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Genetic Evidence for Reproductive Isolation Among Sympatric *Epichloë* Endophytes as Inferred from Newly Developed Microsatellite Markers

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Abstract Reproductive isolation is central to the maintenance of species, and especially in sympatry, effective barriers to prevent interspecific crosses are expected. Host specificity is thought to constitute an effective mechanism for the formation of barriers in different genera of Fungi, but evidence for endophytes is so far lacking. Sexual *Epichloë* species (Ascomycota, Clavicipitaceae) represent an ideal study system to investigate the mechanisms underlying speciation as mediated by host specificity because they include species complexes with several host-specific taxa. Here, we studied genetic differentiation of three host-specific *Epichloë* species using microsatellite markers that were newly in silico identified on the genome of *Epichloë poae*. Among these, 15 were experimentally tested and applied to study an extensive sampling of isolates representing *Epichloë typhina* infecting *Dactylis glomerata* and *Epichloë clarkii* infecting *Holcus lanatus* from a site with sympatric populations in Switzerland, as well as a reduced sampling of *E. poae* infecting *Poa nemoralis* to create a three-taxon dataset. Both principal coordinate analysis and Bayesian clustering algorithm showed three genetically distinct groups representing the three host-specific species. High pairwise F_{ST} values among the three species, as well as sequencing data of the *tefA* gene revealing diagnostic single nucleotide polymorphisms (SNPs), further support the hypothesis of genetic discontinuities among the taxa. These results provide genotypic evidence of the maintenance of reproductive isolation of the species in a context of sympatry. In silico testing of 885 discovered microsatellites on the genome of *Epichloë festucae* extend their applicability to a wider taxonomic range of *Epichloë*.

Keywords Endophytes · Microsatellites · Population structure · Reproductive isolation · Sympatry

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Introduction

Speciation in fungi can occur through numerous mechanisms resulting in the formation of isolating barriers among divergent lineages. Compared to speciation in plants and animals, where geographic separation has long been hypothesized to be a strong driver in the formation of current biodiversity, the mechanisms underlying speciation in fungi have been less amenable to a general consensus [1–3]. Indeed, as eukaryotic microorganisms typically have global geographic ranges, geographic isolation has been questioned as a promoter of divergence [4]. Similarly, the process of sympatric speciation has often been regarded as unlikely because minimal gene flow is sufficient to prevent differentiation [5]. However, recent advances facilitated by the use of genome-wide data in well-established model systems [6, 7] are greatly contributing to the understanding of speciation in fungi, and evidence is emerging for both types of speciation processes. Several instances have been described, where divergence originated in a context of allopatry, but speciation remained cryptic because of undistinguishable phenotypes within species complexes [8–10]. On the other hand, speciation in sympatry may indeed

occur following the formation of isolating barriers, and in this context, adaptation to different hosts has been proven to be a strong driver of this process [11–13]. Because of the difficulty to separate between development and mating, mutations providing adaptation to a new habitat can pleiotropically affect both the fitness and the ability to mate in this habitat, which can thus be sufficient to restrict gene flow in sympatry [2, 14]. However, whether host specificity is indicative of different species in a context of sympatry depends on the efficiency of the isolating barriers that prevent admixtures of genotypes, which should be proven with population genetic data.

Sexual species of *Epichloë* (Ascomycota, Clavicipitaceae) constitute an ideal study system to investigate the genetics behind reproductive isolation, as they form host-adapted species complexes with pooid grasses (Poaceae) that are maintained in sympatric populations under a strong depletion of natural hybrids [15–17]. The endophytes grow in plant apoplasts primarily in shoot apical meristems and leaf sheaths [18] with little or no negative effects on the vegetative tissues of their hosts. However, during their sexual life cycle, they may form external reproductive structures, i.e., stromata, that partially or completely inhibit host flowering and seed production. The effect of such interaction on the plant is known as "choke disease" [19]. Sexual species of *Epichloë* are heterothallic and possess a bipolar self-incompatibility mating system [20]. Successful mating, therefore, requires transfer of spermatia (male gametes) between stromata of opposite mating types, usually mediated by specialized flies of the genus *Botanophila* (Anthomyiidae) in a process similar to pollination [21].

