene sequence data. This assumes, of course, and besides the accuracy and precision of branch length estimates, that the phenotypes of interest evolved in perfect synch with, say, a cpDNA sequence. This assumption, of course, is patently absurd. Possible significance of a linear regression coefficient notwithstanding, there is no *biological* cause for such congruence, other than their inherent positivity.

For purposes of reconstruction and statistical comparative analysis, Pérez2020 used an *ultrametric* tree calibrated, using an unspecified method and parameters, from the still unpublished and undocumented phylogram of Jara2017. Such procedures stretch and shrink "jagged" phylogram branches (e.g., Figures 10 & 12) proportionally such that the distance from the root to each terminal tip is equal. But in this case, this *ultrametric* tree was derived using an incorrect phylogeny based on poorly aligned, incomplete, and otherwise "dirty" sequence data, moreover combining data from loci whose individual trees, in *that* analysis, were significantly incongruent. For this reason, among *others*, Pérez2020 must be

considered invalid. But for additional statistical and epistemological reasons, I would regard the work as invalid even if their ultrametric tree were absolutely accurate.

But a peculiar feature of the Pérez2020 ultrametric tree is the disproportionate length of the terminal relative to internal branches. For statistical comparative analytical purposes, this means that phenotypic differences among the modern species are relatively ancient. But this seems to be an artifact of the inaccuracy of the Jara2017 misalignments and sequence errors, demonstrated in Figures 1–8. Much of the variation in the combined Jara2017 alignment corresponds to the six noisy and misaligned untrimmed 5' and 3' portions for each of the three loci. Additional significant variation is introduced by the by the other artifacts: **(1)** spurious infralocus "recombinations;" **(2)** interlocus incongruencies, whether accurate or inaccurate, because they distort both phylogenetic relations and evolutionary distance estimates in combined locus analysis (see above); and **(3)** spurious distances consequent to misalignment and sequence inaccuracy. Collectively, these artifacts will increase estimated evolutionary distance between *sister* species that are actually not so or even at all *truly* divergent genetically. And this will render their terminal branches spuriously long, which, in turn, will distort the results of phylogenetic comparative analysis.

As an example of the preceding, consider the relations of *L. rosea* in Jara2017 and in the ultrametric tree of Pérez2020. There, this "cold-steppe" species situates in the basal divergence of "Division 2" of Subclade III (see above). This is a consequence of interlocus incongruence in this sample, viz. the ITS sequence is nearly identical to certain Subclade III species, while the cpDNA sequences are nearly identical to certain Subclade II species. Thus, in either tree, the terminal branch is very short to nil. In the combined analysis of Jara2017, this leads to an irrational result, in which the relations of this taxon differ from that inferred from *either* locus separately. The consequence is that, in the Pérez2020 ultrametric tree, its phylogenetic relations are not only wrong, but its divergence appears to be "ancient" rather than very recent. Its branch is exceedingly but *spuriously* long. The separate locus trees situate this species towards the "tips" of Subclade II or III, not at the base. As noted, this analytical (viz., not biological) phenomenon is well-known in phylogenetics, but evidently unknown to the authors of Jara2017 and Pérez2020.

But, as reported above, an independently-obtained *trnL-trnF* sequence for *L. rosea* pertains to Subclade III and has highest similarity with the Jara2017 *L. gayana* sequence. The ("de-noised") Jara2017 ITS sequences for these species are identical. In any case, removing the evidently spurious *L. rosea* branch from the Pérez2020 tree would redistribute the "weight" of the depicted cold-steppe Subclade III ancestry in the direction of warm-lowland. Returning to the Pérez2020 tree, as noted above, the position of *L. gayana* differs from that of Jara2017. As noted, correcting the arrangement also would shift weight towards an ancestral warm-lowland habitat (see also Hershkovitz, 2024a).

Just as importantly, Figures 10 and 12 render clear that in the corrected alignment, interspecific sequence divergences within clades are mostly very small. For each locus, many species have identical sequences. Thus, I consider highly probable that, with additional sampling, even the small observed sequence differences between many species would prove to be variously shared interspecific polymorphisms (e.g., as in Hershkovitz, 2006; see especially Hershkovitz, 2021b). Correcting for the artifacts listed above, a corrected combined-data ultrametric tree for *Leucheria* should show rather short terminal branches and several instances of habitat transition occurring over *very* short evolutionary distances rather than the spuriously long ones indicated in Pérez2020.

Figures 10 and 12 still show a few taxa having inordinately long terminal branches compared to closely related taxa. I emphasize again that, while I corrected the alignment and removed from it clearly spurious sequence, I cannot vouch for the complete accuracy of the remaining sequences. Indeed, a few, upon simple inspection of the alignment, manifest numerous base differences from closely related

sequences. Interestingly, these differences seem to concentrate in 5' and 3' regions, precisely those that seem to be otherwise problematic. As I noted, I did not include *L. polyclados* in the cpDNA analysis, because the *rpl32-trnL* sequence (there is no *trnL-trnF* sequence) was so highly and obviously incorrect that its inclusion in the analysis greatly reduced bootstrap values of branches supporting the major clades. Its branch of course, was exceptionally long. This simply confirms empirically my conclusion above that both the Jara2017 tree topology and, even ignoring topology, the ultrametric tree branch lengths in Pérez2020 are highly erroneous.

As noted above, Lavandero2020 reanalyzed the Jara2017 data and published a partial phylogeny that showed only relations among Clade A species. They reported that this showed only "slight differences" with the Jara2017 Clade A topology. But as I noted above, simple inspection reveals that the topologies are 75% incongruent and that the conflicting branches are strongly supported in the respective analyses. In other words, Lavandero2020 does not show support for relations poorly resolved in Jara2017, but *contrary* relations *strongly* supported in Jara2017. Also as noted, the Clade A relations in Lavandero2020 are highly compatible with the present ITS Clade A results. Based on this, it seems highly likely that the *same* would be true for their *unreported* results for Clade B. In any case, with or without the Clade B results, this means that Lavandero2020 *also* invalidates Pérez2020, since the latter was based on Jara2017. But Lavander2020 did not mention Pérez2020, which is very peculiar, if not disturbing (see below).

Conclusions

The present phylogenetic analysis of *Leucheria* represents a great improvement over the error-ridden and nonrigorous analysis of Jara2017. Still, it remains inadequate owing to uncorrectable inaccuracies in the Jara2017 data, as well as missing data for several taxa, and still inadequate taxon sampling given both the taxonomic complications introduced by Katinas et al. (2022) and, in general, the manifest inadequacy of single per species sampling for molecular phylogenetic purposes (Hershkovitz, 2021b).

The errors in Jara2017 owe to those at essentially every step of the molecular phylogenetic laboratory and analytical process, viz.: **(1)** excessive reliance on herbarium material for DNA sampling; **(2)** consequent low-quality DNA preparations that failed to amplify one or more loci in several sampled taxa; **(3)** poor quality DNA sequencing template; **(4)** failure to read and edit sequence chromatographs and recognize their poor quality and consequent artifacts; **(5)** failure to trim chromatograph 5' and 3' noise prior to sequence alignment and consequently including misaligned and noisy sequence in the phylogenetic data set; **(6)** failure otherwise to edit the alignment and detect numerous and fairly obvious by-eye alignment errors; **(7)** exclusive reliance on problematic BE phylogenetic analysis and failure to apply resampling techniques; **(8)** publication only of a *combined* nuclear and plastid loci tree, hence concealing incongruence of the separate data trees; and **(9)** effectively *ignoring* numerous significant incongruencies by merely speculating but not critically analyzing their underlying causes. Moreover, the authors failed to note that *Polyachyrus* sequences for all three loci were reported in a 2008 paper they cited, and they overlooked a 2009 paper that reported the *Oxyphyllum* sequences and demonstrated their close relation to *Leucheria*.

In summary, it is difficult attribute Jara2017's results simply to unfortunate errors and omissions. It is difficult to escape a conclusion that the work was undertaken by researchers *not remotely qualified* to carry out even the most *basic* field, laboratory, bibliographic, and analytical procedures of molecular

phylogenetics research[7] or, if otherwise, negligent, to say the least, for putting their names on the publication without thoroughly inspecting the results. After all, despite whatever it says in fine print in the "author contribution" disclaimer, for practical purposes, *every* coauthor receives *full* credit as though they were the *sole* authors. In other words, *all* authors are *fully* responsible.[8] If my opinion here is mistaken, I respectfully request correction.

My assessment might be judged as unfair given that Jara2017 did, after all, discover the major clades within *Leucheria* (viz. Clade A and Clade B Subclades I, II, & III, with evidence for monophyly of Clade B itself tantalizing but inconclusive). One might say that the authors simply made honest mistakes. I am sympathetic, since I often make mistakes in my published works. But these are of the absent-mindedness or editorial sort, I do not have the benefit of coauthors, reviewers, and editors to help preempt them, and I regularly report and correct my errors and omissions in follow-up work. I probably have made several such errors here.

But more to the point, the data used in molecular phylogenetic analysis, the DNA sequences, are produced by the *organism* and not the researcher. Organisms possess DNA sequences and, per molecular evolutionary theory, these can aid in reconstructing phylogenetic relations. In other words, nominal "phylogenetic signal" is an *intrinsic* property of the organism's genome. Researchers are owed no credit for the existence of this phylogenetic signal, only credit for their scientific abilities to extract, process, analyze, and interpret this signal. Nowadays, much of this procedure is automated to the point where producing a molecular phylogenetic estimate per se *requires* no scientific abilities on the part of the researcher.[9] The "science" now is "color by number."

Effectively, the credibility of the clades recovered by Jara2017 owes nothing *scientifically* to Jara2017. Clearly they did not *know* whether or not their sequences, alignment, or phylogeny were correct or incorrect. Some clades are correct, but their credibility owes nothing to *their* analysis. It owes to confirmation in independent *reanalysis*. And, as noted, it owes to the *organisms* themselves that produced the DNA sequences. The same is true for Pérez2020, whose validity rests first and foremost on the accuracy of topology and branch lengths of the tree they used but failed to evaluate *scientifically*. At the same time, it would have been possible for Jara2017 and Pérez2020 to have generated the correct results, even without *scientific* abilities. But the fact that they could not demonstrates that their *scientific* abilities were lacking, viz. they approached the problem purely *methodologically* without the ability or even inclination to *scientifically verify* each methodological result every step of the way.

