

Uncovering alkaloid chemotype diversity in natural *Festuca–Epichloë festucae* symbioses across Iberian ecosystems

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Epichloë fungal endophytes are characterised by their biotrophic lifestyle and their highly specific and variable interaction with cool-season Pooideae grasses. These endophytes grow systemically in the apoplast of the aerial tissues and can reproduce sexually, asexually or interspersing both lifecycles. Within the cosmopolitan genus *Festuca* (Poaceae), *Epichloë festucae* is one of the most prevalent *Epichloë* endophytes, appearing in up to 30 taxa. This interaction has been thoroughly studied in agricultural species (e.g., *Lolium perenne*) due to the impact of endophyte-expressed secondary metabolites on farming and livestock production. To date, four alkaloid classes have been identified: ergot alkaloids, 1-aminopyrrolizidines (including lolines), indole-diterpenes and pyrrolopyrazines (including peramine), exhibiting toxicity or deterrence to vertebrates, insects or both.

This study characterised the holobionts formed by five fine-leaved *Festuca* species (*F. lambinonii*, *F. nigrescens*, *F. rubra* subsp. *pruinosa*, *F. yvesii*, and *F. rothmaleri*) and their respective *E. festucae* strains across diverse Iberian ecosystems. The specific objectives were: (1) to perform an integrative characterisation of both symbionts using molecular, cytogenetic and morphological approaches; and (2) to quantitatively and qualitatively assess the chemotypes and conduct genetic screenings of endophytes in these holobionts. A wide variety of chemotypes was recorded, finding differences between individuals at intra- and

interspecific levels. For pyrrolopyrazines, peramine was detected in some individuals within the species *F. yvesii* and *F. rothmaleri*, whereas an alternative compound (pyrrolopyrazine 2a) was detected in the remaining individuals of these species and in all *F. rubra* subsp. *pruinosa* and *F. lambinonii* samples. For ergot alkaloids, only *F. rubra* subsp. *pruinosa* showed traces of chanoclavine and quantifiable ergovaline. Indole-diterpenes (e.g., paxilline, terpendole isomers, paspaline and 13-desoxypaxilline) were variably present in *F. nigrescens*, *F. yvesii*, and *F. rothmaleri*, whereas 1-aminopyrrolizidines were absent in all samples. Interestingly, results of the alkaloid analyses were not always consistent with the results of the genetic screens: in some cases, the genes of biosynthetic pathways were present, but the corresponding alkaloids were undetected.

Altogether, the presence of at least two alkaloid classes were detected for all studied species, with the exception of *F. lambinonii*, suggesting that the host species might obtain protection against invertebrates and, for those that produce certain ergovaline or certain indole-diterpenes, also against vertebrates. We believe that these findings evidence the importance of characterising naturally distributed holobionts to expand our comprehension regarding the secondary metabolism associated with this symbiosis and the plasticity of *E. festucae*.