Most sexual *Epichloë* species are circumscribed based on their reproductive incompatibilities with other species following a biological species concept [22]. Based on intersterility tests between species, nine different mating populations corresponding usually to a single taxonomic *Epichloë* species were identified [23]. Each species typically shows a rather narrow host range restricted to grass species of one genus or closely related host genera, with the exception of *Epichloë typhina* that forms a complex with several host-specific taxa [22]. Some of the species of the *E. typhina* complex have been demonstrated to be able to hybridize in artificial experiments of cross-fertilization, but appear to remain reproductively isolated in natural populations [24, 25].

A particular case where this is evident to a large extent includes *Epichloë clarkii* infecting *Holcus lanatus* and *E. typhina* infecting *Dactylis glomerata*. These host-specific species are morphologically well defined [26] and occur scattered at multiple locations of their distribution range throughout temperate Europe and western Asia. In a recent field study, Bultman et al. [27] described sympatric populations of the two species infecting hosts growing intermixed at the site of Aubonne in Canton Vaud, Switzerland. Here, hybrid ascospores were reported in a small fraction (~9 %) of the fertilized stromata; however, capability of these spores to infect hosts seemed to be virtually null, as grasses infected with hybrid *Epichloë* were never found. This scenario suggests that *E. clarkii* and *E. typhina* have developed strong isolating barriers that maintain the species boundaries. However, the mechanisms that underlie the maintenance of the species barriers are largely unknown.

Addressing questions of speciation in sexual *Epichloë* species involves careful examination of the structure and hybridization of natural populations for which efficient molecular tools, such as microsatellites, are necessary. Previously, microsatellite markers have been developed to study the ecology and diversity of *E. bromicola* and *Neotyphodium* spp. [28, 29], and for identifying different sexual and asexual *Epichloë* endophytes in planta [30–32]. Most *Neotyphodium* species, today classified in the genus *Epichloë* [33], are thought to be derived from ancestral *Epichloë* species, often as a result of ancient hybridization events [34, 35]. However, the available microsatellites yielded unsatisfactory results when tested on our samples, probably because of partial primer mismatches among *Epichloë* species.

In this study, we aimed to investigate the genetic signature of species delimitation in the case of the two sympatric populations of *E. typhina* and *E. clarkii* at the Aubonne site in order to gain insight in the genetic differentiation of the fungal species and provide genetic evidence of their effective reproductive isolation. To achieve this goal, we developed a new set of microsatellite markers starting from whole genome sequences of *Epichloë poae* [36] to detect markers that are potentially applicable to a wide range of species in the genus. To our knowledge, this study is the first that addresses the genetics behind reproductive isolation among sympatric *Epichloë* species.

Material and Methods

Computational Workflow for Microsatellite Design

A workflow was developed to find, in silico test and filter microsatellites in the genomic DNA sequences of *E. poae* strain E5819 downloaded from Genome Projects at University of Kentucky (http://www.endophyte.uky.edu). The workflow employed in-house Perl scripts calling the Tandem Repeats Finder v. 4.0.4 [37] to find repeats, Primer3 v. 2.3.0 [38] to construct primers in the flanking regions, and BLAST+ v. 2.2.26 [39] for in silico primer testing on the genome.

Primer pairs were designed in order to obtain PCR products between 100 and 500 bp long. The workflow allowed for filtering of the microsatellites into PCR fragment size classes and motif length classes. Primers with more than one potential PCR product were discarded. Furthermore, the primers were in silico tested on the genome of the grass species

Brachypodium distachyon strain Bd21-1 downloaded from the *Brachypodium* Genome Database (http://www.brachypodium.org) to reduce the potential risk of cross-amplification on the genome of the host plants. This should allow better use of the primers for experiments targeting the endophytes in planta. An additional primer blasting against *E. festucae* strain E894 genome (Genome Projects at University of Kentucky) with up to two mismatches was performed to identify shared repeats with a closely related species and thus create a large dataset of markers potentially applicable to a broad taxonomic range within the genus.