And besides all of this, Jara2017 and Pérez2020 authors' did not undertake these works out of the kindness of their hearts, or even their dedication to science, lest their work would have been *much* better. Their errors were very well-financed and the authors very well compensated with generous *public*[10],[11]

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[7] Jara-Arancio et al. (2014) might suggest otherwise, but circumstantial and anecdotal evidence indicates that this work is primarily that of a "middle" author whose familiarity with the theory and methods is demonstrated in prior and qualitatively similar publications. Critical analytical comparison of Jara-Arancio et al. (2014) and Jara2017 suggests that these could not have been the work of the *same* person.

[8] Ever since the mid-1980s I have encountered coauthors that disclaim responsibility for grievous errors in their multi-authored publications ("It was *them*, not *me*."), while at the same time using the *same* papers to *compete* for employment and funding and project themselves as *accomplished researchers* on whatever it was that was *really* "them, not me." And they compete very successfully against researchers who are more concerned with the quality rather than the quantity of their publications and also take full responsibility for them.

[9] In Hershkovitz (2024a), I described my discovery vis-à-vis *Polyachyrus* as "accidental." It was in the sense that I neither sought nor expected this result. Nonetheless, this emerged from a patently scientific investigation that began with my *scientific* evaluation of the conclusions of Pérez2020 and effort to *scientifically* verify their results.

[10] Although, former UK Prime Minister Margaret Thatcher eloquently and *accurately* quipped, "*There is no such thing as* ***public*** *money. Only* ***taxpayers'*** *money*."

funds. And they *pursued* these resources within a *highly* competitive science funding system in which there are few winners and many losers. But no matter, for career purposes, Jara2017 and Pérez2020 were and *remain* tangible resume assets for *all* of the authors, viz. towards the objective of career advancement and researcher funding. Criticism does *nothing* to change this, and is therefore completely *innocuous*.

Be "all of the above" as it may, a plot twist consequent to Lavandero2020 should not go unnoticed. The similarity between the Lavandero2020 and my Clade A ITS results suggests the *possibility* that Lavandero2020 *themselves* discovered massive errors in Jara2017…but did not *report* them. They reported that their results basically agreed with Jara2017, even though clearly they did not. In this context, it is notable that Lavandero2020 reported that they constructed a *new* alignment, viz. downloaded 141 sequence documents, performed and edited *three new* alignments, and concatenated the three alignments into a *new* combined data alignment. Why would they go to all this trouble when, in perhaps 60 seconds, they could have simply *added* and aligned their three new sequences to the *existing* Jara2017 (3 X 47) sequence alignment, which they clearly had at hand?

Examination of the timeline of events is revealing. Lavandero2020 reported that the specimen of *L. cantillanensis* was collected on 27 December, 2019. Pérez2020 was submitted for publication on 19 January 2020, accepted 6 May, and published 4 June. Ten weeks later, on 12 August, Lavandero2020 was submitted. Ten weeks is more than enough time to have initiated and completed the work of Lavandero2020. But the specimen was *available* more than four months before Pérez2020 was accepted.

If I had been involved in this research, at least out of scientific curiosity, I would have completed the additional laboratory and phylogenetic work within days of discovering the new species.[12] I would have been all the more motivated if the results might affect those of a phylogenetic comparative analysis that I also was coauthoring. But this is "me." I thus give Lavandero2020 the benefit of the doubt and assume that they did not obtain the new results until *after* Pérez2020 was published.

But this is not entirely satisfactory. It remains troubling that the Lavandero2020 Clade A phylogeny was significantly different from that of Jara2017, yet they reported it to be only "slight[ly]" different. And they did not divulge the Clade B interspecific results, which, based on their Clade A results, also probably differed significantly from Jara2017. Again, if it had been me, I would have, first, noticed the significant differences and, second, appreciate that these *invalidated* Pérez2020. As I have noted, this analysis was dependent not only on the topology of the tree, but also its *branch length* estimates.

Thus, as *coauthor* of Pérez2020, I would have published a *retraction* of this work in the same journal. Or at least a *correction*. A correction of the Pérez2020 analysis would have taken perhaps a few days (or hours) to complete. This would have involved reanalysis of the *same* phenotypic and geographic data files, changing only the *tree* file. And pressing "Enter." This reanalysis would have yielded the same or *different* conclusions. Whatever. So be it. If one is a *dedicated* scientist, one wants to know and report the true answer and not simply an expedient one. In any case, this reanalysis and correction would have taken much less time than the *four years* that have transpired since Pérez2020 and more than *seven years* since Jara2017.

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[11] Jara2017 and Pérez2020 acknowledged "public" research grants awarded to these authors and Arroyo totaling (at that time) ca. US$ 400,000. They also cited an "umbrella" research grant to Arroyo that, for that period, totals ca. US$ 5 *million*. These monies, of course, are besides salary and infrastructure support from all of the authors respective universities. Government-funded researchers are immune from, e.g., "consumer protection" laws and, *evidently*, meaningful scientific oversight quality control. A win/win for researchers, but a lose/lose for society.

[12] Of course, if I had been involved in this research, the laboratory and phylogenetic work would not have been done incorrectly in the first place.

At the same time, again, if it were me, I would not be unaware that reporting such a massive error in a recently published paper, in which I was coauthor, would have repercussions for *all* involved authors. In this case, Lavandero2020 was a "by-product" of a large research grant whose *principal* objective and product incarnated in Pérez2020. Very embarrassing, and the funding agency would not be very pleased. But the massive error was not consequent to Pérez2020's data, but to Jara2017. But this *also* was the principal product of an earlier large research grant. So those authors also might have endured repercussions. But possibly the most severe repercussions would affect M.T.K. Arroyo, since she coauthored *both* research grant proposals and *both* publications. Moreover, she had been the *formal* PhD tutor of *both* of the first authors, who undertook their MPE doctoral research in the University of Chile. Nonetheless, again, if it had been *me*, I *would* have reported/corrected the error, "sí o sí." This is not to be per se honest, but to be a *scientist*, whose quintessential quality itself is honesty.

However, it is possible that Lavandero2020 did *not* discover the errors in Jara2017 and really *believed* that their new Clade A phylogeny was essentially the same. Circumstantial evidence for this is that they had used the Jara2017 tree in the first place, reproduced it incorrectly, and misleadingly described it as fully resolved. Technically it was fully resolved, but so is a random tree. The Jara2017 tree was not *well-supported*. So, as noted above, Jara2017 and Pérez2020 demonstrate that these researchers simply were not qualified to undertake MPE research. Lavandero2020 then would follow from the "Principal of Uniformitarianism." Evidently, the authors still are not qualified, lest *by now* they certainly *would have* discovered their errors and published a *correction* of their earlier work.

But even the second narrative is unsatisfactory. And the reason is that this question is *not* about the phylogeny of some genus in southern South America. It is about the accuracy and integrity of research articles published in peer-reviewed and "impact-indexed" science journals. And this is important because, every year, human civil liberties and even "universal rights" become ever more encroached upon by policies purportedly based on "science." Need I remind anyone what it was like to be locked up in one's home for weeks on end, obliged to wear face masks, and to be forced to submit to injections with poorly tested vaccines? And need I remind anyone how much of the now-debunked "science" had been challenged by scientists who *initially* were censored by social media giants or, in China, disappeared (Shir-Raz et al., 2023)?

In these cases, the policy-makers exhort citizens to "follow the science," by which they mean the conclusions of articles in peer-reviewed journals authored by the most distinguished of researchers from the most distinguished institutions. Jara2017 and Pérez2020 would qualify as examples of the latter. It would seem to be contrary to the interest of the scientific institution to undermine its own credibility. An explanation is needed. We need to peel back the layers here. Why were Jara2017 and Pérez2020 accepted for publication? Was acceptance influenced by the authors' nominal qualifications? But if the authors were not qualified to do the research, why were they awarded generous funds to do it? Here, it is critical to note that both grant proposals were coauthored by Arroyo, who, evidently, *also* was unqualified. Or she would not have put her name on the paper. In *theory* at least. If I am making an error here, I will correct it correspondingly.

But we need to peel back another layer to see that Arroyo was the formal PhD tutor of both Jara-Arancio and Pérez in the University of Chile, and she oversaw their MPE dissertation research. *Admission* to this PhD program is conditioned partially on the student's record and partially on the *mentoring* qualifications of the *PhD tutor*.[13] Once admitted, the student then competes *again*, for a *national* PhD fellowship, and later *again* for a *national* PhD research grant. And again, these competitive awards are

[13] Actually, I was on this admission committee one time. The other members told me that by "qualification," they meant the mentor's demonstrated ability to obtain *funding* for the student.

conditioned on the *PhD tutor's* qualifications. So, in each of these "quality-control" steps, what was the *basis* for *accepting* Arroyo's qualifications to mentor, specifically, MPE research? This is a critical question, because the present analysis demonstrates that Arroyo and her former PhD students were *not* qualified, years *later*, to undertake the MPE research published in Jara2017 and Pérez2020.

Again, these questions are *not* just about a few botanical researchers in Chile. Arroyo is *extremely prominent*, *proactive* and *influential* in science, educational, environmental…and *social*…policy, both at the *national* and *global* scale.[14] For example, Gleick et al. (2010) published an editorial in the preeminent journal Science, entitled "Climate change and the *integrity* of science [italics mine]," asserting that human-induced climate change was an established fact. The article includes as coauthors 254 members of the US National Academy of Sciences, among these, *Arroyo*. I am in no way qualifying here what this editorial asserted. In fact, I do not have the qualifications to do so. And that is the point. *Did Arroyo have the qualifications*?[15]

The answer to the above is that Arroyo was highly qualified in *several* disciplines according to her *publication* record. Unfortunately, over the past half century, publications have transformed from a medium of scientific communication to the "cryptocurrency" of scientific *career advancement*.[16] This is because, for *qualification* purposes, sole-authored, first-authored, and secondary-authored publications are *equivalent* and take *no* account of the *contribution* of the author to the research.[17] And it takes no account of the *quality* of the research other than that presumed by its de facto publication.[18]

This has created a sort of "black market" in which academically valuable *co-authorships* are *bartered* in exchange not for legitimate and essential scientific collaboration, but for economic, political, and personal favors. This, in turn, has made both highly feasible and *lucrative* the possibility of *acquiring* an impressive research trajectory in a discipline in which a researcher has practically no theoretical knowledge, experience, or *legitimate* scientific accomplishment.[19,20] Because this mechanism is

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[14] *More* importantly, her manifestly *like-minded* academic cohorts and offspring *dominate* academic positions and research throughout Chile. She *trained* them in the "art" of academic success.

