

Phylogenetic relationships of the genera *Anthoxanthum*, *Ataxia*, *Hierochloë*, and *Phalaris* (*Poaceae*)

Филогенетические отношения родов *Anthoxanthum*, *Ataxia*, *Hierochloë* и *Phalaris* (*Poaceae*)

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<https://doi.org/10.31111/novitates/2025.56.08>

Abstract. Phylogenetic relationships of genera *Anthoxanthum* L., *Hierochloë* R. Br., *Ataxia* R. Br., *Phalaris* L., and related genera were studied by sequencing the internal transcribed spacer region (ITS) of nuclear ribosomal DNA (rDNA) and the *trnL*–*trnF* region of chloroplast genomes. The ITS1–5.8S rDNA–ITS2 region was sequenced and analyzed in 30 species and subspecies previously assigned to the tribe *Phalarideae* Kunth. The tRNA-leu intron (*trnL*) and the intergenic spacer between the *trnL* and tRNA-phenylalanine gene (*trnF*) were sequenced in 19 *Phalarideae* species. Phylogenetic hypotheses indicating the most probable divergence pathways of *Anthoxanthum* and *Hierochloë* species were tested. It was shown that both *trnL* intron and *trnL*–*trnF* intergenic spacer carry some species-specific and genus-specific synapomorphic insertions and deletions that allow us to clarify the divergence pathways in the *Anthoxanthinae* A. Gray subtribe. In particular, the *Anthoxanthum* species previously attributed by Brown to the genus *Ataxia* R. Br. have at least three genus-specific indels, form a separate clade that occupies an intermediate position between *Hierochloë* and *Anthoxanthum*, which allows these species to be restored at the rank of the genus *Ataxia*, thereby preserving the division of the traditional genera *Hierochloë* and *Anthoxanthum*. Following the Löve's genomic concept of the genus, the unique composition of the *Anthoxanthum*, *Hierochloë*, and *Ataxia* genomes is a strong basis to consider these three genera as intrinsically distinct. On the phylogenetic tree, *Hierochloë australis* (Schrad.) Roem. et Schult. is located separately from the other species of the genus *Hierochloë* at the base of the *Anthoxanthum* branch. In addition, three synapomorphic indels bring it closer to *Anthoxanthum*. But according to morphology this species is typical of the genus *Hierochloë*. In our opinion, it is possible to retain *H. australis* in the paraphyletic genus *Hierochloë*.

Keywords: *Poaceae*, *Anthoxanthinae*, *Phalarideae*, molecular phylogeny, 35S rRNA genes, ITS1–5.8S rDNA–ITS2, *trnL*–*trnF*, duplication hot spots, incongruence of plastid and nuclear markers.

Аннотация. Филогенетические взаимоотношения родов *Anthoxanthum* L., *Hierochloë* R. Br., *Ataxia* R. Br., *Phalaris* L. и родственных родов были изучены путем секвенирования внутренних транскрибируемых спейсеров ITS1 и ITS2 генов 35S рРНК и региона *trnL*–*trnF* хлоропластных геномов. Регион ITS1–5.8S рДНК–ITS2 был секвенирован и проанализирован у 30 видов и подвидов, ранее отнесенных к трибе *Phalarideae* Kunth. Интрон тРНК-лейцина (*trnL*) и межгенный спейсер между геном *trnL* и геном тРНК-фенилаланина (*trnF*) были секвенированы у 19 видов *Phalarideae*. Были проверены филогенетические гипотезы, указывающие на наиболее вероятные пути дивергенции видов *Anthoxanthum* и *Hierochloë*. Показано, что как интрон *trnL*, так и межгенный спейсер *trnL*–*trnF* содержат видоспецифические и родоспецифические синапоморфные вставки и делеции, позволяющие уточнить пути дивергенции родов и видов в подтрибе *Anthoxanthinae* A. Gray. В частности, виды рода *Anthoxanthum*, ранее отнесенные Р. Брауном к роду *Ataxia* R. Br., имеют по меньшей мере три видоспецифические вставки и делеции, образуют отдельную кладу, занимающую промежуточное положение между *Hierochloë* и *Anthoxanthum*, что позволяет восстановить *Ataxia* в ранге рода, тем самым сохраняя разде-

Поступила в редакцию | Submitted: 27.06.2025

Принята к публикации | Accepted: 24.12.2025

Опубликована онлайн | Published online: 29.12.2025 (Страницы | Pages: e08: 1–16)

ление традиционных родов *Hierochloë* и *Anthoxanthum*. Следуя геномной концепции рода А. Лёве, уникальный геномный состав *Anthoxanthum*, *Hierochloë* и *Ataxia* является веским основанием для того, чтобы считать эти три рода принципиально различными. На филогенетическом дереве *Hierochloë australis* (Schrad.) Roem. et Schult. расположен отдельно от других видов рода *Hierochloë* в основании ветви *Anthoxanthum*; кроме того, с видами *Anthoxanthum* его сближают три синаноморфных инделя в районе *trnL–trnF*. Однако по морфологии этот вид является типичным видом рода *Hierochloë*. По нашему мнению, возможно сохранить *H. australis* в парафилетическом роде *Hierochloë*.

Ключевые слова: *Poaceae*, *Phalarideae*, *Anthoxanthinae*, молекулярная филогения, гены 35S rRNA, ITS1–5.8S rDNA–ITS2, *trnL–trnF*, горячие точки дупликаций, неконгруэнтность хлоропластных и ядерных маркеров.

Introduction

Phylogenetic relationships among *Anthoxanthum* L., *Ataxia* R. Br., *Hierochloë* R. Br. and *Phalaris* L. have been discussed for a long time. Kunth (1829) and his successors placed these genera within the broadly defined tribe *Phalarideae* Kunth which encompassed not only *Hierochloë*, *Anthoxanthum*, and *Phalaris* but also such genera as *Lygeum* Loefl. ex L., *Zea* L., *Coix* L., *Alopecurus* L., *Beckmannia* Host, *Phleum* L., *Holcus* L., and some others (Kunth, 1829, 1835; Endlicher, 1836; Ledebour, 1853, and others). Since the early 1840s, a narrower circumscription of *Anthoxanthum*, *Hierochloë*, and *Ataxia* began to take place. These genera continued to be placed within *Phalarideae*, but the tribe was defined sensu stricto, including only *Anthoxanthum*, *Hierochloë*, *Phalaris* and, occasionally, some small genera occurring in Southern Hemisphere (Bosch, 1841; Wimmer, 1841; Moritz, 1844; Patze et al., 1848; Hackel, 1887; Roshevitz, 1934, and others). Due to their morphological similarities, Krause (1908) proposed to merge all species of these genera into a single genus, *Phalaris*. However, Avdulow (1931) emphasized the differences in chromosome number among *Phalaris*, *Anthoxanthum* and *Hierochloë*, arguing that these cytogenetic barriers make hybridization between the species of these genera unlikely. Based on karyological data, Avdulow (1931) excluded several genera from the *Phalarideae*, retaining only *Phalaris*, *Anthoxanthum*, and *Hierochloë*.

