Peter Englmaier[1], Thomas Wilhalm[2]

Phylogenetic remarks on hexaploid varicoloured fescues (*Festuca* sect. *Eskia*, Poaceae) in the Southern Alps

[1] OECONSULT, Expert Consultancy for Ecological Sciences, Vienna, Austria and University of Vienna, Faculty of Life Sciences (retired)
[2] Museum of Nature South Tyrol, Bolzano, Italy

Introduction

Varicoloured fescues (*Festuca varia*-group, *Festuca* sect. *Eskia*) are widespread throughout the South European and Southwestern Asian mountain ranges. Although many different taxa were described in the early days of intensive botanical research, the knowledge of their characteristics is rather limited. Most of the taxa have been confused either among each other or with other species with varicoloured spikelets.

Ploidy levels and reliable data on distribution of several taxa throughout the Alps are known even for a quarter of a century (Wallossek 1999, 2000). First data on molecular phylogeny exist since 2002 (Torrecilla & al. 2002; Catalán & al. 2004; de Nova & al. 2006).

In addition to the diploid *Festuca acuminata*, two hexaploid taxa (*F. scabriculmis* subsp. *luedii* and subsp. *handel-mazzettii*) occur in the Southern Alps, whose morphology suggests relationships to each other and to the Western Alpine *F. scabriculmis* subsp. *scabriculmis*. Based on these considerations, an adapted taxonomic concept has already been presented (Englmaier 2020).

However, questions about the origin of these hexaploid taxa and their ancestors remain unresolved.

Thus, they were target of a high resolution molecular genetic exploration, which included not only nuclear (ITS) and plastid markers (*trn*L-F) but also other markers already tested on the genus *Festuca* (especially microsatellite loci, Segarra-Moragues & Catalan 2011). This required extensive methodological considerations, which ultimately led to a selection of 9 primer pairs already tested on *Festuca* or *Lolium*. 7 of these provided amplicons whose sequences have now been subjected to an initial evaluation.

Materials and methods

Plant collection, cultivation and sampling

Accessions of each of the two hexaploid taxa (*Festuca scabriculmis* subsp. *luedii* (West), *F. scabriculmis* subsp. *luedii* (East) and *F. scabriculmis* subsp. *handel-mazzettii*) were selected, plus accessions of each of the diploid species *F. acuminata* and *F. alpestris*, as well as of a further hexaploid taxon, *F. varia* subsp. *winnebachensis* for comparative purposes. *F. laevigata* was selected as an outgroup (see Figure 1 for sampling sites).

Each accession represents one single plant. Parts of the huge tufts were removed for cultivation in the Botanical Garden (HBV), University of Vienna and conserved as a herbarium voucher.

Fresh leaves were taken from cultivated plants for analysis and stored at approx. 200 K up to processing.