[15] Ironically, if *any* of the coauthors were *not* qualified to *scientifically evaluate* the principal conclusion, then the article's *own* integrity was severely compromised (see also Lüdecke, 2023). Authors of scientific publications should make a *clear* distinction between what they merely *believe* to be true and what they actually can defend *scientifically*.

[16] This, in turn, reflects the disproportionately increased *socioeconomic* and *political* benefits afforded to scientists. In Chile, many scientists receive government compensation totaling 6–12X the average Chilean salary. Arroyo, I have been told, receives even *more*. In "developed" countries, this disparity would be ca. 3X. And the personal income is *besides* "fringe benefits" unavailable to common laborers *and* government funding and support comparable to the *privately* funded capitalization and operating costs of a small business. It seems paradoxical that biodiversity science researchers generally predicate to be "socialists" or even Marxists. But living among Chilean academics for 25 years has taught me that there are none so capitalistic in society than Marxists. *Both* seek to "own the means of production." But the capitalists *buy* it. The Marxists simply *steal* it.

[17] Effectively, a publication is an academic "asset" whose value, *irrationally*, is *not* diluted by its division. Thus, an author incurs zero *cost* and only *benefit* for frivolous coauthor addition, while the frivolous coauthor realizes the *same* benefit.

[18] E.g., Jara2017 and Pérez2020.

[19] See, e.g., Givnish et al. (2015, 2016), two prominent and *highly-cited* works on orchid evolution based on complex phylogenomic data. Arroyo is coauthor of both. Givnish et al. (2015: 179) states her *author contribution* as providing (*two*) Chilean orchid specimens. Givnish et al. (2016: 1916) does not mention her contribution *at all* and, moreover, described the paper as "*a product of an international collaboration among* ***specialists*** *in* ***orchid*** *systematics, phylogenetics, ecology, and biogeography* [***emphases mine***]." Arroyo was not a specialist in orchids, much less in phylogenomics. That is, *before* she put two samples of a reasonably common Chilean plant into an envelope and mailed them to the US. *Now*, per her resume, she "qualifies" as *both*.

"irrational," *legitimate* scientific research *cannot be sustained*. By a mechanism analogous to "Muller's Ratchet,"[21] it *bound* to render the *entire* population of *nominal* scientific researchers *maladapted* for scientific research, yet *highly* adapted towards whatever *unscientific* objectives they chose to pursue. *Legitimate* researchers become marginalized, even *criminalized*.[22]

As a second-generation biodiversity science researcher who first worked in a museum sorting skulls at age five (60 years ago), I would be the last person in the world to deny that humanity faces multiple existential threats, that policy should address those threats, and that policy should "follow the science." But I also recognize that even the most computationally sophisticated biodiversity science remains, at its core, arcanely metaphysical and difficult to *verify*. Its *reliability*, if it has any at all, thus depends on *scrutiny*, and much *more* scrutiny than for the physical sciences or even medical sciences. I am not merely disconsolate, but literally nauseated by the specter of there being no greater existential threat to biodiversity than…*biodiversity* "*science" itself*. If biodiversity science can aid in averting global disasters, it will not owe to "funding" or "publication resumes," or journal "impact" indices. Scientific problems are solved by *science*, not "*scientism*," and by *persons*, not "*personajes*." And the *quintessential quality* of science and scientists are, necessarily, *integrity* and *honesty*.[23]

Addendum

Following publication of the first version of this work, I realized that Katinas et al. (2022) included *Leucheria congesta* in *L. runcinata* D.Don. The latter is a Subclade III species. The *L. congesta* sequences analyzed here pertain to Subclade II and the cpDNA sequences are identical to *L. bridgesii* Hook. & Arn. Katinas et al. (2022: 26) noted that *L. runcinata* and *L. bridgesii* are superficially similar and share the same habitat. It seems likely, therefore, that *L. congesta* here and in Jara2017 and Pérez2020 is actually *L. bridgesii*. At the same time, the possibility of gene flow between these species becomes intriguing, especially because *L. tomentosa* sensu Katinas et al. (2022) includes plants pertinent to both Subclades II and III. Also, as I noted, the ITS1 portion of the ITS sequence of *L. congesta* reported by Jara2017 pertains to Clade A, most likely consequent to researcher error.

Acknowledgments

Elaboration of my 33 publications since 2018 and many more to follow would not have been possible except for the generous support and dedicated efforts of Fundación Reshet in Chile (www.reshet.cl). I acknowledge assistance from additional individuals, whom I will thank privately.

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[20] A few of my older publications include unavoidable superfluous authorships, but *none* my own authorships are superfluous. I later *refused* to participate in patently illegitimate authoring schemes. This is *precisely* why my *academic* career was terminated, and why I later supported my research by eating out of the garbage (see Letelier Parga, 2017: 2). In fact, as a matter of personal *dignity*, I preferred *not* to be included as author of a student research project I directed and significantly funded, viz. Jara-Arancio et al. 2012. I have *no idea* what the four additional authors contributed scientifically to this work.

[21] https://en.wikipedia.org/wiki/Muller%27s_ratchet

[22] E.g., https://en.wikipedia.org/wiki/Nikolai_Vavilov. But the government-coordinated censoring of legitimate and eventually corroborated scientific opinion regarding Covid19 also amounts to *criminalization* of legitimate science, because it deprived the scientists of their basic civil liberties, not to mention seriously aggravating the global-scale consequences of the pandemic itself (Shir-Raz et al., 2022).

[23] Science approximates truth by rejecting what is false. Mathematical "proofs" are no exception, because these are not scientific "truths," but (albeit scientifically very useful) *tautologies* rooted in *assumptions*. In any case, this "proves" that science quintessentially is *honesty*, viz., to paraphrase Samuel Taylor Coleridge, "an *unwillingness* to suspend *disbelief*."

Conflict of interest

The author declares no conflict of interest, viz. this research received no support from a government funding agency or any so-funded research project or organization, except, perhaps indirectly, those of Jara2017 and Pérez2020, since these provided me with the raw (to say the least) data. The present work is entirely that of the author whose authorship therefore is neither "vicarious," nor otherwise a political or economic transaction.

Supplemental file

The data set in NEXUS format for this work is available at https://osf.io/xmnhf/files/osfstorage/663e243c4664da60d3ed6b4a/?pid=xmnhf

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Figures 1–8. Cropped screenshots of DNA sequence alignments as visualized in BioEdit, with those of the phylogenetic data set of Jara2017 (Online Resource 5) on the left side juxtaposed with alignments used in the present analysis on the right side. For the Jara2017 alignment, all but two of the outgroups (viz., Marticorenia and Moscharia) were removed and the alignment condensed to remove the consequent empty columns. The sequences are identified (center column) as pertaining to *Leucheria* species according to Jara2017 (cf. Katinas et al., 2022), with the lowermost sequence pertaining to *Marticorenia*.

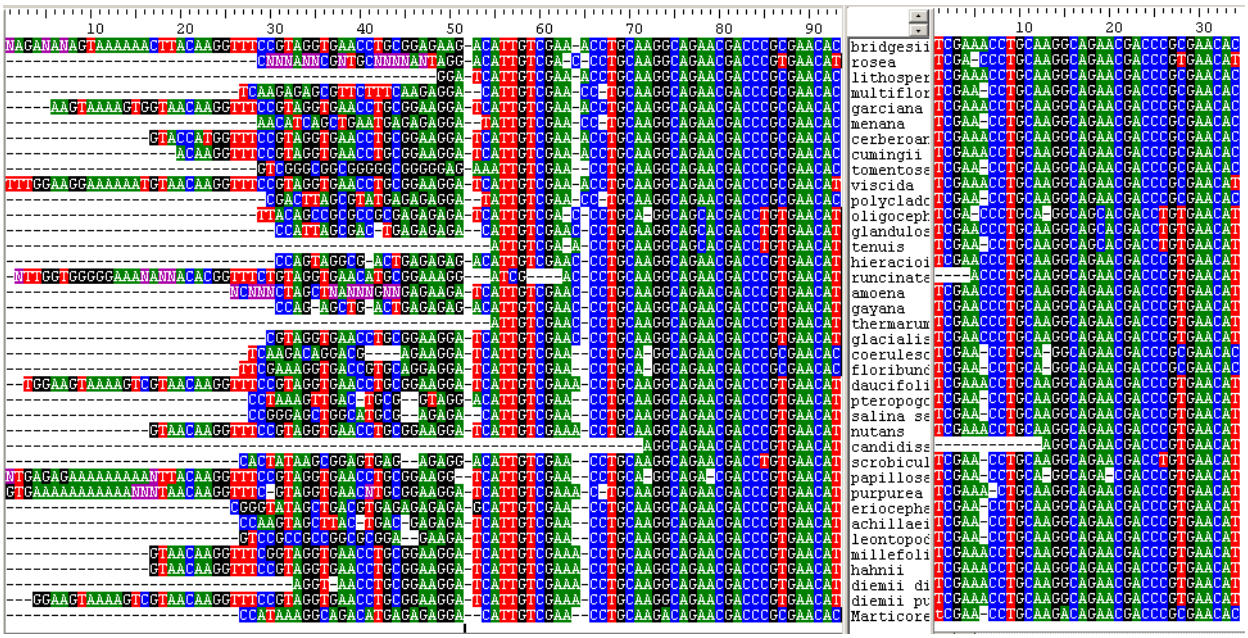

Figure 1. 5'-end of the rDNA ITS region (viz., ITS1). The Jara2017 alignment (left side) includes considerable but varying length of 18S rDNA sequence plus chromatographic noise. The sequences appear poorly aligned, even though the highly conserved 3'-end of the 18S locus should align perfectly at this phylogenetic level. The alignment used here (right side) begins with the canonical 5' ITS1 initiation motif, TCGAAACCT… or similar variant (Hershkovitz et al., 1999). Note that the *Leucheria* sequences manifest considerable length and sequence variability in this motif. In my experience, while such variants do occur among angiosperms, the motif or variant thereof generally is highly conserved at the interspecific level. But also in my experience, the motif often is compressed in sequence chromatographs, which must be carefully compared in both sequencing directions to determine the correct number of A's and C's. Given the overall poor quality of both sequences and alignment in Jara2017, the variation here in this motif might owe to chromatographic artifacts.