The placement of *Phalarideae* within *Poaceae* Barnhart has undergone numerous revisions. Bentham (1881) believed that the *Phalarideae* is closely related to *Oryzeae* Dumort. Lamb (1912) suggested a close relationship between the *Phalarideae*, *Oryzeae* and *Panicaceae* R. Br. In particular, he speculated that the *Panicaceae* have arisen from the *Phalarideae* by reduction of the spikelet. Later, Hutchinson (1973) wrote that characteristic features of *Anthoxanthum* and *Hierochloë* suggest relationship with the *Aveneae* Dumort. In the monograph “Grasses of the USSR”, Tzvelev (1976) included *Phalaris*, *Anthoxanthum*, and *Hierochloë* in the subtribes *Anthoxanthinae* A. Gray and *Phalaridinae* Fr. of the tribe *Phalarideae*. Later, he reclassified *Phalaris* as part of the diverse tribe *Phleaeae* Dumort. and transferred *Anthoxanthum* and

Hierochloë to subtribe *Anthoxanthinae* of the large tribe *Poeae* R. Br. (Tzvelev, 1989). In his final work “Grasses of Russia”, a monograph co-authored with Probatova (Tzvelev, Probatova, 2019), Tzvelev once again revised the placement of *Phalaris*, assigning it and *Phalaroides* Wolf to the subtribe *Phalaridinae* of the tribe *Poeae*, while *Anthoxanthum* and *Hierochloë* remained in the subtribe *Anthoxanthinae* within the same tribe.

This view is generally consistent with the results of molecular phylogenetic studies using plastid and nuclear DNA markers. *Phalaridinae* and *Anthoxanthinae* are currently considered as two widely separated subtribes of the *Poeae* (Soreng et al., 2015, 2017; Saarela et al., 2017; Tkach et al., 2020; Gallaher et al., 2022; Tkach et al., 2025; Grass Phylogeny Working Group III, 2025).

A particularly relevant contribution to this issue is Tzvelev’s short paper (Tzvelev, 2011), in which the section “On the genera *Hierochloë* R. Br. and *Anthoxanthum* L.” addresses the proposal by Schouten and Veldkamp (1985) to unite *Hierochloë* and *Anthoxanthum* into a single genus, which was accepted in “Flora of North America” (Allred, Barkworth, 2007), “Flora of China” (Wu, Phillips, 2006), other taxonomic treatments of *Poaceae* (Soreng et al., 2015; Tkach et al., 2020), as well as in some other papers (Villalobos et al., 2019; Chepinoga et al., 2020; de Lange, James, 2024). However, it remains far from being universally accepted (see, e. g. Tzvelev, 2006, 2011; Connor, 2008, 2012; Tikhomirov, 2010; Fedoronchuk et al., 2013; Węglarz et al., 2015; Perić et al., 2017; Tzvelev, Probatova, 2019; Jia et al., 2019; Lema-Suárez et al., 2021). For example, Tzvelev (2006, 2011) argued that the basic chromosome number differences ($x = 5$ in *Anthoxanthum*, $x = 7$ in *Hierochloë*) and clear morphological gap between them justified maintaining them as separate genera.

Molecular phylogenetic hypotheses based on comparisons of chloroplast genes showed that plants previously classified as *Athaxia* belong to the same clade as *Anthoxanthum odoratum* L., a sister clade to *Hierochloë*. On the other hand, in a tree based on the nuclear genome markers ITS and ETS, two subclades, *Athaxia* and *Hierochloë* sensu stricto, formed a clade, as sister to the *Anthoxanthum* sensu stricto (Pimentel et al., 2013; Tkach et

al., 2020). The authors suggested that *Athaxia* ancestor arose as a hybrid between *Hierochloë* and *Anthoxanthum*. Later it was shown, that *H. austarlis* Roem. et Schult. occupied an unusual position on the tree – it was closer to *Anthoxanthum* spp. than to other *Hierochloë* (Rodionov et al., 2017; Tkach et al., 2020). Even before the publication of these interesting results, Tzvelev (2011) and Tzvelev, Probatova (2019), taking into account molecular phylogenetic data of the Rayko's (2011) thesis, agreed that preserving the generic name *Ataxia* R. Br. for intermediate forms of *Anthoxanthum* would resolve the issue of transitional morphotypes, allowing the traditional approach to accept *Anthoxanthum* and *Hierochloë*. Selected parts of our research devoted to phylogeny of *Anthoxanthum*, *Ataxia*, and *Hierochloë* (Rayko, 2011) were previously published and discussed at conferences (Rayko et al., 2007, 2008; Rayko, Glusker, 2008; Rayko, 2011; Rayko, Rodionov, 2011; Rodionov et al., 2017). In this paper we present the full experimental portion of our study on these phylogenetic relationships.

Materials and methods

The study material was collected during expeditions conducted in the Leningrad and Arkhangelsk regions, the Teberda Nature Reserve (Karachay-Cherkessia, the North Caucasus), and the Altai Republic (South Siberia). Additional specimens were obtained from the herbarium of the Komarov Botanical Institute (LE) and the Central Siberian Botanical Garden (NS). Herbarium specimens of *Anthoxanthum* from Spain were kindly provided by Dr. M. P. Pereira of the University of A Coruña, Spain. Specimens of *Anthoxanthum* and *Hierochloë* from New Zealand were provided by Prof. G. Connor (University of Canterbury) and Dr. E. Cameron (Auckland Museum of Natural History). Specimens from the Main Botanical Garden RAS were provided by Dr. I. A. Schanzer. The list of studied species is presented in the [Appendix](#) (Table A1). Totally 43 samples were used for analysis, including 16 samples of *Anthoxanthum* spp. sensu stricto, 17 samples of *Hierochloë* spp., and 9 ones of *Phalaris*.

To determine the phylogenetic placement of *Anthoxanthum*, *Hierochloë*, *Phalaris* and *Ataxia* species within the *Poaceae*, we compared our sequences with those in GenBank, including representatives from closely related genera (see [Appendix](#): Table A2). Totally, the samples of 60 species and subspecies were used for phylogenetic analysis.