Primer Testing

Lab protocols of newly developed microsatellites were initially tested on a set of samples, hereafter named "lab strains," which included pure cultures of *E. poae*, *E. typhina*, *E. clarkii*, and *Epichloë bromicola* and F1 progeny of four crosses (i.e., *E. clarkii*×*E. clarkii*, *E. typhina*×*E. typhina*, *E. clarkii*×*E. typhina*, and *E. typhina*×*E. clarkii*; Table 1) grown on potato dextrose agar (PDA; BD Comp., Sparks, MD, USA) to test the detectability of hybrids, should they occur in natural populations. Crosses were performed in the greenhouse on freshly emerging stromata on the grasses from which the parental fungal strains were collected.

Microsatellite sequences were in silico screened for 100 % perfect di-, tri-, tetra-, penta-, and hexanucleotides on the genome of *E. poae* with an optimal annealing temperature between 58 and 60 °C [40]. In total, 75,432 potential primer pairs amplifying 8451 microsatellites were generated by our workflow from *E. poae* whole genome sequences. Out of these, 8136 primer pairs covered 1084 microsatellites with motifs repeated 5–20 times. Fifty-one primer pairs were randomly chosen across repeat types and labeled with cost-efficient M13 [41] to sort out non-working microsatellites in preliminary tests performed on the lab strains. The PCR volume of 10 μl contained approximately 1 ng of genomic DNA, with 5× PCR Buffer (Promega), 5 U/μl Go-Taq Polymerase (Promega, Madison, WI, USA), 25 mM $MgCl_2$, 2.5 mM of each dNTP, 2.5 μM of forward primer, and 2.5 μM of each reverse and universal FAM-labeled M13 primers. Amplification was carried out separately for each locus on Sensoquest labcycler (Witec AG, Luzern, Switzerland) with initial denaturing at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, followed by 8 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s with a final extension of 60 °C for 10 min. All PCR products were run with GeneScan-500 LIZ as size standard on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and electropherograms were analyzed using Geneious 6.1.8 [42].

Out of 51 tested M13 primer pairs, 38 successfully amplifying were retained (Table 1). Sixteen of the microsatellites consisted of dinucleotide repeats, 12 of trinucleotide repeats, eight of tetranucleotide repeats, one of a pentanucleotide motif, and one of a hexanucleotide motif. Successful amplification of these primers across our lab strains ranged from 82 to 100 % (Table 1). The targeted PCR fragments containing microsatellites averaged 294 bp in length and ranged between 107 and 478 bp.

Eventually, 15 polymorphic markers were chosen based on clean and consistent peaks without stuttering in order to decrease the error rate. To create PCR multiplex sets, primer pairs were selected according to their different product sizes and labeled with one of three fluorescent labels (6-FAM, HEX, and ATTO0550). Finally, markers were arranged in four multiplex sets (Table 2) and tested on the lab strains using a PCR volume of 10 μl containing approximately 1 ng of genomic DNA, 2× Multiplex Mastermix (Multiplex PCR Kit, Quiagen), and 10× Primermix (2 μM). Amplification was carried out on Sensoquest labcycler (Witec AG) with initial denaturing at 95 °C for 5 min, followed by 28 cycles of 95 °C for 30 s, 58 °C for 90 s, and 72 °C for 30 s, with a final extension of 60 °C for 30 min.