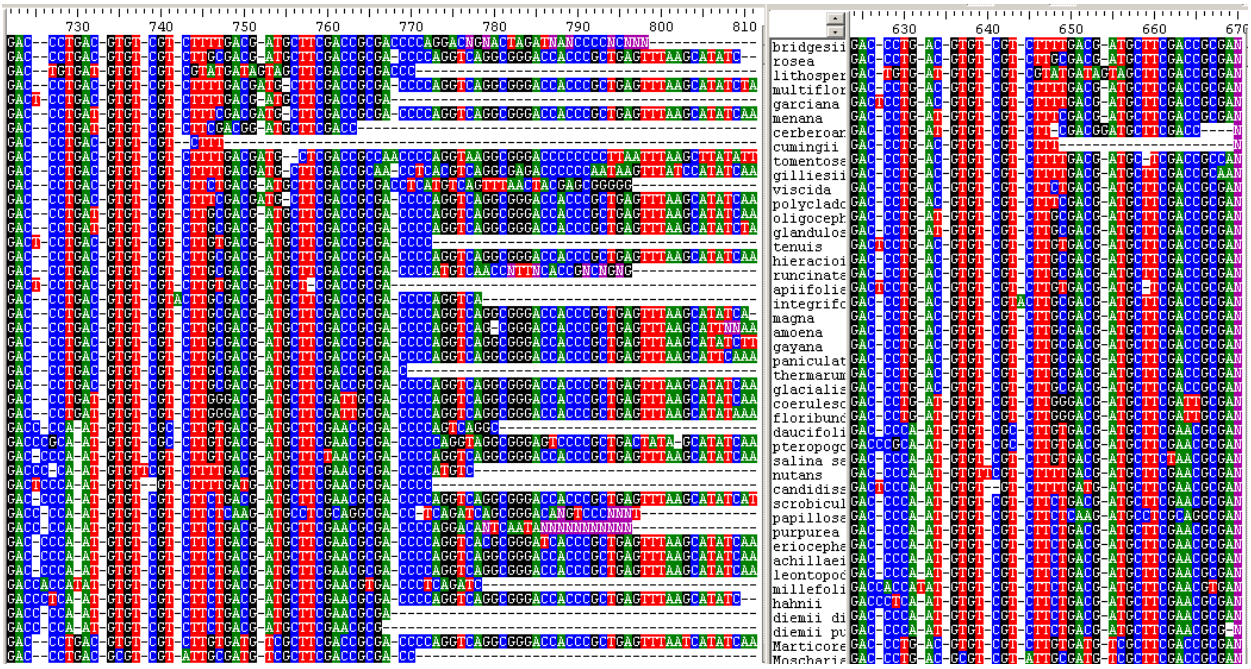

Figure 2. 3'-end of the rDNA ITS region (viz. ITS2). The Jara2017 alignment (left side) includes variable lengths of the highly conserved 5'-end of the 26S gene, with some sequences showing misalignment and/or chromatographic noise. The ITS portion shows significant misalignment of the *L. menana* and *L. gilliesii* sequences on the upper right, as well as poor but less consequential misalignment of the ACCC[C] motif on the lower left (conserved motif 6 in Hershkovitz & Zimmer, 1996). The alignment used here (right side) terminates just upstream of the ITS2 terminus, GCGACCCC. The single base inserts in some sequences, especially those within the ACCC[C] motif, are "suspicious" and may be chromatographic noise.

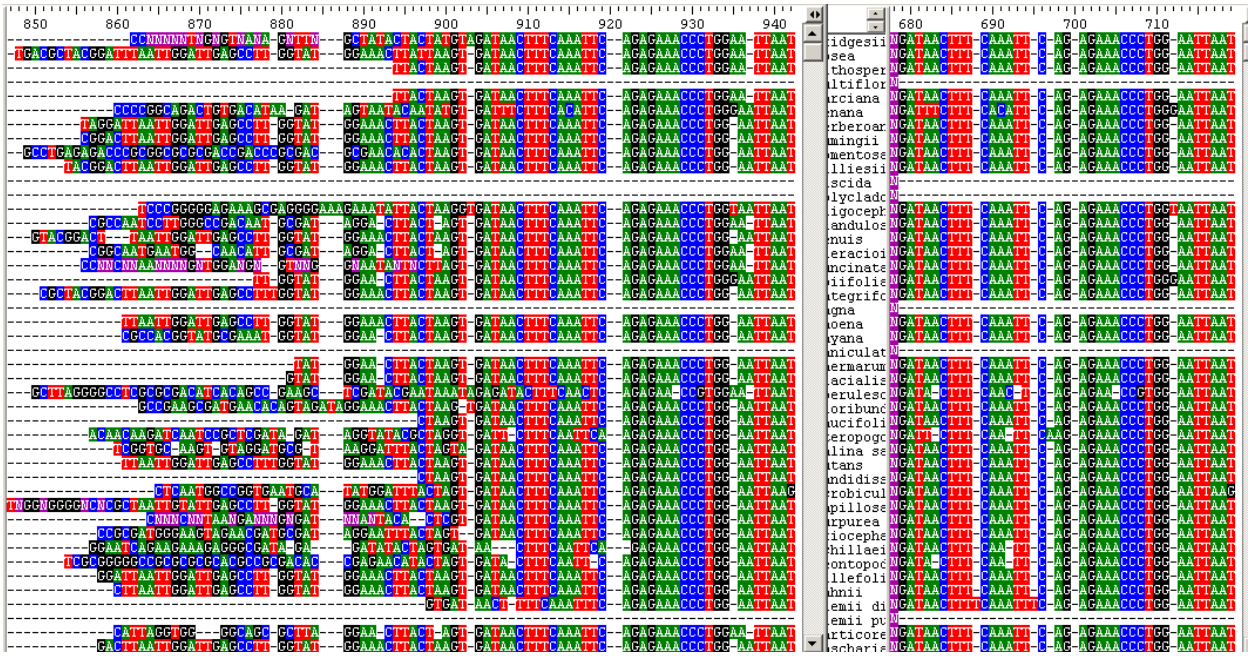

Figure 3. 5'-end of the *trnL-trnF* alignment of Jara2017. The sequence shown here pertains to the *trnL-trnF* intron. The 5'-side of the Jara2017 alignment (left side) is noisy and poorly aligned, while significant misalignment also is evident on the better-aligned 3'-side. The alignment used here (right side) was trimmed to a conserved motif on the 5'-side and realigned. As with the 5'- and 3'-ends of all loci in the Jara2017 alignment, several "suspicious" single-base indels are in evidence. These may reflect chromatographic noise. No *trnL-trnF* sequences were available for some taxa.

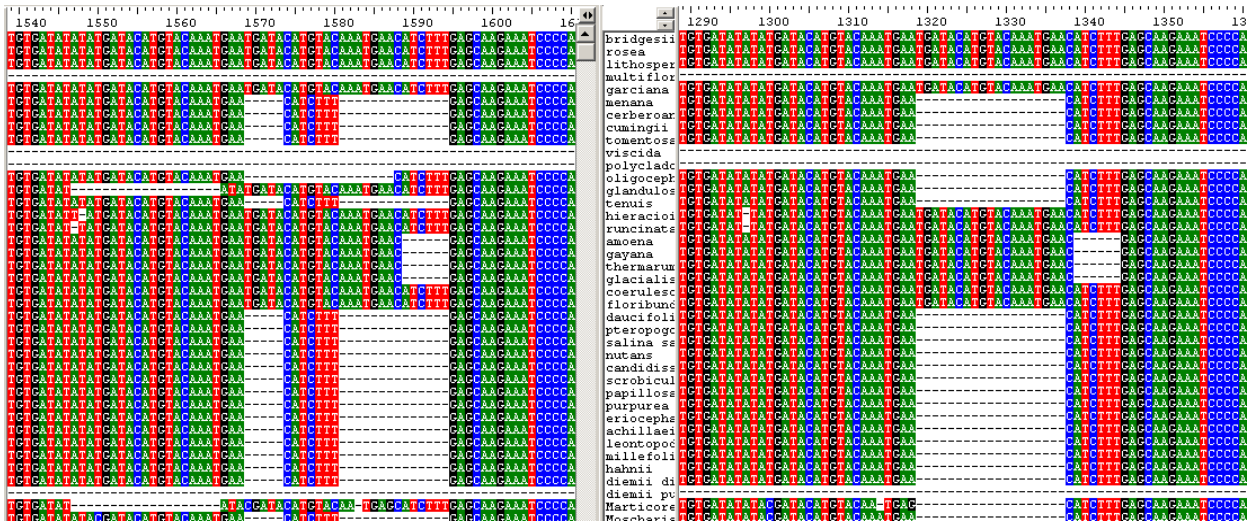

Figure 4. Length-variable region of the *trnL-trnF* locus. The Jara2017 alignment (left side) shows misalignment that yields three spurious "substitutions." The alignment used here (right side) correctly positions the length-variable regions. No *trnL-trnF* sequences were available for some taxa.