Genomic DNA was extracted following the Doyle and Doyle (1987) protocol with minor modifications. DNA quantity and purity were assessed using 1% agarose gel electrophoresis and a Nanodrop spectrophotometer. The ITS1–5.8S–ITS2 region was amplified using the primers ITS1P 5'-aaccttatcatttagaggaagg-3' (Ridgway et al.,

2003) and ITS4 5'-tctcgcgcttattgatatgc-3' (White et al., 1990). The PCR amplification protocol was: 1 cycle at 94 °C for 10 min; 35 cycles of 94 °C for 1 min, 48 °C for 1 min 30 s, 72 °C for 2 min; and a final elongation at 72 °C for 1 min. DNA fragments were purified from gels using the QIAquick Gel Extraction Kit 250 (Qiagen), following the manufacturer's protocol.

The chloroplast genome region *trnL-trnF* was amplified using the primers *tabC*, *tabD*, *tabE*, and *tabF* (Taberlet et al., 2007). Amplification conditions with primer pairs *tabC/D* and *tabE/F* were the same as for ITS. For the outer primer pair *tabC/F*, the protocol was: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 52–56 °C for 1 min 10 s, and 72 °C for 1 min 10 s; final extension at 72 °C for 10 min. Sequencing was performed on an ABI Prism 3130 (Applied Biosystems, USA) using fluorescently labeled terminators at the Core Facilities Center “Cell and Molecular Technologies in Plant Science” at the Komarov Botanical Institute (St. Petersburg, Russia).

Initial sequence processing and alignment were done in MEGA, using ClustalW and MUSCLE algorithms (Tamura et al., 2021). Aligned sequences were exported in FASTA format and further aligned using MAFFT (Kato et al., 2009). Phylogenetic trees were reconstructed using the Neighbor-Joining (NJ), Maximum Parsimony (MP), and Bayesian inference methods. Tree robustness was assessed via bootstrap analysis with 500 replicates. The Kimura-2-parameter model with pairwise gap deletion was used for distance calculations. For MP and Bayesian analyses, the optimal substitution model was determined using ModelTest 3.7 based on the Akaike Information Criterion (AIC), identifying the GTR+I+G (General Time Reversible with invariant sites and gamma distribution) model as the best fit. MP trees were constructed in PAUP 4.0b10, and Bayesian trees were inferred using MrBayes 3.1 (Ronquist, Huelsenbeck, 2003). Bayesian analysis ran for 500,000 generations using the GTR+I+G model.

Results and discussion

Intraspecific and interspecific variability of the ITS1–5.8S–ITS2 DNA barcode region

Among representatives of the tribe *Phalarideae* examined in this study, the length of the ITS1 region – from the TCGW to the AATCCN motif – ranged from 215 bp (*Anthoxanthum hookeri* (Griseb.) Rendle) to 223 bp (*Anthoxanthum ovatum* Lag.). The 5.8S rRNA gene was 163 bp in length, while ITS2 – from the HWAAYACGCT motif to the YYHGACS motif – varied between 212 bp (*Phalaris coeruleascens* Desf.) and 218 bp (*Hierochloë rariflora* Hook. f.). For comparison, in all *Poaceae* taxa we analyzed, ITS1 length ranged

Intraspecific variation in *Hierochloë* was found to be substantially lower than that observed in *Anthoxanthum*. The ITS sequences we obtained for *H. alpina* Roem. et Schult., *H. equisetata* Zotov, and *H. fusca* Zotov were identical to those already deposited in GenBank (Table 2). Similarly, the sequences of *H. repens* P. Beauv. from the Zhiguli Reserve and from the Volgograd Region matched each other exactly, as did the sequences from *H. australis* (Schrad.) Roem. et Schult. collected in the Leningrad Region, Russia, and in Finland.

The ITS sequences of *Hierochloë odorata* aggr. (*H. hirta* (Schrank) Borbás, *H. hirta* subsp. *arctica* (J. Presl) G. Weim., *H. odorata* (L.) P. Beauv.) (Tzvelev, Probatova, 2019), as well as *H. repens* (Host) P. Beauv. were identical (Table 2). The first three taxa make a polyploid series and may be more accurately described as eco-geographic races of a single species, *H. odorata*. Note that *H. repens*, which shares the same ITS sequence, was also once treated as a subspecies of *H. odorata* (Chrtek, Jirásek, 1964).

Among the *Phalaris* specimens we sequenced, the interspecific p-distances were markedly greater than in *Anthoxanthum* or *Hierochloë*, ranging from 3.2% to 9.1%. This suggests that *Phalaris* diverged earlier in evolutionary time than the other *Phalarideae* genera. Interestingly, the ITS1–5.8S–ITS2 sequences of *P. canariensis* L. and *P. brachystachys* Link (sect. *Phalaris*) were found to be identical, indicating either extreme similarity or possible conspecificity (Table 3). At the same time, the ITS sequences of the third species in this section, *P. truncata* Guss. ex Bertol., differed significantly from those of *P. canariensis* (Table 3).

Phylogenetic relationships of *Anthoxanthum*, *Hierochloë* and *Phalaris* inferred from the ITS1–5.8S rDNA–ITS2 sequences

Phylogenetic trees based on comparisons of ITS1–5.8S–ITS2 sequences were estimated using the Neighbor-Joining (NJ), Maximum Parsimony (MP), and Bayesian inference (BI) methods. As outgroups, we used *Secale cereale* L. and *Brachypodium distachyon* (L.) P. Beauv. All approaches yielded similar topologies; Fig. 1 shows the Bayesian tree to illustrate relationships among the species of the former tribe *Phalarideae*, with posterior probabilities indicated. The GTR+I+G model of sequence evolution was used, and the standard deviation of split frequencies was 0.018237.

Species of the former tribe *Phalarideae* were clearly divided into two well-separated, monophyletic groups: the first one comprises all *Phalaris* species, and the second includes all species of *Anthoxanthum*, *Ataxia* and *Hierochloë* (Fig. 1). The first clade corresponds to the subtribe *Phalaridinae*, and the second to the subtribe

Anthoxanthinae. This is completely consistent with the data of other authors (Soreng et al., 2015; Saarela et al., 2017; Tkach et al., 2020; Gallaher et al., 2022; Grass Phylogeny Working Group III, 2025). It is suggested that a maternal ancestor of both *Phalaridinae* and *Anthoxanthinae* was close to the supersubtribe *Agrostidodinae* Soreng, while their paternal ancestors were different. It is suggested that *Phalaridinae* had seemingly the same paternal ancestor as *Scolochloinae* Tzvelev, whereas the nrDNA donor of *Anthoxanthinae* belonged neither to *Agrostidodinae* nor to *Aveninae* J. Presl but was close to both (Tkach et al., 2020).