Table 1 Species with their respective hosts and experimental hybrid progeny with numbers of isolates used in this study and the number of microsatellites out of 38 tested that successfully amplified with M13

Fungal species	Host	No. of isolates	No. of microsatellites
E. poae	*Poa nemoralis*	3	38
E. typhina	*Dactylis glomerata*	4	37
E. clarkii	*Holcus lanatus*	3	36
E. bromicola	*Bromus erectus*	2	31
E. clarkii×*E. clarkii*	Progeny	6	35
E. typhina×*E. typhina*	Progeny	4	38
E. clarkii×*E. typhina*	Progeny	4	37
E. typhina×*E. clarkii*	Progeny	4	37

Genotyping of Natural Populations

Sympatric host grasses infected by *E. typhina* and *E. clarkii* were sampled in Spring 2013 at a natural grassland site of approximately 2 ha in Aubonne, Canton Vaud, Switzerland. One stroma of each infected plant occurring along eight transects across the population were collected every 5 m resulting in 27 stromata of *E. typhina* and 50 of *E. clarkii*. Approximately 10 % of the estimated number of individuals of *E. typhina* and 25 % of those of *E. clarkii* were thus sampled. Additionally, in order to compare genetic distances between the two sympatric species to those of a third taxon, 22 samples of *E. poae* from three different locations were included in the analysis. The host of *E. poae* occurs in sympatry with *E. typhina* and *E. clarkii* at one edge of the population in Aubonne, but the rare occurrence of the fungus at this site allowed collecting only three samples. Eighteen additional

Table 2 Repeat motifs, primer sequences, and size ranges of 15 selected microsatellite loci from *Epichloë* spp. used in four (M1–M4) fluorescent labeled multiplexes

Mix	Locus	Dye	Repeat motif	Primer sequence (5′–3′)	Size range (bp)
M1	E29	H	$(AGC)_9$	F: TTCCAGCAGCTCTTCAATACC R: ACAGTGGTTCCTGAGGTTTGA	129–201
	E8	F	$(AC)_{14}$	F: CATGGACCAAGTTGTGAGACC R: AGCAAGTCTCGTAACGGTCTG	226–266
	E50	H	$(TTG)_{12}$	F: TCGTCTTGGACTTTGCCTTT R: TTGAGGTTGTCGAGATACACG	315–378
	E39	F	$(GTTTC)_{12}$	F: GTAGCACATGCATCGAATCAG R: ACCCACTAAAGACGGATGACA	425–500
M2	E47	H	$(CTCA)_9$	F: GCCTGTTGAGAAAGACGTGAT R: GATCGAAACACGGGATCATAC	286–354
	E32	A	$(CAG)_{11}$	F: AGATGAATGGTCAGCAGTTCC R: GGACCATACTTCGTCAACGTC	326–347
	E45	A	$(GT)_{15}$	F: TTGACGTCGGGAGGTAGTAGA R: CTGGTTACGGAAAGCGAGATA	376–466
	E13	F	$(GA)_{11}$	F: GTTCTCCAAGGCTTCCAATTT R: GAGAAACGATATTCGCATTGG	485–551
M3	E4	F	$(AG)_9$	F: ATTGACCTGTAGCGCGAGTAG R: CAGAACCAATTCGAATCCATC	126–170
	E33	F	$(TCG)_{11}$	F: TGCCAGATGTTTCAATGACTG R: AACCCATACTCAGCTTTGCAG	329–338
	E36	H	$(TGC)_7$	F: ATTCGAGAATGGATGACCTGA R: AAGAAAGGAATGGGATTGCTC	393–417
M4	E22	F	$(TGGA)_{10}$	F: GCAAGGATTGGTTGGTGATAA R: GCGGATCACTCTGTAGGCTAA	124–152
	E27	H	$(GA)_8$	F: TATAAATGACGCTGGGCTTGT R: TGCACTTGAAGAAGCCATGTA	370–428
	E11	F	$(CT)_{11}$	F: GTCAGAGGGCAGTAGTGACG R: ATGTAATGCTCTGCCTGCTTC	265–289
	E46	A	$(AG)_9$	F: TCGTGACACCTTCTTCGGTAT R: AGAGGTTGTCGTGAGCATCAT	382–418

H Hex, *F* 6-Fam, *A* ATTO0550

samples came from the Botanical Garden in Zurich and one from Zollikerberg in Zurich. DNA was extracted from cultured fungi and, in particular, from each stroma using mycelium from the interior part of the stroma split open under sterile conditions. DNA extraction followed the cetyltrimethyl ammonium bromide (CTAB) standard protocol [43]. Extracted DNA was subjected to electrophoresis to confirm the presence of template for PCR and quantified using a Nanodrop spectrophotometer (Thermo Scientific, Inc., Waltham, MA, USA).