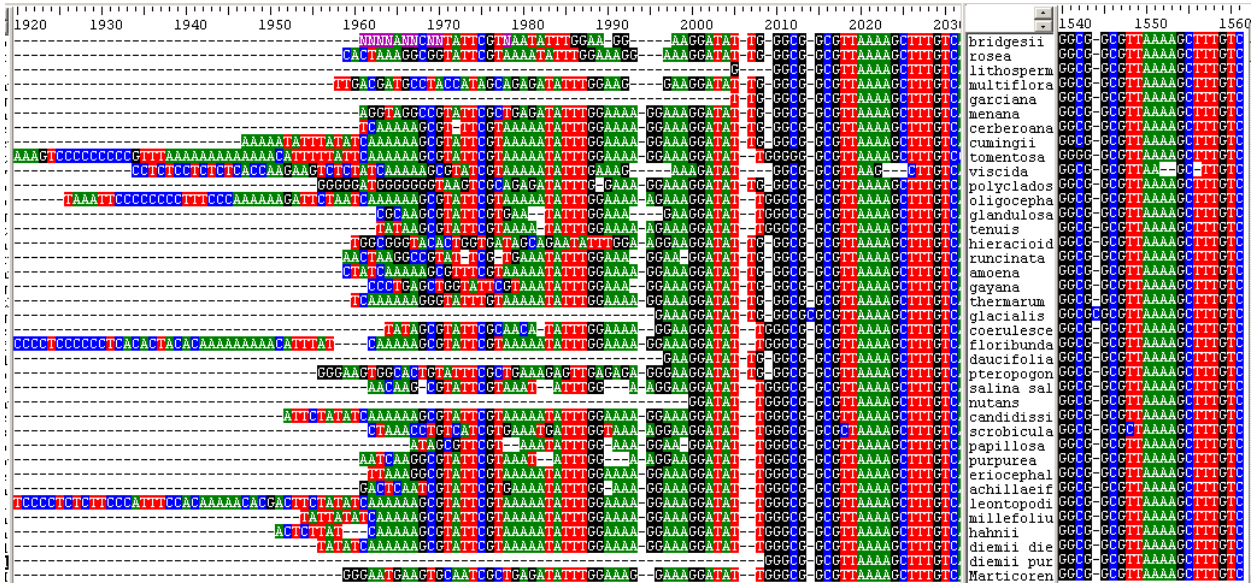

Figure 5. 5’-end of the *rpl32-trnL* locus. As with the 5’- and 3’-ends of all loci, the Jara2017 alignment includes variable lengths of poorly aligned and noisy sequence. The sequence used here (right side) trims the 5’-end to a perfectly conserved and aligned motif that is misaligned in the Jara2017 alignment, yielding a spurious “substitution.”

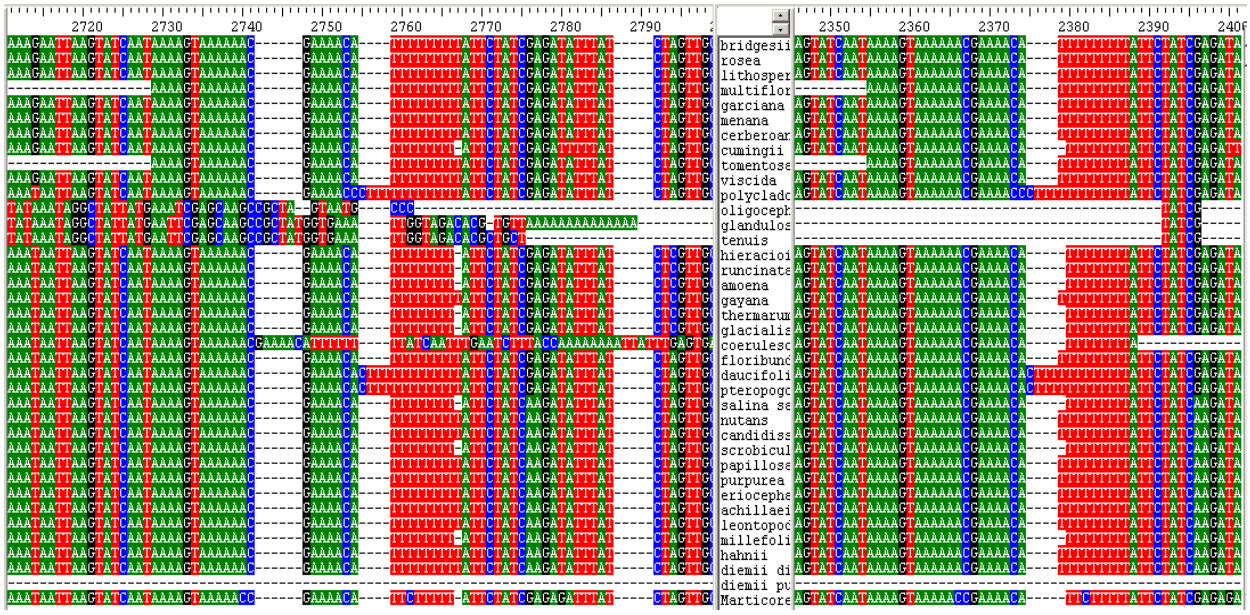

Figure 6. Length-variable region of the *rpl32-trnF* locus. The Jara2017 alignment (left side) shows misalignment and premature termination of three sequences (*L. oligocephala* J.Rémy, etc.) and, below that misalignment on the 3'-side of the *L. coerulescens* J.Rémy sequence. The alignment used here (right side) shows that the former misalignment owes to a large deletion in the three sequences that begins upstream of the framed region (cf. Fig. 7), and that the latter misalignment owes to a large deletion in the *L. coerulescens* sequence that begins on the 3'-side of the framed region (cf. Fig. 7). Note that there is no sequence available for one of the taxa in this portion of the alignment.

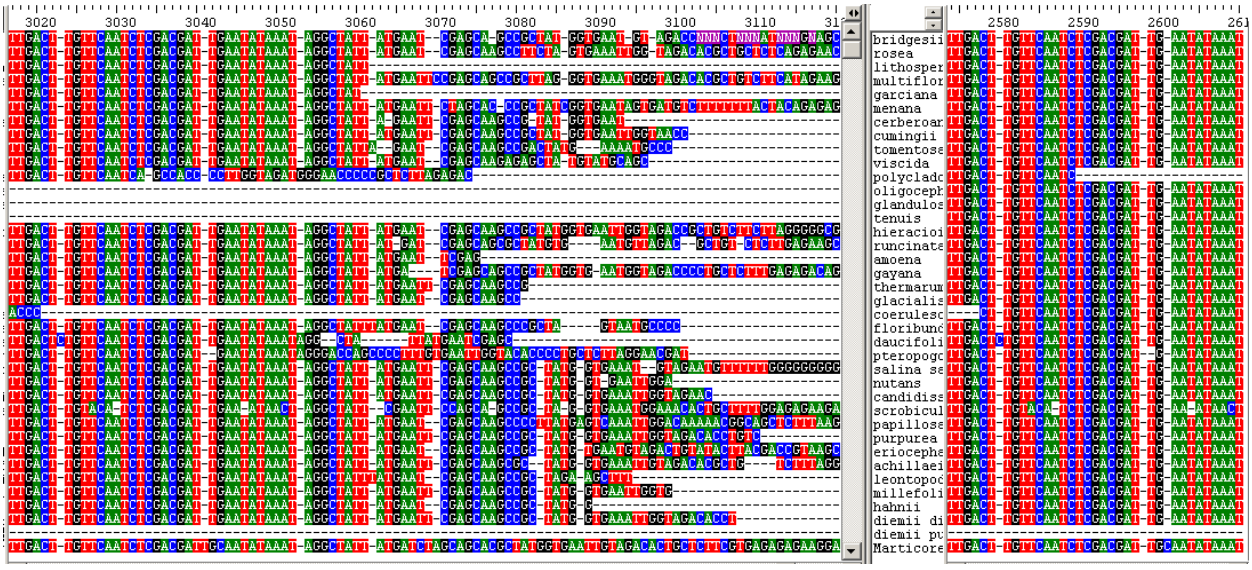

Figure 7. 3'-end of the *rpl32-trnL* locus. As with the ends of all loci, the 3'-end in the Jara2017 alignment (left side) includes variable lengths of unaligned and noisy sequence. The three sequences described in Fig. 6 (*L. oligocephala* etc.) appear absent and the *L. coerulescens* sequence appears to terminate prematurely. Also, the 3'-end of the *L. polyclados* sequence does not align with the other sequences. The alignment used here (right side) demonstrates that indeed the corresponding aligned 3' sequence exists in all four of the sequences terminating upstream on the left. The unaligned portion of the *L. polyclados* sequence is random noise that retrieved no other sequence when submitted to BLAST. Thus, this sequence was trimmed in the alignment at the point where the unaligned sequence begins. Note that there is no sequence available for one of the taxa in this portion of the alignment.

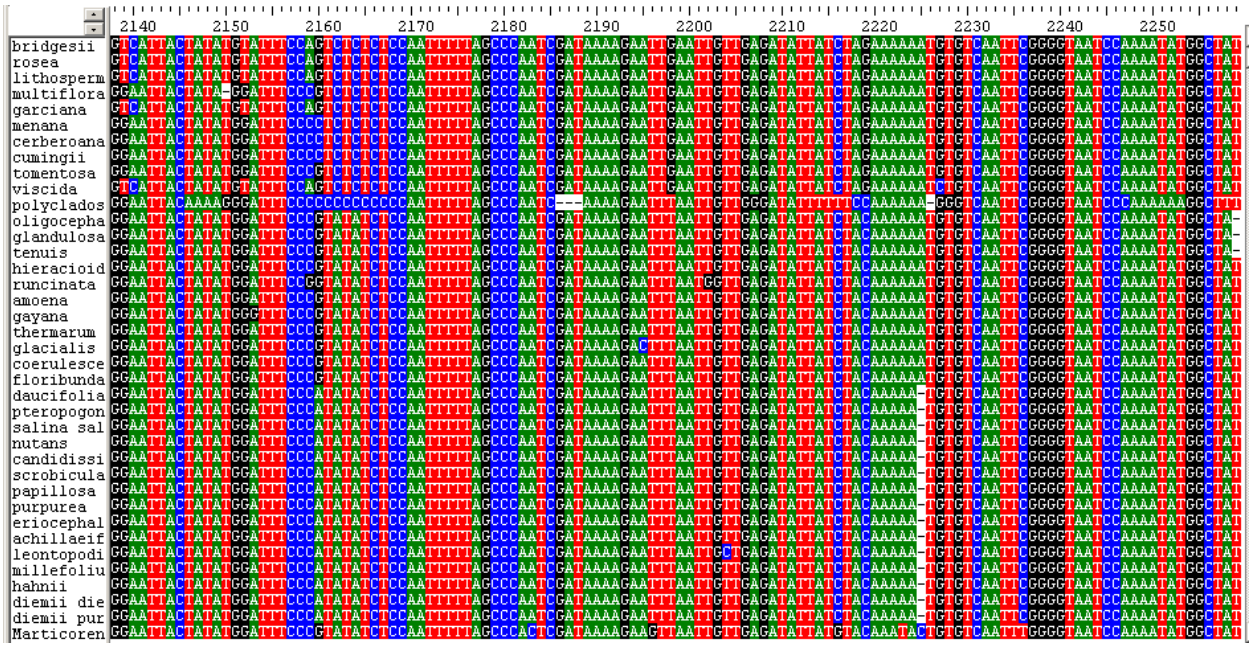

Figure 8. Aligned portion of the *rpl32-trnL* locus demonstrating chromatographic "smear" in the sequence of *L. polyclados*. These smears comprise extended mononucleotide repeats in regions where all of the other sequences show highly conserved nucleotide diversity. See text for an explanation of these smears.