Within the *Anthoxanthinae* clade, five distinct subclades were identified with high posterior probabilities: (1) the diploid *Hierochloë australis* consistently occupied the basal, sister position to all other *Anthoxanthinae*, supported by both strong bootstrap and posterior probability (P = 1.0); (2) the *Ataxia* group, a distinct lineage composed of species formerly classified in the genus *Ataxia* — *Anthoxanthum hookeri* and *A. siamense*; (3) “core” *Anthoxanthum* clade, which includes *A. alpinum*, *A. nipponicum*, *A. odoratum*, and the Mediterranean *A. puelii* Lecoq et Lamotte (synonym of *A. aristatum* Boiss.); (4–5) two *Hierochloë* clades with unclear relationships (posterior probability P = 0.53): clade 4 includes three Southern Hemisphere species (*H. equisetata*, *H. novae-zelandiae* Gand., and *H. rariflora*), and clade 5 includes the Eurasian group (*H. odorata* aggr., *H. hirta*, *H. glabra* subsp. *sibirica* (Tzvelev) Tzvelev, *H. glabra* subsp. *sachalinensis* (Printz) Tzvelev, *H. glabra* subsp. *bungeana* (Trin.) Peschkova) and the Pacific Rim group (*H. occidentalis* Buckley, *H. pauciflora* R. Br., *H. alpina*, *H. fusca*).

An intriguing result is that the New Zealand *H. fusca* is closely related to the North Pacific *H. alpina*. This pattern, although seemingly paradoxical, has also been observed in *Festuca* L. (Inda et al., 2008), *Poa* L. (Rodionov et al., 2010), and in various genera across families *Apiaceae* Lindl., *Boraginaceae* Juss., *Brassicaceae* Burnett, *Caryophyllaceae* Juss., *Ranunculaceae* Juss., and *Scrophulariaceae* Juss. (Winkworth et al., 2005). In fact, gene flow from Northern Hemisphere plant taxa into New Zealand and subantarctic islands ~1–5 million years ago is increasingly well-supported (Winkworth et al., 2005; Rodionov et al., 2010).

Phylogenetic relationships between *Anthoxanthum*, *Hierochloë*, *Ataxia*, *Phalaris* and some other grasses inferred from the chloroplast sequence of *trnL–trnF*

The *trnL* intron and the adjacent *trnL–trnF* intergenic spacer evolve rapidly and are frequently used in phylogenetic studies of plants (Taberlet et al., 2007; Soreng et al., 2015; Tkach et al., 2020). The *trnL* gene

encodes Leu-tRNA and consists of one intron and two short, highly conserved exons. The *trnL* intron is the only Group I intron in plant chloroplasts. These introns act as ribozymes, capable of self-splicing without of other enzymes (Wang et al., 2022).

In our case, the aligned length of the *trnL* gene (including the intron) was 644 bp, and the *trnL-trnF* spacer was 494 bp. Structural changes were found in the intron, including insertions and deletions (indels) that affected the RNA secondary structure, particularly in loops L1–L9. Notably, three indels were found in the L6 loop (Taberlet et al., 2007), though none altered its overall folding:

1. A 5-nucleotide deletion (-AAAAC-) in loop L6 observed in *Alopecurus* and *Phleum*. According to GenBank data, it also occurs in all sequenced species of *Puccinellia* Parl. and *Hordeum* L. Interestingly, this deletion appears only in *Avena* L. species with A-genomes, not those with C-genomes (e. g., *A. macrostachya* Balansa ex Coss. et Durieu, *A. eriantha* Durieu, *A. ventricosa* Balansa ex Coss., *A. clauda* Durieu), despite the *Avena* A/C genomes divergence being dated to ~20 Mya (Fu, 2018).

2. A 4-nucleotide genus-specific insertion (AAAG) unique to the genus *Dactylis*.

3. A 5-nucleotide insertion (TCGAA) characteristic of *Calamagrostis* Adans., *Briza* L., *Ammophila* Host, and *Polypogon* Desf., and also found in *Gymnachne* Parodi and *Agrostis* L.

Additional large indels were found in loop L8 – the longest and most variable hairpin structure in the *trnL* intron. In all *Hierochloë* species, this loop was 297 bp long. In contrast, species of *Agrostis*, *Avenula* (Dumort.) Dumort., *Ammophila*, *Briza*, *Calamagrostis*, and *Polypogon* exhibited a large deletion, reducing L8 to 174 bp. An even larger deletion (down to 97 bp) was observed in *Avena*, *Arrhenatherum* P. Beauv., *Grapphephorum* Desv., *Koeleria* Pers., and *Trisetum* Pers. These major deletions appear to be synapomorphic for “core” *Aveneae*. By contrast, *Anthoxanthum*, *Hierochloë*, *Phalaris*, and all other sampled *Poeae* retained the ancestral full-length L8 hairpin.

Within species of the former tribe *Phalarideae*, we found several noteworthy *trnL* indels: ACAT insertion in *Phalaris*; GAAATTA deletion in all *Hierochloë* except *H. australis*; TTGAAA deletion in *Phalaris aquatica* L. and *P. minor* Retz. (partially overlapping the above); CCCCA duplication at the tRNA stem base in all *Anthoxanthum* spp., *Ataxia* spp., and in *H. australis* but not in other *Hierochloë* species (Fig. 2).

In the *trnL-trnF* spacer, a 16-nucleotide duplication (GTATGCCATATACAAT) was found in all *Anthoxanthum* species except *A. hookeri*, *A. siamense*, and *A. mexicanum* (Rupr. ex E. Fourn.) Mez (Figs. 2; 3).