The 15 selected markers in four multiplexes described above were used to study the population structure of *E. typhina*, *E. clarkii*, and *E. poae* at the Aubonne site. In order to test whether genetic diversity in the three species was also detectable in gene sequences, a portion of *tefA* nuclear region was sequenced for ten, 15, and seven representative samples of *E. typhina*, *E. clarkii*, and *E. poae*, respectively. The *tefA* gene is commonly used for distinguishing described sexual *Epichloë* species, as it is rich in polymorphic introns allowing for sufficient resolution [22]. PCR reactions were performed using primers 5′-GGG TAA GGA CGA AAA GAC TCA-3′ (forward) and 5′-CGG CAG CGA TAA TCA GGA TAG-3′ (reverse) for *tefA* as previously described [44], and sequenced using BigDye Terminator v. 3.1 on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Forward and reverse strands were assembled in Geneious v. 6.1.8 [42], and consensus sequences were aligned using the default plugin of the software. The obtained nucleotide sequences have been submitted to GenBank under accessions KP064360–KP064391.

Data Analysis of Natural Populations

Number of alleles, allelic richness at each locus, gene diversity, as well as genotypic linkage disequilibrium based on 10,500 permutations were determined using the program FSTAT v. 2.9.3 [45]. In the analyses of linkage disequilibrium, the data were coded as diploid data, where single-allelic loci were considered to be homozygous. The genetic differentiation among species was assessed in POPGENE v. 1.31 [46] using Fisher's exact tests and pairwise F_{ST} statistics [47]. The software program GENALEX v. 6.2 [48] was used to analyze the molecular variance (AMOVA) among and within species of *E. typhina*, *E. clarkii*, and *E. poae* with 9999 permutations.

Sequential Bonferroni corrections were performed for simultaneous multiple tests [49].

To infer population structure of *E. typhina*, *E. clarkii*, and *E. poae*, PCO-MC analysis was used, a method that combines principal coordinate analysis (PCO) with testing of significant clusters for population structure [50]. Following the authors' recommendations (http://lamar.colostate.edu/~reevesp/PCOMC/PCOMC.html), stability of clusters was first assessed with a cut-off set to 15, and significance was then tested at the 0.05 level. Furthermore, the Bayesian model-clustering algorithm implemented in the program STRUCTURE v. 2.3.2 [51, 52] was also employed. Iteration parameters were set to 950,000 Monte Carlo Markov Chain (MCMC) iterations preceded by a burn-in period of 50,000 iterations. Ten independent simulations were performed to test for the consistency of the results for *K* ranging from 1 to 5. To infer the number of clusters, i.e., *K*, that best fit the data the method described by Evanno et al. [53] as implemented in STRUCTURE HARVESTER was used [54]. Membership coefficients estimated by STRUCTURE were visualized using the software programme DISTRUCT v. 1.1 [55].