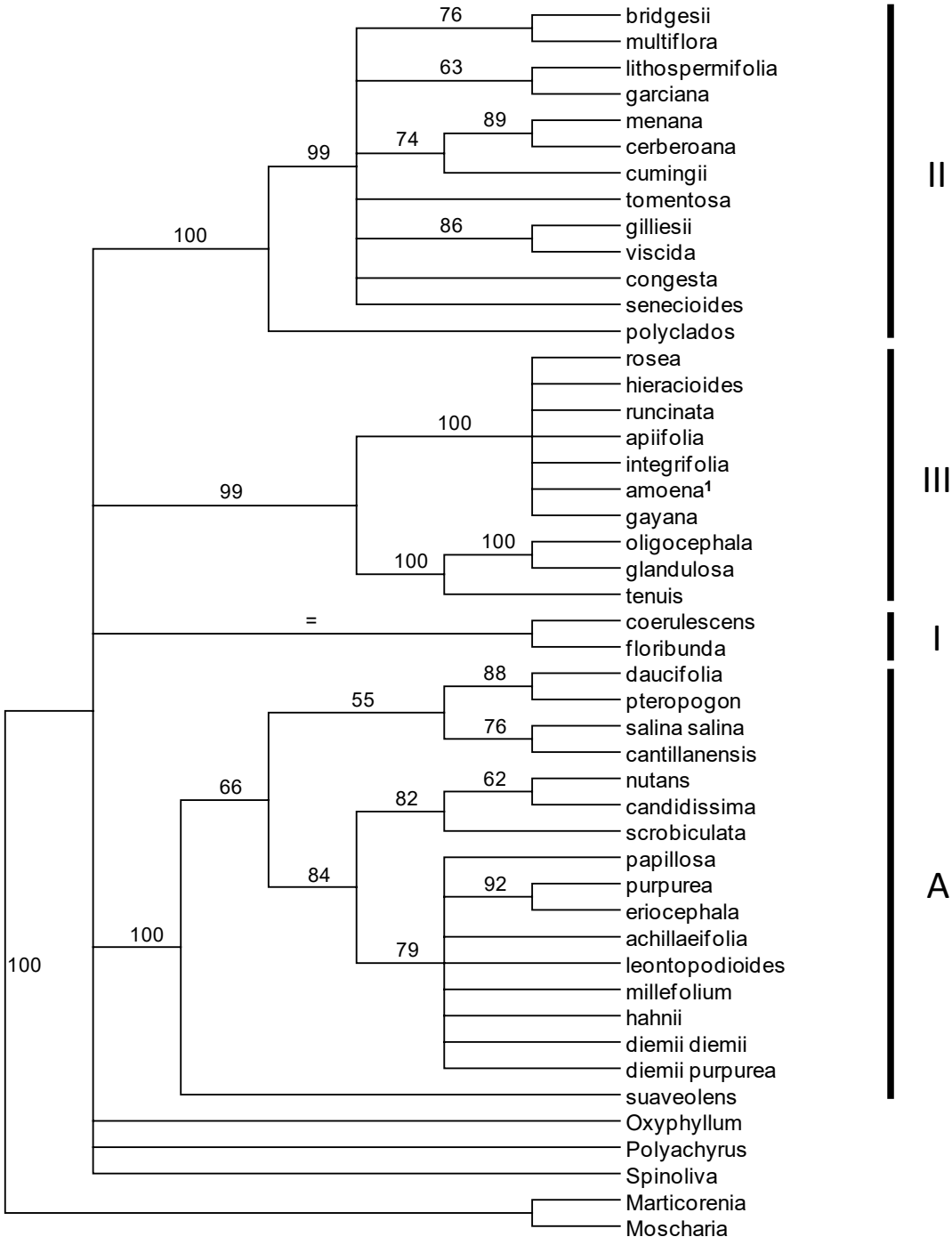

Figure 9. MP bootstrap consensus for the ITS data. Numbers indicate bootstrap %. "=" means that the sequences are identical. The superscript 1 refers to other taxa with identical sequences, in this case *Leucheria glacialis*, *L. magna*, *L. paniculata*, and *L. thermarum*. Graphics on the right side delimit *Leucheria* Clade A and Clade B Subclades I–III of Jara2017. Taxonomic identity of the sequences follows Jara2017 and Hershkovitz (2024a).

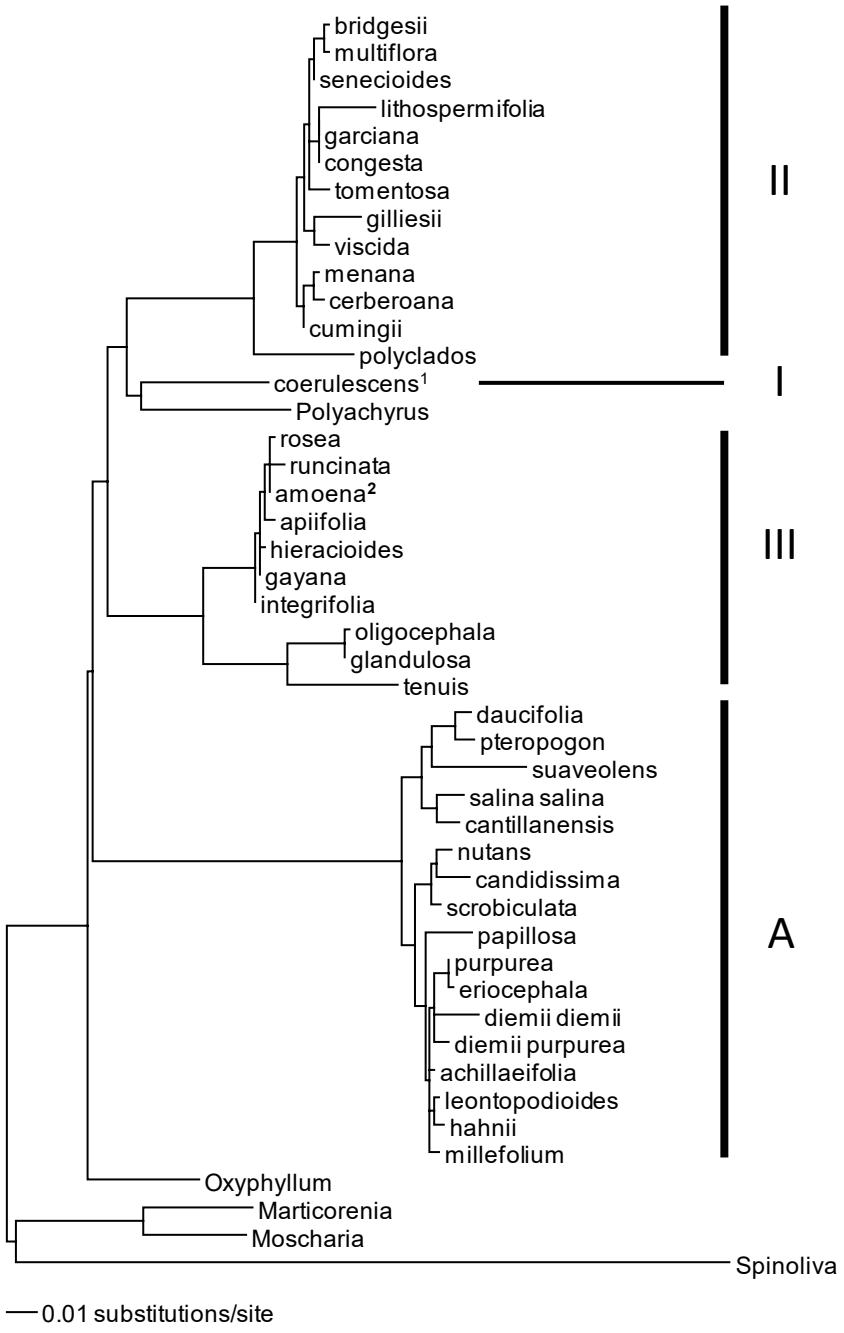

Figure 10. One of 25 ML phylograms for the ITS data. Superscripts refer to other taxa with identical sequences as follows: 1, *Leucheria floribunda*; 2, *L. glacialis*, *L. magna*, *L. paniculata*, and *L. thermarum*. Graphics on the right side delimit *Leucheria* Clade A and Clade B Subclades I–III of Jara2017. Taxonomic identity of the sequences follows Jara2017 and Hershkovitz (2024a).