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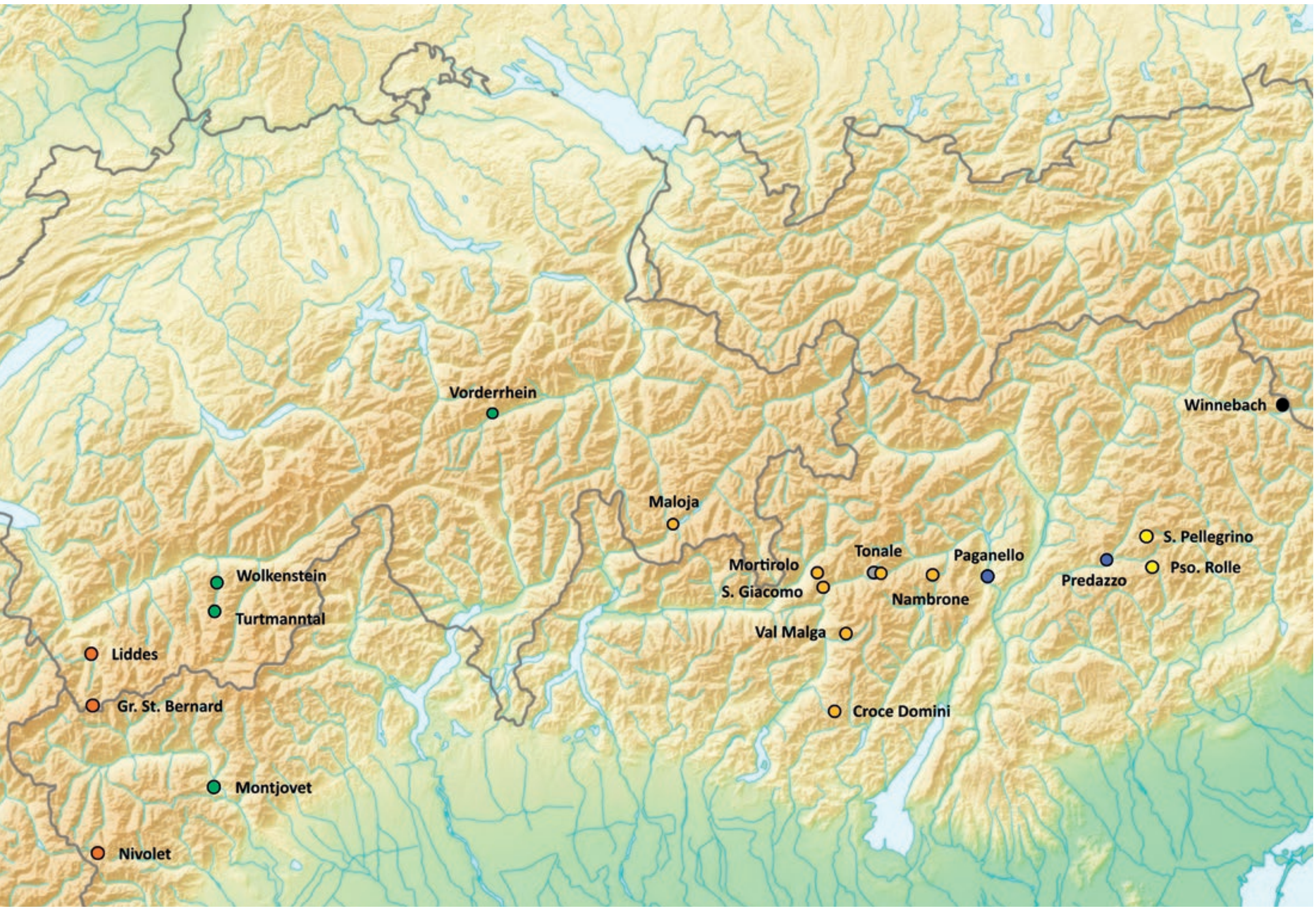

Figure 1: Taxa and sampling sites.
Festuca alpestris (blue signs): 2 populations, 10 accessions
Festuca acuminata (green signs): 4 populations, 15 accessions
Festuca scabriculmis subsp. *luedii*, western area (red signs): 3 populations, 11 accessions
Festuca scabriculmis subsp. *luedii*, eastern area (orange signs): 7 populations, 11 accessions
Festuca scabriculmis subsp. *handel-mazzettii* (yellow signs): 2 populations, 10 accessions
Festuca varia subsp. *winnebachensis*, for comparison (black sign): 1 population, 5 accessions
Festuca laevigata s. l., outgroup (grey sign): 1 population, 5 accessions
(Basemap source: Wikimedia commons, Lencer/Pechristener, CC-BY-SA3.0)

Sample preparation, sequencing and data analysis strategy

The following regions were selected for comparability with published studies:
Plastid *trn*L-F, using already tested primers „c“, and „f“ (Taberlet & al. 1991: 1106).
Nuclear ITS, using primer „18S-KRC“ (Torrecilla & Catalan 2002: 243) and „ITS-p4“ (Cheng & al. 2016: 14;, Kolter & Gmeinholzer 2021: 135), with a 4-bp-shift towards 5’ compared with common ITS4 as used by Torrecilla & Catalan (2002), to avoid interferences caused by endophytic DNA.
In addition, microsatellite targets were selected, according to Segarra-Moragues & Catalan (2011: 361) and Tomczyk & al (2020): A101, A 104, A115, A117, B101, B104, and C 105.
After DNA extraction, indexing and amplification, sequencing was done on an AVITI instrument (Element Biosciences, San Diego, CA) using 300-bp paired-end mode.
All labwork was done by IGAtech, Udine, Italy.
Results were generated by analysis of amplicon sequence variants (ASV).