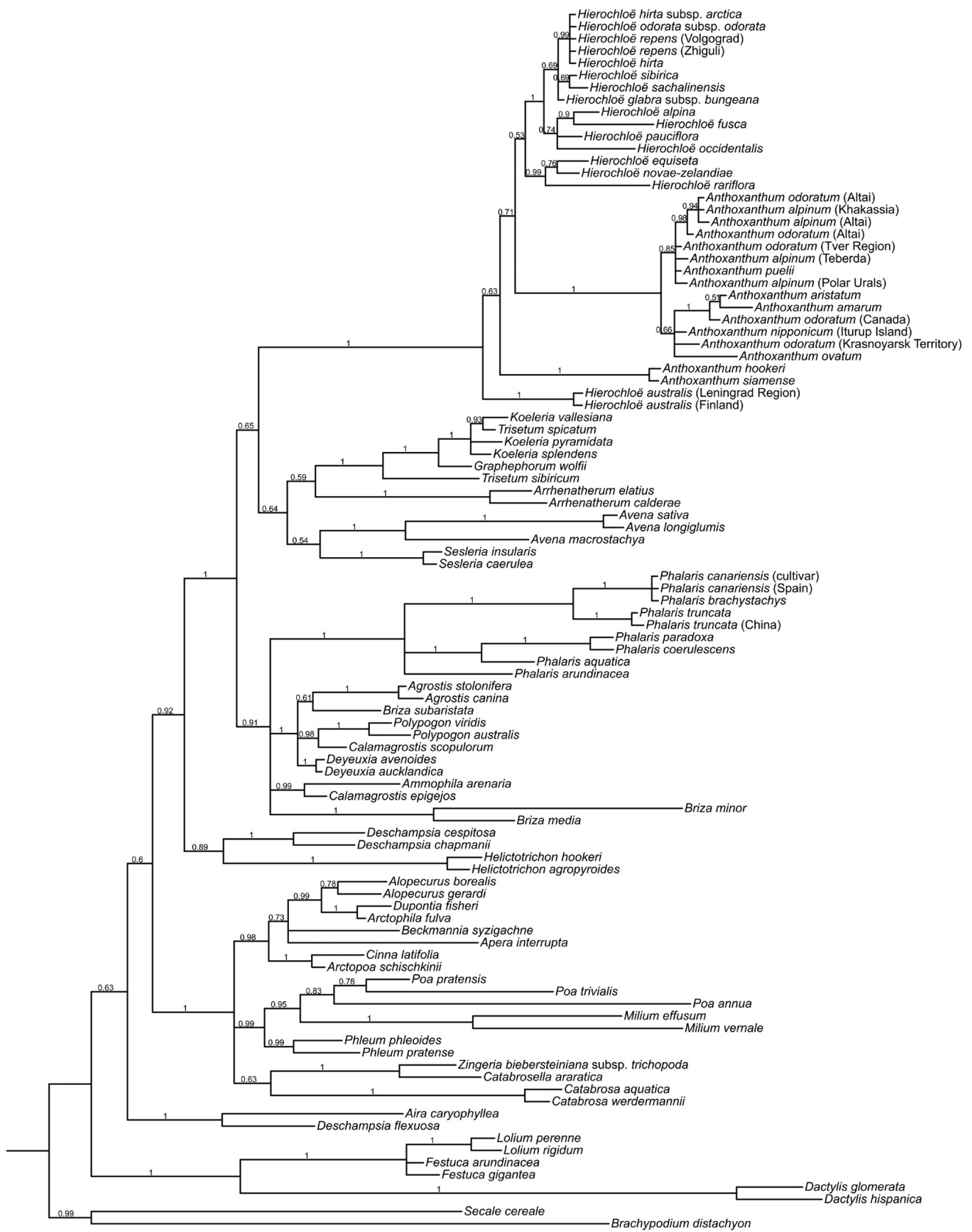
Interestingly, in the duplicated region, *Anthoxanthum* and *Hierochloë* shared a derived A→T substitution relative to the ancestral sequence (Fig. 3). It is important to emphasize that this substitution is absent from all other *Aveneae* and *Poeae* species analyzed, including the genus *Phalaris*.

In the same genomic region of *Grapphephorum wolfii* (Vasey) Vasey ex Coult., a similar duplication is found – but it is two nucleotides longer and displaced toward the 3'-end (Fig. 3). This pattern allows us to make informed inferences about the structural history of this chloroplast genomic region. It is likely that the transversion A-to-T at the site of the future duplication arose in the maternal ancestor of all *Anthoxanthinae*, and that the 16-nucleotide duplication occurred later, after the divergence of the lineages corresponding to *Hierochloë*, *Ataxia*, and *Anthoxanthum* sensu stricto. The presence of the transversion A→T exclusively in *Anthoxanthinae*, combined with differing lengths of the duplicated segments in *Anthoxanthum* and *Grapphephorum*, indicates that these are two independently originated duplications in what appears to be a structural “hotspot” of the chloroplast genome.

Phylogenetic tree was constructed based on the alignment of chloroplast *trnL-trnF* sequences. In this tree, all studied species – excluding the outgroup (*Secale* L. and *Brachypodium* P. Beauv.) – fall into two large, well-supported clades. This division has been also reported in many previous studies of chloroplast genes in the *Aveneae* and *Poeae* (e. g., Quintanar et al., 2007; Soreng et al., 2015; Tkach et al., 2020). Following Soreng et al. (2015), we refer to these two clades as the Chloroplast Group 1 (*Aveneae*-type) and the Chloroplast Group 2 (*Poeae*-type). These groups correspond broadly to the *Aveneae* and *Poeae*, though with some exceptions (Tkach et al., 2020). In our dataset, *Anthoxanthum*, *Hierochloë*, and *Phalaris* all clustered within the Chloroplast Group 1 (*Aveneae*-type) (Fig. 4). As with the nuclear ITS tree, the *Phalarideae* split cleanly into two well-separated, monophyletic lineages: subtribe *Anthoxanthinae* (*Anthoxanthum*, *Ataxia* and *Hierochloë*), and subtribe *Phalaridinae* (*Phalaris* spp.).

It turned out that the large deletions in the *trnL* intron clearly marked several taxonomically meaningful groups – the Agrostidinae + Brizinae clade (characterized by a 76-bp deletion in the L8 hairpin and a 5-bp insertion (TCGAA) in the L6 hairpin) and the Aveninae + Koeleriinae clade with a 209-bp deletion in the L6 hairpin. These structural features correspond well with clades also resolved in the ITS-based phylogeny.