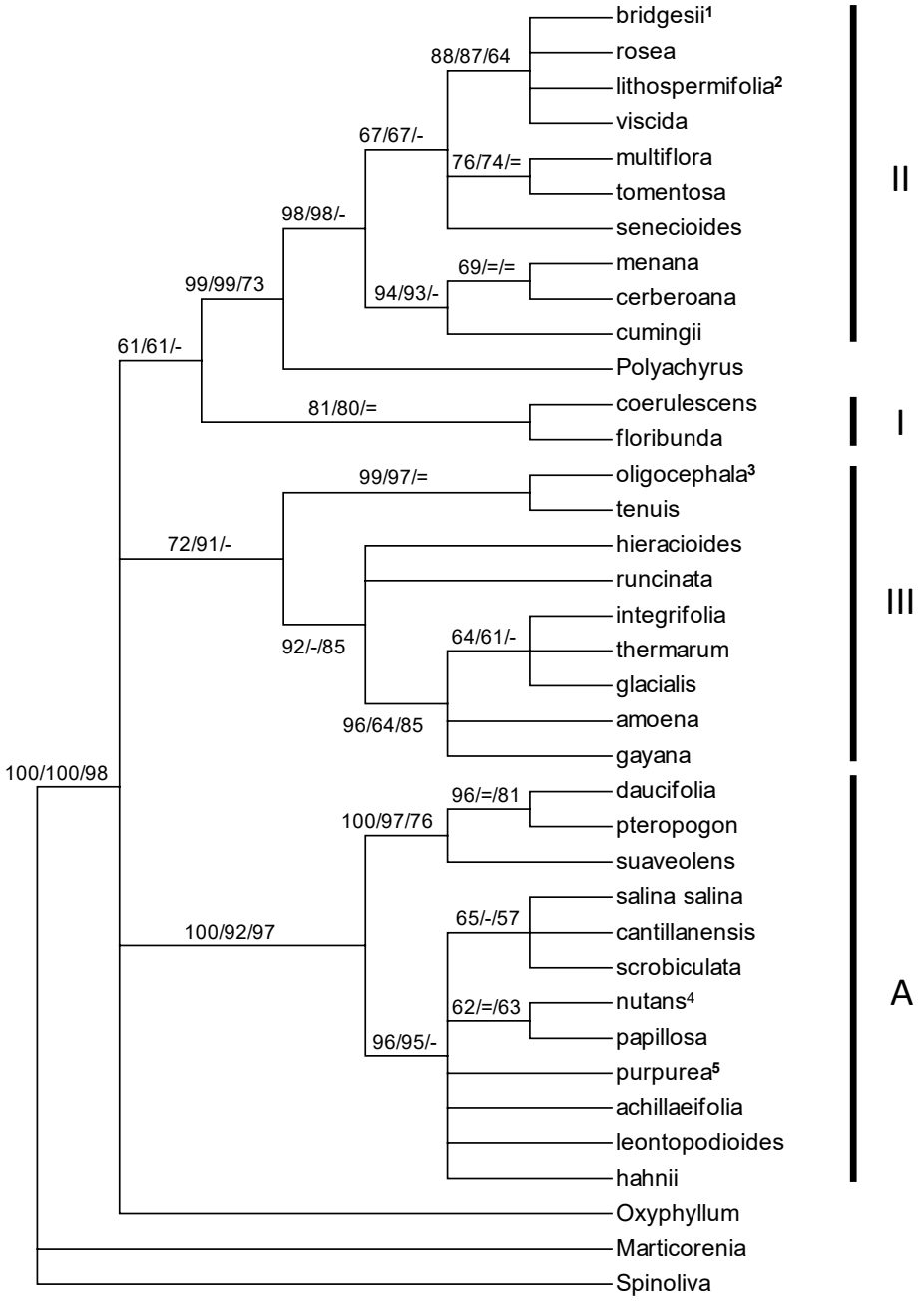

Figure 11. MP bootstrap consensus for the combined cpDNA data. Numbers indicate bootstrap % for, respectively, combined, *rpl32-trnL*, and *trnL-trnF* data. "-" means < 50%; "=" means that the sequences are identical. Superscripts refer to other taxa with identical sequences as follows: 1, *Leucheria congesta*; 2, *L. garciana*; 3, *L. glandulosa*; 4, *L. candidissima*; 5, *L. diemii* var. *diemii*, *L. eriocephala*, and *L. millefolium*. Graphics on the right side delimit *Leucheria* Clade A and Clade B Subclades I–III of Jara2017. Taxonomic identity of the sequences follows Jara2017 and Hershkovitz (2024a).

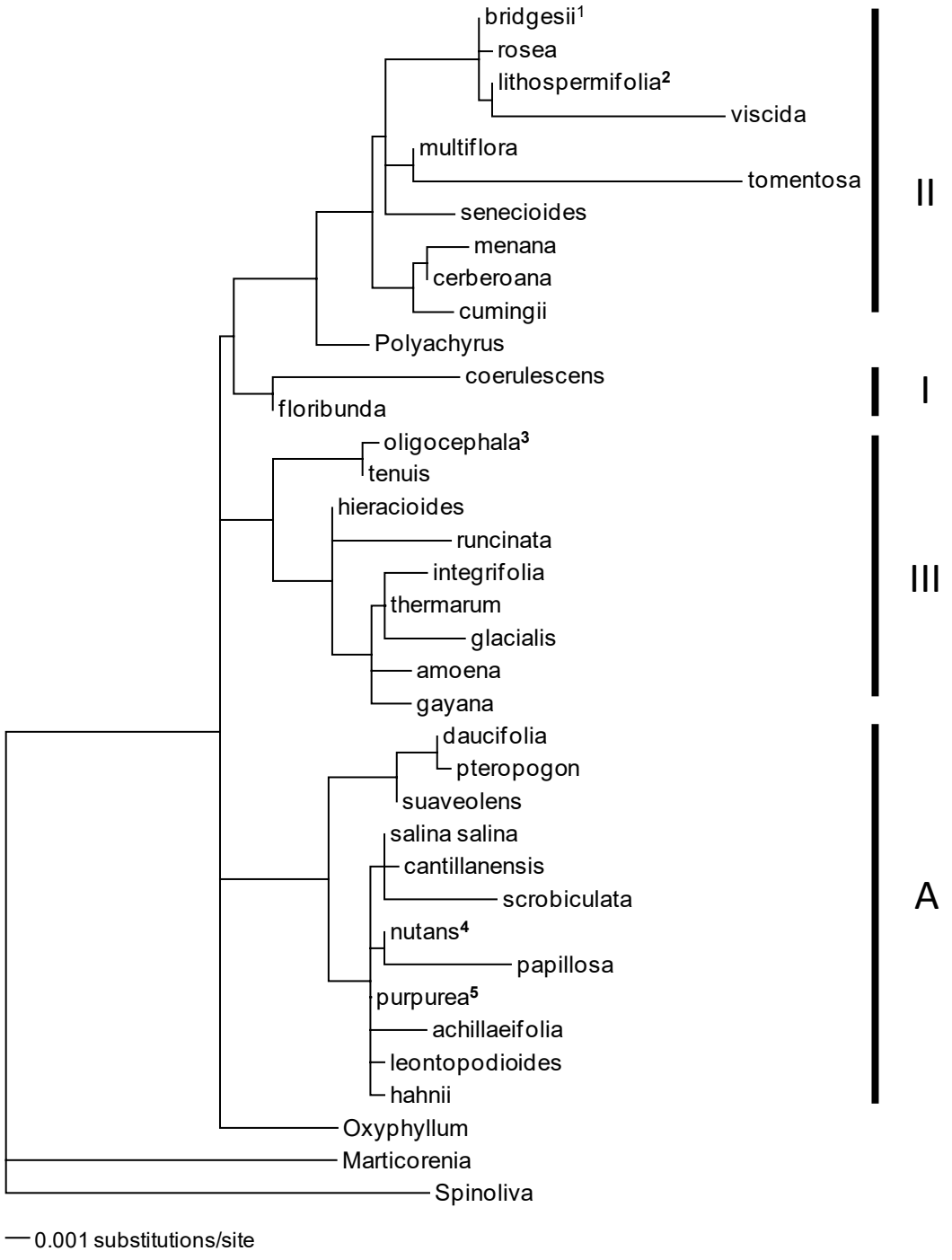

Figure 12. Single ML phylogram for the combined cpDNA data. Superscripts refer to other taxa with identical sequences as follows: 1, *Leucheria congesta*; 2, *L. garciana*; 3, *L. glandulosa*; 4, *L. candidissima*; 5, *L. diemii* var. *diemii*, *L. eriocephala*, and *L. millefolium*. Graphics on the right side delimit *Leucheria* Clade A and Clade B Subclades I–III of Jara2017. Taxonomic identity of the sequences follows Jara2017 and Hershkovitz (2024a).

Appendix 1. Summary of theoretical and operational characteristics of phylogenetic methods.

The following summarizes and adds to information discussed in Hershkovitz (2021b). I do not pretend proficiency in statistics, mathematics, or computer science. My interpretation of methods and their performance bases on my study of the relevant literature and empirical experimentation.

The present results were derived using MP, frequentist ML, and the bootstrap applied using MP. The effectiveness and limitations of MP, frequentist ML, and also BE ML were discussed in Hershkovitz (2021b). To encapsulate, as is "common knowledge," MP yields trees with the smallest number of changes, in this case substitutions of DNA bases (and indels as the case may be) presumed to be homologous. In the case of the former, unweighted parsimony takes no account of statistically-inferred probabilities of substitution classes. To a degree, it inherently takes into account among-site rate variation, but in a peculiar way. Similar to the ML invariant sites model, it excludes sites that are invariant empirically. It includes two classes of variable sites, those that are parsimony-informative and those that are not. Logically, those that are not parsimony-informative should evolve more slowly than those that are. ML analysis distributes these unique substitutions stochastically across the *entire* tree. Thus, they occurred at no *particular* time during phylogeny. MP distributes them among the *external* tree branches. Thus, they are the substitutions that occurred most *recently* during phylogeny.

ML (and ML-based distance methods, which I do not discuss here) incorporate statistically-inferred probabilities of substitution classes and also inferred differential probabilities of substitution among sites. Estimated model parameter values have variance and this variance multiplies across parameters, but the standard ML phylogenetic analysis treats estimated parameter values as fixed and ignores this variance. For this reason, less parameterized models can perform as well or better than more parameterized models, even if more parameterized models are statistically superior. Also, increased parameterization effectively approaches the assumption of MP. Furthermore, standard ML assumes that the estimated substitution dynamics have been constant throughout the phylogenetic history, whereas empirical analysis often shows that these dynamics are not only nonstationary, but that they are "unreal," viz. they represent mean dynamics estimated from *different* dynamics that occurred during the course of evolution. In practice, standard ML analysis assumes that each sequence site in an alignment evolves independently of other sites, albeit with shared substitution dynamics. Again, empirical data suggest that this assumption is false. Some ML models can correct for this, but, in practice, such models rarely are used, partially for the burden on variance.

It generally is not appreciated that, in phylogenetic practice, ML is computed not analytically, but rather using a variety of approximations that render its analysis computationally feasible. Programs such as PAUP allow the user to select the approximation type and other parameters, but users (me included) generally use the preselected defaults. Most users – systematists – lack the mathematical/statistical background to understand the different methods or even ML itself. Additionally, during the course of (default) heuristic tree search, the default parameter for saving/discarding trees is much more stringent in ML than MP, such that "optimal" trees are infinitesimally better than discarded trees. The consequence is that, for a given dataset, the ML procedure will save one to a few trees for further optimization or search termination, whereas MP (unless limited) might find many thousands of equally parsimonious trees with no upper bound. Commonly, the -log likelihoods of the MP trees can vary at up to the third digit (i.e., –XXXX.XXXX vs. –XXYY.YYYY). Thus, ML trees have not only false precision, but, given the data sample, model inaccuracy, and parameter value variance, they also may be less reliable than an MP tree. For this reason, it must be emphasized that trees produced by any method are estimates of the true tree, and they must be evaluated in terms of how the method parses the data set of interest.