Results

Analysis of the plastid-*trn*L-F region revealed a total of 7 ASV. One is exclusively represented in *F. acuminata* (with exception of the Vorderrhein population [c1-c5], 85 %), exclusively in *F. s.* subsp. *handel-mazzettii* [r, s], in subsp. *luedii* (exclusively, with the exception of the Gran Paradiso population [h], 85 % and populations from the Pso. Mortirolo area [l, m], 65 %) and in *F. v.* subsp. *winnebachensis* [t], 30 %. Another one is represented in small proportions in *F. s.* subsp. *luedii* (from Gran Paradiso [h], 15 %, from the area around P.so Mortirolo [l, m], 30 %) and in *F. acuminata* (from Vorderrhein [c1-c5], 15 %. Four ASV are exclusively represented in *F. alpestris*, one of them exclusively in the Predazzo population [a1-a5].

Analysis of the nuclear ITS region also revealed a total of 7 ASV. One is present in all taxa and all accessions, in varying frequencies: in *F. alpestris* [a1-5, b1-5] exclusively, in *F. acuminata* (from the Aosta Valley [f1-f5 50 %, all others exclusively), in *F. scabriculmis* subsp. *handel-mazzettii* 60 %, in subsp. *luedii* generally 25–60 % (from the Maloja Pass [n] 80 %), in *F. varia* subsp. *winnebachensis* [t] 80 %, in *F. laevigata* [u, outgroup] 85 %. Others were detected in individual taxa or populations.

Among microsatellite loci, A104 showed by far the greatest diversity and was therefore preferred for analysis. 18 ASV were identified. Two of these were exclusively found in each of the two diploid taxa (*F. alpestris* [a, b] and *F. acuminata* [c-f]. Five of these were found in individual populations of all taxa analyzed, the others are characteristics of different provenances of certain taxa. Clear differentiations between the two subareas of *F. s.* subsp. *luedii* are recognizable neither in this nor in other ms loci used in this study.

For all taxa, variability of all markers within populations was significantly lower than even between neighboring populations.

Discussion

Our results clearly point out, that *F. scabriculmis* subsp. *luedii* and subsp. *handel-mazzettii* are closely related to each other and to *F. acuminata* and result from hybridization with *F. acuminata* as the only maternal ancestor, as they have nearly identical plastid ASV composition. In contrast, the third hexaploid, *F. varia* subsp. *winnebachensis*, is clearly distinct. According to Mucko & al. (2024), a connection to taxa occurring in the Eastern Alps (*F. calva* and *F. varia* subsp. *varia)* is probable, but requires further confirmation.

It is also noticeable that there is a remarkable variability of microsatellite markers within *F. scabriculmis* subsp. *luedii*, which is seen both in spatial (populations around the Adamello Mts.) and in altitudinal scales (adjacent populations up to the Mortirolo Pass and along the Valle dell'Orco up to the Col de Nivolet), but not any significant differences were recognizable between the two conspicuous sub-areas of subsp. l*uedii*. Only the isolated population around the Maloja Pass is clearly separated.

Within *F. acuminata*, the northern (Rhine valley) and southern (Aosta valley) populations are slightly different from the Valais populations.

Outlook

Further investigations should show to what extent the populations in the Southern Alps are embedded in the overall Alpine diversity of varicoloured fescues, i.e., whether the *F. acuminata* populations of the Western and Maritime Alps, as well as *F. scabriculmis* subsp. *scabriculmis* occurring sympatrically there, correspond to the related Southern Alpine taxa.

Likewise, the position of *F. pumila* in relation to these taxa will also be of interest, whether the findings of Mucko & al. (2024) will be confirmed on closer examination and whether this morphologically variable taxon also shows a relatively high intraspecific genetic variability.

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