Within *Phalaris*, the most basal lineage consisted of *P. canariensis* and *P. brachystachys*. Oram (2004) shown that *P. canariensis* is a domesticated derivative



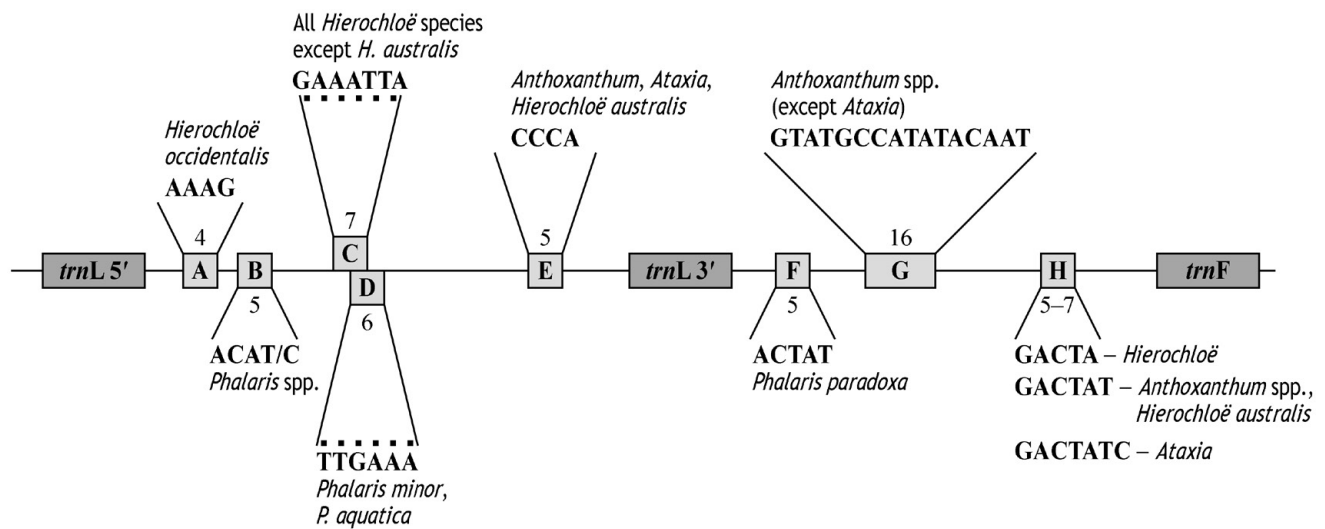


Fig. 2. Structural changes in the intron of the *trnL* gene and the *trnL*–*trnF* intergenic spacer characteristic of species in the tribe *Phalarideae*. Deletions are shown with dotted lines. Numbers indicate the lengths of insertions and deletions in regions A–H.

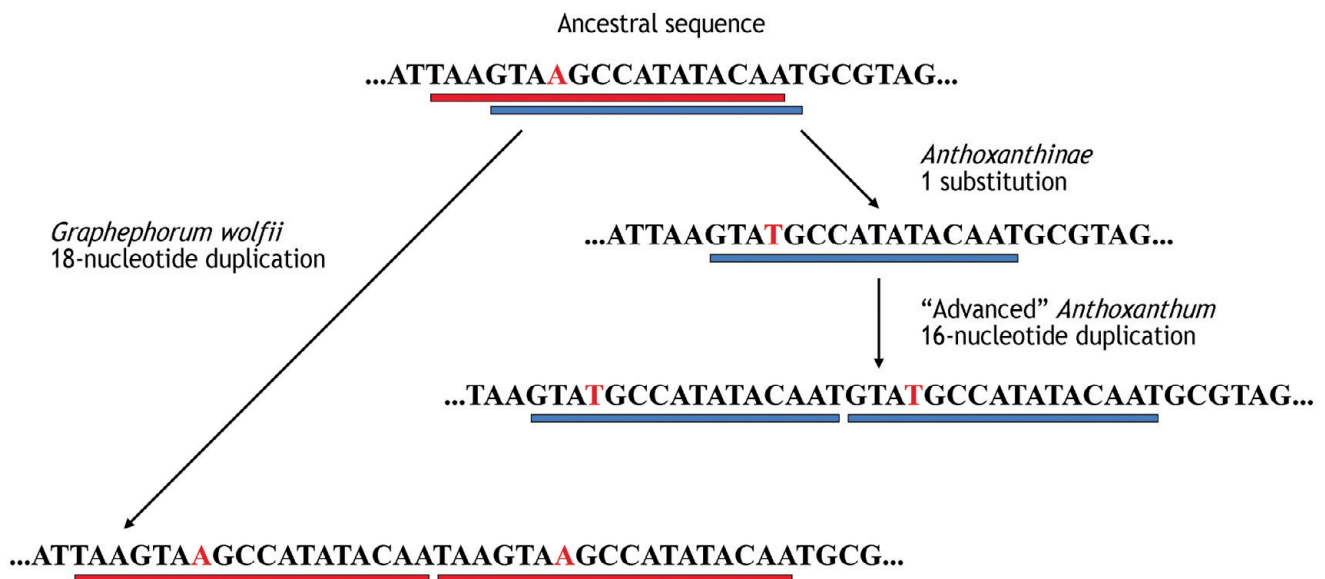


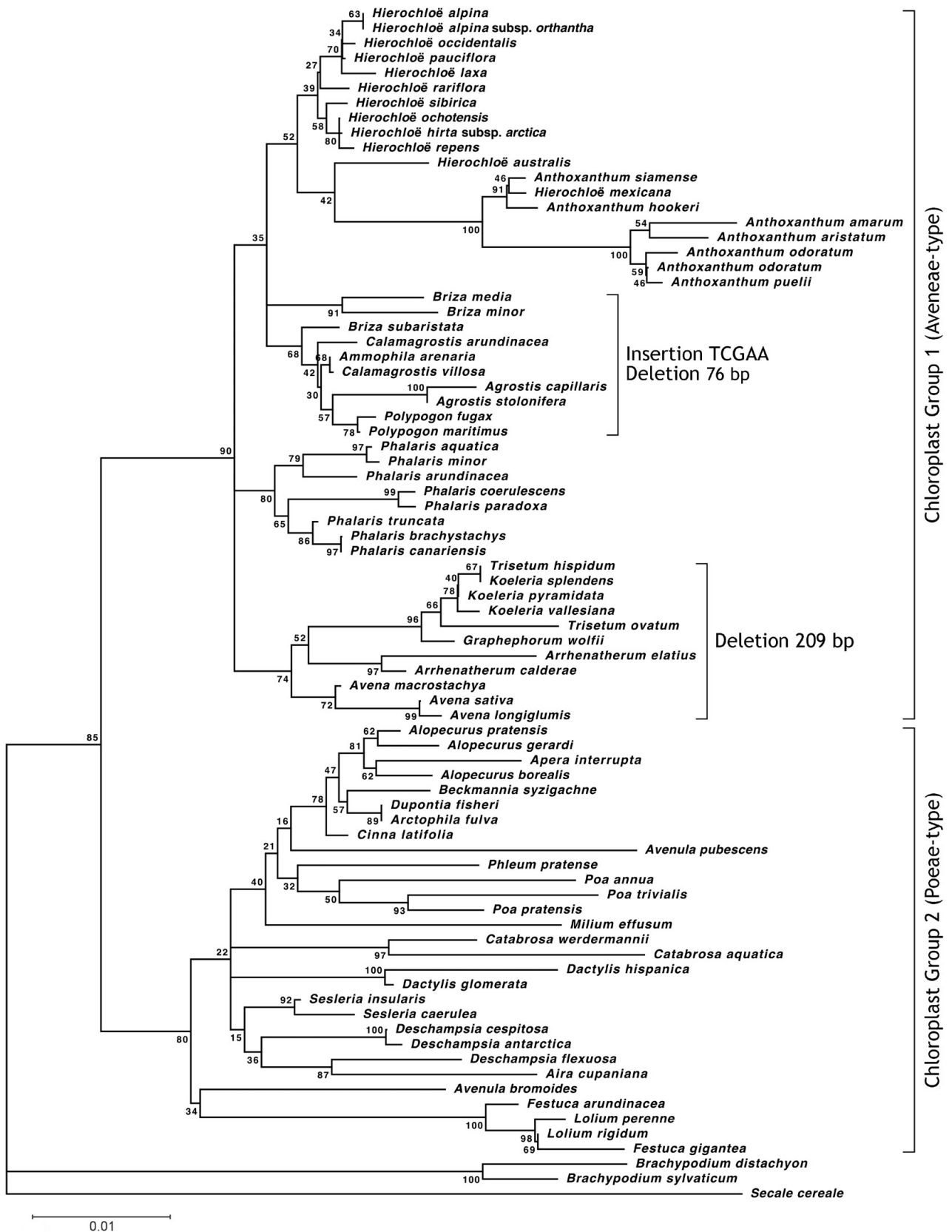
Fig. 3. Duplications in the intergenic spacer *trnL*–*trnF*.