Results

Primer Design

Blasting of the total 75,432 primer pairs generated in this study against *Epichloë festucae* revealed that 10,406 primer pairs covering 2022 microsatellites with 100 % perfectness are potentially suitable also for application on other *Epichloë* species. By restricting the repeat number of the microsatellite motifs to 5–20, 885 primer pairs covering 180 microsatellites remained. The full list of these candidate loci with respective descriptions and primer combinations is available as supplementary material (Tab. S1). Within the full set of microsatellites, the motif of hexanucleotide repeats (67 %) was the most abundant class, followed by trinucleotide (15 %), pentanucleotide (12 %), tetranucleotide (5 %), and dinucleotide repeats (1 %; Fig. 1a). In contrast, when filtering for 5–20 motif repeats, trinucleotide repeats (79 %) were the most abundant, followed by dinucleotide (15 %), tetranucleotide (3 %), pentanucleotide (2 %), and hexanucleotide repeats (1 %; Fig. 1b). Among the 15 fluorescent labeled primers developed on *E. poae*, seven were recovered also in the genome of *E. festucae* and yielded successful PCR products when tested in vitro. Of the remaining eight microsatellites, three yielded successful PCR products in *E. festucae*, despite the fact that they were not found in silico in this species. This indicates that microsatellites can be missed if a draft version of the genome is used and that unsuccessful blasting does not necessarily translate into negative in vitro results.

Genetic Diversity and Structure of Sympatric *Epichloë* Species

The allelic diversity of 99 isolates of *E. typhina*, *E. clarkii*, and *E. poae* was analyzed within each species at 15 polymorphic loci. The number of alleles per locus ranged from 2 to 12 for *E. typhina*, from 1 to 14 for *E. clarkii* and from 1 to 4 for *E. poae*. The allelic richness corrected for uneven sample size ranged from 2 to 11.87 per locus for *E. typhina*, from 1 to 12.86 for *E. clarkii*, and from 1 to 4 for *E. poae* (Table 3). The mean allelic richness across the three species was 9.58, of which 6.07 alleles were observed across isolates from *E. typhina*, 6.55 alleles in *E. clarkii*, and 2.33 alleles were observed among isolates from *E. poae*. All loci from *E. typhina* were polymorphic, whereas *E. clarkii* and *E. poae* had 93 and 80 % polymorphic loci, respectively. Overall, 91 % of the loci from the three species were polymorphic. Significant linkage disequilibrium (P_{adjust}<0.0005) was detected in one, two and four of the 105 possible pairwise combinations of loci in *E. typhina*, *E. clarkii*, and *E. poae*, respectively. The mean total gene diversity (*H*) calculated using the allele frequencies across all isolates was 0.53. This was similar to the values obtained for gene diversity estimates, namely, 0.664 and 0.628, in isolates from *E. typhina* and *E. clarkii*, respectively, and 0.345 observed in isolates from *E. poae* (Table 3).

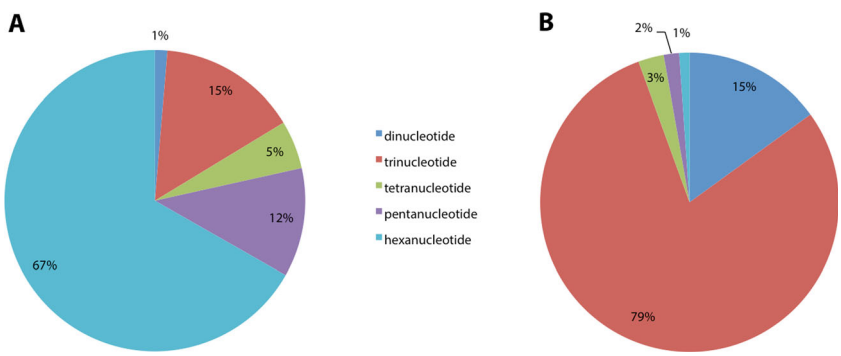

Fig. 1 Frequencies of different classes of nucleotide repeats for microsatellites compatible in *E. poae* and *E. festucae*. **a** Microsatellites (2022) with any number of motif repeat. **b** Microsatellites (180) with motifs repeated 5–20 times

Table 3 Number of alleles, allelic richness, and gene diversity per locus at 15 microsatellite loci for *E. typhina*, *E. clarkii*, and *E. poae*