ML analysis also *effectively* assumes that base substitution is a linear Gaussian process, such that substitution likelihood along a branch is calculated as a function of branch length. This, in turn, is a

likelihood function of all substitutions estimated to have occurred along that branch. In standard substitution models, this also assumes that each site in a rate class evolves independently via a Gaussian process. The past few years have seen research demonstrating that *phenotypic* data, when mapped on a calibrated molecular phylogeny, fit better a Cauchy or "jump" rather than Gaussian process (Bastide & Didier, 2023). But it seems to me from uncalibrated phylograms that substitution itself *also* may fit better a Cauchy process, which is exactly what phenotypic and other empirical genetic data would thus predict. In other words, phylograms render clear that the relation between total substitution and time is not linear, yet this assumption underlies likelihood calculation of branch lengths and, in turn, tree topology, and, in turn, phenotypic evolution.

For purposes of reconstructing phenotypes and clade ages, phylograms are calibrated to effectively "average" disproportionate sister branches, so that the distance between the root and all terminals is equal, viz. so that the tree is ultrametric. A conundrum arises, however, because the molecular tree calibrated assuming a Gaussian process is then used to test whether the phenotypic data evolved under a Cauchy process. What happens to phenotypic evolution if the molecular data evolved under a Cauchy process is not clear to me. I suppose that Bayesian estimation could be used to accommodate all possibilities, but I have a difficult time believing that the result will be unimodal, i.e., it seems most likely that multiple and very different solutions will be equally likely. Ironically, MP, notwithstanding its performance inconsistency (i.e., long branch attraction) in the case of Gaussian process evolution, may well perform better than ML in the case of Cauchy process evolution.

I add to the above that the discovery that much published phylogenetic comparative data presumed, for analytical purposes, to have evolved under a Gaussian model actually fit significantly better to a Cauchy model. This validates empirically Eldredge and Gould's (1972) punctuated equilibrium model, then regarded as heretical by the statistical population genetics paradigm. But more than that, the model actually is consistent with Hershkovitz' (2021b and earlier references) "Principle of Evolutionary Idiosyncraticity" (PEI) which bases upon the Natural Drift theory of evolution (Maturana & Mpodozis, 2000; Mpodozis, 2022), which bases, in turn, upon the definition of life as autopoietic (Maturana & Varela, 1972).

In particular, *both* the Gaussian and Cauchy models are *stochastic* (and thus probabilistic) and effectively assume that organisms are *passive* actors whose evolution is *indeterminate* and driven by (classical) *genetic* mutation and environmental stimuli. Autopoiesis and Natural Drift, in contrast, emphasize the *primary* role of the *organism* itself and its "*total*" genotype (viz. *any* trait, "genetic" or not, that a parent transfers to its offspring) in *determining* its evolutionary destiny. This imparts a chaos-like component to the evolutionary process. This led Hershkovitz to describe the evolutionary process as neither chaotic, nor stochastic, but *idiosyncratic*. A chaotic (or idiosyncratic) process, of course, cannot be reconstructed statistically as though it were a stochastic process. But, notably, a chaotic process may *appear* to behave similar to a stochastic process *locally*, indeed to a Gaussian or, alternatively, Cauchy process. Thus, the discovery that empirical evolutionary data might fit a Gaussian *or* Cauchy model or *both* (multimodally) at least demonstrates that it does not fit any *single* stochastic model, which is precisely what PEI states and what autopoiesis predicts.

Moving on, the present work bases its conclusions in part on results of MP bootstrap analysis. Hershkovitz (2021b) attempted to describe the meaning of bootstrap values, in particular as representing confidence intervals and not (although related to) probabilities. The bootstrap resamples data in order to generate pseudosamples that yield a pseudovariance. This, in turn, permits estimation of the reliability of a phylogenetic tree in relation to a hypothetically larger data sample that might counterevidence the tree derived from actual data.

Hershkovitz (2021b) explained intuitively a basis for the 70% bootstrap support cutoff for high confidence that is commonly dogmatically accepted but never explained. The 70% support means that the maximum bootstrap value for any conflicting branch is only 30% (sort of "two standard deviation" thing going on here). But, in practice in analyses of "numerous" taxa, conflicting branches have less than 10% bootstrap support, and most much less than that. Consequently, the 70% branch usually is on the order of ten times less likely than any alternative to be *counterevidenced* by adding empirical data. And *a lot* of empirical data, at that. At the same time, it must be emphasized that the bootstrap cannot overcome methodological inconsistency or bias. To the contrary, in such cases, it can yield high confidence in wrong results. Thus, one must consider not only the bootstrap value, but also its underlying cause, as I did in the present work.

I did not bother with BE. BE PPs commonly are interpreted as being at least qualitatively equivalent to bootstrap values, but they are not. Unlike the bootstrap, Bayesian analysis does not represent the degree of *data* support for the tree. It resamples parameter value space, including the tree parameter. Hershkovitz (2021b) conjectured that BE in phylogenetics is nothing more than a heuristic optimization search algorithm. The PPs are merely the probability that the branch exists in the ML tree optimized using the parameter model. Since the parameter model is unrealistically simplistic, it overestimates accuracy/reliability of the tree, including branches with unitary PPs. To appreciate this, ML will resolve a branch supported by asymptotically infinitesimal character data. The BE PP for that branch under the same model would approach unity. Indeed, if that branch were supported by an unequivocally shared substitution, the PP almost certainly would be unity. But bootstrap support would probably be < 70%, because a large number of bootstrap replicates would fail to sample that single site. In fact, BE operationally is known to support with unity PP branches that do not exist in the true tree. And this really sucks.

BE in phylogenetics seems to have become popularized partially in response to the computational demands of frequentist ML analysis, but also because of the dilemma famously described by Yang et al. (1995). In particular, optimization scores of MP and distance trees can be compared directly, but those of ML trees cannot. This is because the topology of the tree is itself a parameter used in its likelihood calculation. In ML, alternative hypotheses are tested using the likelihood ratio. But this requires that one alternative be nested within the other, in this case that a branch exists or does not exist (viz., H_0 vs. H_1). But alternative tree topologies with 5 or more terminals are not so-nested, hence the likelihood of one cannot be compared with that of another, at least not calculated over the same model parameters and parameter values. At the same time, it remains true that the optimal tree indeed is the one that has the highest likelihood, but considering all parameters and parameter values. BE is a clever workaround, since it is designed to sample all of parameter space in order to find the "true" ML tree. But, again, the PP reflects only an overestimate of the probability that the BE tree is the "true" ML tree, and it is in no way equivalent or even comparable to a bootstrap value.

In empirical phylogenetics practice, it seems that many researchers have little or no understanding of the theory behind the analytical methods they use. Since I am not a qualified statistician, my own comprehension is constrained, although I do consult the theoretical literature and perform empirical tests. For many researchers, BE seems to be preferred often for its "aesthetics." A *million* or more "Metropolis-Coupled Markov-Chain Monte Carlo generations" sounds much more scientific and rigorous than a mere *thousand* or less "bootstrap" replicates. This sounds like something one does to herd cattle. And of course, BE trees appear to be better resolved than bootstrap trees, with more branches showing PP values > 0.95. This *looks* much better than a tree with fewer branches supported by bootstrap values merely > 70%. Even though the bootstrap tree probably is more reliable.

The meaning and "significance level" of PPs in phylogenetic analysis is even more mysterious than for the bootstrap. As noted in Hershkovitz (2021b), Alfaro & Holder (2006) pointed out PPs in

phylogenetic analysis are not equivalent to probabilities in frequentist statistics, viz. p = 0.05, because those test whether a distribution is different from a null distribution. Indeed, a relation between p and PP can be estimated in the case of null distribution testing. But in phylogenetic BE, there is no null distribution and none is possible, except in reference to a completely unresolved tree. Here, branch PPs mean that, in the analysis as modeled and parameterized, a branch occurs in some proportion of the total posterior tree distribution. Axiomatically, it represents a *single* distribution, never more. This distribution comprises trees saved during the Metropolis-Coupled Markov Chain Monte Carlo procedure and excludes those that are rejected. I have inferred this to mean "the probability of that branch in the 'true' ML tree optimized per that model." In this sense, BE is essentially an ML algorithm.

Bootstrap proportions and BE PPs are not related linearly. A branch might be supported by PP = 1.0 but bootstrap % < 70 and, conversely, bootstrap values > 70% with PP much less than 0.95. This is context-dependent, because, as noted above, bootstrap % and PPs are unrelated *qualitatively*. High PPs with low bootstrap % often (but not always) occur on exterior branches, because the PP is related to the ML, regardless of data support, while the bootstrap % relates to the amount of corroborating data. The opposite relation might exist along interior branches, where PP is more sensitive to tree space exploration and bias.

To appreciate the last notion, consider the tree space imbalance flanking the branch that separates two terminals from the rest of the tree. In practice the sister terminals often (but not always) represent very similar to identical sequences. Certainly that is true for *most* sister terminals in *Leucheria*. Biased sampling in the rest of tree space will not alter this similarity, hence neither their relation in an ML tree nor their BE PP. In contrast, the "middle" branch in a tree separates two large trees, each comprising terminals highly divergent in sequence. Here, the reliability of that branch is more sensitive to tree space exploration, viz., whether that branch is present in the incalculable true as opposed to algorithmically approximated ML tree. In the case where the difference in likelihood of trees having versus lacking that branch is small, the PP also may be small. Because both flanking trees are large, there is a correspondingly much larger number of rearrangements of each of them to be tested in order to test the internal branch. The BE algorithm probably will spend a lot of generations swapping between branch-present and branch-absent rearrangements, not settling on either one of them.

There is no magic value of either bootstrap % or PP that separates significance from insignificance. For PP, I prefer the more conservative 0.95 as the value necessary to consider the branch as highly likely. This takes into consideration the meaning of the PP together with its implications. It means that there are other trees having a likelihood sufficiently close to that of the BE tree such that they could be preferred by adding only a small amount of additional data. At PP = 0.90, of course, even less additional data could overturn the results. And, besides this, it has been known now going on a quarter century that PPs, even when 1.0, overestimate tree accuracy.