of *P. brachystachys*. In our analysis, these two species shared identical ITS1–5.8S–ITS2 sequences and differed in chloroplast markers by only two nucleotides – supporting their very close relationship. Three species of the subgenus *Phalaris* section *Phalaris* (*P. canariensis*, *P. brachystachys*, and *P. truncata*), characterized by

a chromosome number of $2n = 12$, formed a well-supported clade on both chloroplast and ITS-based trees (Voshell et al., 2015).

A second *Phalaris* group, subgenus *Phalaroides* (Wolf) Voshell, Baldini et Hilu, consisted of the species with a base chromosome number of $x = 7$. Within this

Fig. 1. Bayesian phylogenetic tree based on comparative analysis of the ITS1–5.8S–ITS2 rDNA region. Posterior probability values are indicated on the nodes. Substitution model: GTR+I+G. First published in Rodionov et al. (2017).



group, *P. arundinacea* ($2n = 14$) emerged as an early-diverging lineage. In the ITS tree, *P. arundinacea* formed a sister branch to the rest of the genus. Tzvelev and Probatova (2019) proposed transferring it to a separate genus, *Phalaroides*, based on its rhizomatous habit, lax panicles, and glabrous glumes.

Another clade included *P. paradoxa* and *P. coerulescens*, both native to the Mediterranean, with only *P. paradoxa* occurring in Russia (European part and the Caucasus). Tzvelev (1976) classified these under section *Heterachne* Dumort., which is defined by $x = 7$ and panicles with one well-developed spikelet and 4–7 reduced ones. He also noted some affinity between *P. coerulescens* and *P. aquatica* (Tzvelev, Probatova, 2019). Our and Voshell's et al. (2015) ITS trees support this relationship, although their positions on the chloroplast tree are less congruent — *P. paradoxa* and *P. coerulescens* appear more closely related to each other than to *P. aquatica* based on cpDNA markers.

South Asian and American species *Anthoxanthum hookeri*, *A. siamense*, *A. mexicanum*: *Anthoxanthum* or *Ataxia*?

We now turn to the phylogenetic placement and chloroplast marker characteristics of several South Asian and American *Anthoxanthum* species, namely *A. hookeri*, *A. siamense*, and *A. mexicanum*. Morphologically, these species differ markedly from both the core *Anthoxanthum* (*A. odoratum* group) and from *Hierochloë*, occupying an intermediate position. In these taxa, the lower floret is male and the second is reduced — a structure distinct from that of *Anthoxanthum* sensu stricto, where both lower florets are reduced, and from *Hierochloë*, where both are male. Moreover, their lemmas are long-awned and lack lodicules — traits that correspond with a historical definition of the genus *Ataxia* R. Br., which was later subsumed under *Anthoxanthum*.

In our phylogenetic reconstructions, based on both nuclear (ITS) and chloroplast (*trnL-trnF*) markers, we observed four major taxonomic groups within the subtribe *Anthoxanthinae*, namely *Hierochloë australis*, the core *Hierochloë* species group, the *Ataxia* group (*A. hookeri*, *A. siamense*, *A. mexicanum*), and the “advanced” *Anthoxanthum* species group (*A. odoratum*, *A. alpinum*, etc.). However, the branching order among these four groups varied between the nuclear and chloroplast trees, and the relevant nodes were poorly supported (bootstrap support about 40–60%). The same clades were shown by Pimentel et al. (2013) and Tkach et al. (2020).

The topology of the phylogenetic tree inferred from ITS sequence analysis appears to be the result of reticulated evolution among genera in the subtribe *Anthoxanthinae* (Pimentel et al., 2013; Tkach et al., 2020). The following scenario of taxon origin in *Anthoxanthinae* can be proposed: their common ancestor was a diploid with a basic chromosome number of $n = 7$ and morphologically close to *Hierochloë australis*. From this ancestral lineage, a group of species diverged, to give rise to the genus *Ataxia*, followed by the phylogenetic split between the polyploid lineages of *Hierochloë* and *Anthoxanthum* sensu stricto (Fig. 1). We know of only one report of chromosome number in *Ataxia*. Spies and Voges (1988) found that gametophytic (n) chromosome number in *Anthoxanthum tongo* (Trin.) Stapf (syn. *Ataxia tenuis* Trin.) is 20 plus 0–5 B chromosomes. In addition, it was shown that the genome size of *A. mexicanum* ($2C = 18–19$ pg) (Lema-Suárez et al., 2018) is the same as that of the presumably tetraploid species *Anthoxanthum nitens* (Weber) Y. Schouten et Veldkamp ($2C = 19.5$ pg) (Tkach et al., 2025). It is very likely, that this and other *Ataxia* species are allopolyploids.