Locus	*E. typhina*			*E. clarkii*			*E. poae*		
	Na	R	H	Na	R	H	Na	R	H
E8	5	4.937	0.709	7	6.346	0.711	3	3	0.325
E39	2	2.000	0.262	8	6.938	0.712	2	2	0.455
E29	10	9.872	0.875	14	12.185	0.885	4	4	0.571
E50	9	8.904	0.855	9	7.751	0.820	2	2	0.247
E13	12	11.871	0.909	11	10.225	0.796	2	2	0.485
E47	6	5.968	0.672	8	7.368	0.839	2	2	0.519
E32	5	4.999	0.658	4	3.370	0.223	2	2	0.247
E45	10	9.873	0.892	10	8.566	0.770	4	4	0.519
E4	3	2.969	0.544	8	6.946	0.758	3	3	0.606
E33	2	2.000	0.359	4	3.906	0.643	1	1	0.000
E36	6	5.937	0.744	2	1.689	0.040	2	2	0.247
E22	2	2.000	0.484	6	5.190	0.618	3	3	0.394
E11	5	4.936	0.567	1	1.000	0.000	1	1	0.000
E27	12	11.840	0.892	11	9.797	0.817	1	1	0.000
E46	3	2.969	0.544	8	7.032	0.783	3	3	0.558
Mean	6	6.072	0.664	7	6.554	0.628	2	2.333	0.345

Na number of alleles per locus, *R* corrected allelic richness, *H* gene diversity

The observed levels of genetic differentiation (Fisher's exact test $P<0.001$) among *E. typhina*, *E. clarkii*, and *E. poae* reflect a clear structuring of the three species. These results were supported by high pairwise F_{ST} values of 0.446 between *E. typhina* and *E. poae*, 0.453 between *E. clarkii* and *E. poae*, and 0.280 between the more closely related *E. typhina* and *E. clarkii* (Table 4). The AMOVA confirmed these results by showing that 62 % of the molecular variance is found within species and 38 % among species (Table 5).

PCO-MC analyses revealed three stable and statistically significant clusters ($P<0.05$) that corresponded to the three host-specific species (Fig. 2). In this analysis, 19 % of the variation was explained by the first axis, 11.6 % by the second axis, and 4.3 % by the third axis. No samples were found in between the clusters indicating that all samples are assigned to the three genotypically separate species. The STRUCTURE analysis yielded results consistent with those obtained from PCO-MC. The distribution of the maximum likelihood was the highest for $K=3$. The first cluster included all genotypes of

Table 4 Pairwise F_{ST} values among *E. typhina*, *E. clarkii*, and *E. poae*

	E.typhina	*E.clarkii*
E. clarkii	0.280*	
E. poae	0.446*	0.453*

An asterisk indicated the F_{ST} value is highly significantly different from 0 ($P<0.001$). *P* values were adjusted using the Bonferroni procedure

Table 5 AMOVA results testing the genetic structure among *E. typhina*, *E. clarkii*, and *E. poae* at 15 microsatellite loci (9999 permutations)

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation	*P value*
Among species	2	172.57	2.67	38	<0.001
Within species	96	414.53	4.32	62	
Total	98	587.10	6.98		

isolates representing *E. typhina*, while the second cluster consisted of genotypes of *E. clarkii* and the third cluster of *E. poae*. The assignment of the individuals in the plot showed very reduced levels of admixture.

The alignment of the sequences of the nuclear gene *tefA* was ~620 bp in length and showed a considerable number of species-specific fixed single nucleotide polymorphisms (SNPs) for all three species of the study. In particular, four, three, and eight SNPs were found to be fixed among representatives of *E. typhina*, *E. clarkii*, and *E. poae*, respectively. Hence, these SNPs have diagnostic value for the identification of *E. typhina*, *E. clarkii*, and *E. poae* (Fig. S1).

Discussion

Genetic Diversity and Structure of Sympatric *Epichloë* Species

Host specificity is hypothesized to promote reproductive isolation between sympatric fungal species when mating is constrained to occur within the host [1]. Evidence of the role of host specificity in maintaining species boundaries has emerged from a number of systems including *Ascochyta* species, *Mycosphaerella graminicola*, and *Microbotryum* species; hence, this mechanism is hypothesized to play an important role in the speciation of these fungi [12, 13, 56]. To date, no in-depth study has addressed the validity of species boundaries in host-specific endophytes, and we contribute to fill this gap by presenting evidence from sympatric populations of *Epichloë*.