If this scenario is correct, then the reduction of the second floret in *Ataxia* species and the reduction of both lower florets in *Anthoxanthum* species must have occurred independently in two separate branches. Furthermore, the duplication of the motif CCCCA in the *trnL* intron (Fig. 2) must either (a) have arisen independently in *H. australis* and later in the common ancestor of the branch *Anthoxanthum* + *Ataxia*, after their divergence from the polyploid *Hierochloë* branch, or (b) a reversion must have occurred in the *Hierochloë* lineage, restoring the ancestral non-duplicated state of this motif. This is possible, but a more plausible explanation is a different sequence of floret reduction events and changes in the *trnL* region of the chloroplast genome, assuming that the branching order of the *Ataxia*, *Anthoxanthum* sensu stricto, and *Hierochloë* lineages follows the topology of the chloroplast-based phylogenetic tree (Fig. 4).

In this alternative scenario, a mutation A→T occurred in the ancestor of *Anthoxanthinae* in the region that would later be duplicated (a 16-nucleotide duplication), and possibly a duplication of GACTA or GACTAT appeared in the *trnL-trnF* intergenic spacer (Fig. 3). After that, the divergence between *Anthoxanthum* sensu stricto and the polyploid *Hierochloë* lineage took place. Later, in the *Hierochloë* lineage, a deletion of the GAAATTA motif occurred in the *trnL* intron (Figs. 2; 5), whereas in the branch leading to *Anthoxanthum* and

Fig. 4. Phylogenetic tree constructed using the *trnL-trnF* region (Neighbor-Joining method). Bootstrap values from 500 replicates are shown.

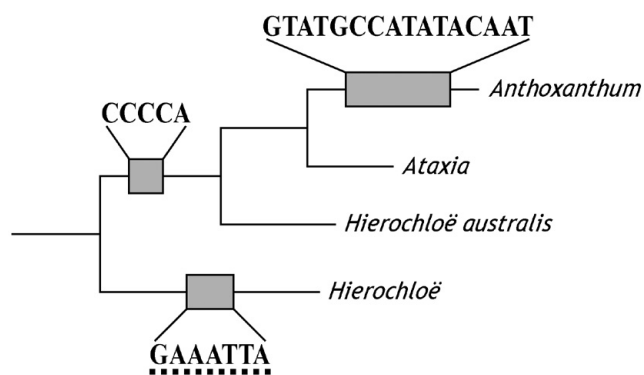


Fig. 5. Changes in the *trnL*–*trnF* region of the chloroplast genome during the divergence of *Anthoxanthinae* taxa.

H. australis, the CCCCCA motif was duplicated in the same region (Figs. 2; 5). Then, the ancestors of *Anthoxanthum* + *Ataxia* diverged from those of *H. australis*. In the former, reduction of the second floret occurred in the intergenic spacer, and a subgroup (*Ataxia*) retained this trait. Later, in the lineage of northern Eurasian *Anthoxanthum*, further reduction of the second lower floret took place, a distinctive 16-nucleotide duplication emerged in the *trnL*–*trnF* spacer, and the base chromosome number was reduced to $x = 5$.

According to the first scenario, one might propose to separate *H. australis* into independent genus; under the second scenario, it could be combined with *Anthoxanthum*. However, morphologically *H. australis* is a typical *Hierochloë*, with a three-flowered spikelets containing two lower staminate florets, short awns, and membranous glumes. Therefore, the only viable conclusion is to accept the paraphyly of the genus *Hierochloë* as a given.

Additional support for the phylogenetic scenario presented in the chloroplast tree comes from shared insertions and deletions between *H. australis* and *Anthoxanthum*, which may be interpreted as synapomorphies (Figs. 2; 3; 5). Among these indels, only the duplication in the right portion of the *trnL*–*trnF* intergenic spacer presents an ambiguous pattern: in polyploid *Hierochloë*, this is a $(GACTA)_2$ duplication; in *Anthoxanthum* sensu stricto and *H. australis*, it is $(GACTAT)_2$; and in *Ataxia* species, it is $(GACTATC)_2$. A simple evolutionary scenario is not apparent in this case – it is likely that this region represents a hotspot where duplications of these short oligonucleotides arose independently in the lineages of polyploid *Hierochloë*, *Ataxia*, *Anthoxanthum*, and *H. australis*, similarly to the independent emergence of the 16- and 18-nucleotide duplications in the *trnL*–*trnF* intergenic spacer of *Grappophorum* and *Anthoxanthum* sensu stricto (see Fig. 3).

Thus, the molecular phylogenetic analysis of 18S rDNA supports the views of Brown (1823: 35) and Tzvelev (2011) regarding the taxonomic justification for recognizing *Ataxia* as a distinct genus. This genus occupies an intermediate position between *Hierochloë* and *Anthoxanthum* and, together with them, forms a natural and well-defined taxonomic group.

Conclusions

We agree with the opinion of distinguished agrostologists (Connor, 2008, 2012; Tzvelev, 2011; Lema-Suárez et al., 2018) that the fusion of *Anthoxanthum* and *Hierochloë*, proposed by Schouten and Veldkamp (1985), does not seem productive. Connor (2012) and Tzvelev (2011) believed that both genera, *Hierochloë* and *Anthoxanthum*, should be maintained because of their floral distinctions and differences in base chromosome numbers (*Anthoxanthum*, $x = 5$, *Hierochloë*, $x = 7$). In addition, Pimentel et al. (2013) suggested the likely origin of an ancestral *Ataxia* by hybridization between *Hierochloë* and *Anthoxanthum*. If this is the case, then according to the genomic criterion of the genus proposed by Löve (1984), unique nuclear and cytoplasmic genome compositions of *Anthoxanthum*, *Hierochloë* and *Ataxia* are a strong basis to consider these three genera as intrinsically distinct. These arguments, in our opinion, convincingly testify in favor of keeping a separate genus *Ataxia* R. Br., as proposed by Tzvelev (2011).

Acknowledgements

We are grateful to Dr. Irina Sokolova and Ivan Tatanov of the Komarov Botanical Institute, to the Editor and anonymous reviewers for their very helpful comments and suggestions on the first version of the paper. This study was supported by the Russian Science Foundation, grant 24-24-00326 and conducted as a part of the program research “Molecular phylogenetic studies and karyosystematics of flowering plants”, state registration number 124020100136-0, with using equipment of the Core Facilities Center “Cell and Molecular Technologies in Plant Science” at the Komarov Botanical Institute (St. Petersburg, Russia).

Supplementary materials

Appendix

Table A1. List of samples whose ITS1–5.8S rDNA–ITS2 and *trnL* sequences were sequenced and analyzed in this study.

Table A2. Species from the closely related genera obtained from the GenBank.

https://www.binran.ru/files/journals/Novitates/2025_56/NSPV_56_08_Rayko_Rodionov_Appendix.pdf

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