Our study provides clear evidence of genetic discontinuity between *E. typhina* and *E. clarkii*, indicating that reproductive isolation mechanisms exist to keep sympatric populations both morphologically and genetically distinct. Moreover, although the third species (*E. poae*) was represented by only three samples from Aubonne, these had clearly distinct genotypes that clustered with additional samples of this species from two different geographical regions. This offers preliminary evidence that also *E. poae* is maintained isolated from *E. typhina* and *E. clarkii*. In fact, both PCO-MC and Bayesian

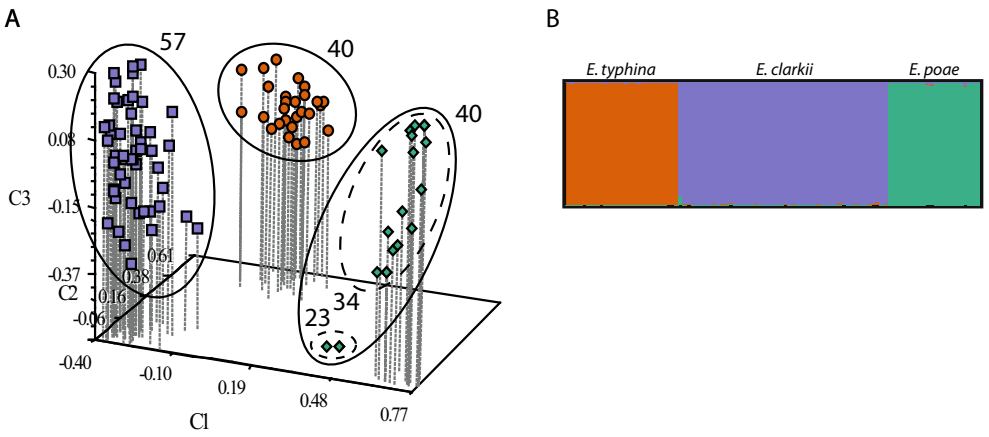

Fig. 2 **a** PCO-MC analysis based on 15 microsatellite loci for *E. typhina* (*circles*), *E. clarkii* (*squares*), and *E. poae* (*diamonds*). *Solid lines* indicate stable and statistically significant ($P<0.05$) clusters, whereas *dashed line* indicate stable clusters that do not pass the significance threshold. **b** Bayesian clustering of *E. typhina*, *E. clarkii*, and *E. poae* at 15 microsatellite loci as inferred from STRUCTURE under the assumption of $K=3$ populations

analyses placed the isolates representing the three host-specific species in separate clusters. High pairwise F_{ST} values and the evidence provided by sequencing data from the nuclear gene *tefA*, where several species-specific fixed SNPs for each species were recovered, further support these findings. Consistently, *E. typhina*, *E. clarkii*, and *E. poae* represent separate evolutionary lineages as previously shown in phylogenetic trees reconstructed from *actA*, *tubB* and *tefA* sequences [22]. Overall, our results indicate high genetic differentiation among the three species possibly maintained by very limited gene flow.

To date, studies that have assessed the genetic structure of *Epichloë*/*Neotyphodium* populations have focused on asexual species. Because asexual endophytes do not form wind-dispersed ascospores and are vertically transmitted through the infection of developing seeds of their host grasses, gene flow may only be mediated by the process of plant seed dispersal. As this is expected to have a small effect on geographically distant populations [57, 58], high levels of F_{ST} are expected among endophyte populations. However, results of F_{ST} analyses indicate that asexual reproduction is hardly a good predictor of levels of F_{ST} among geographically isolated populations. Low levels of gene flow were found among populations of asexually reproducing *Neotyphodium sibiricum* ($F_{ST}=0.6799$) and *Neotyphodium gansuense* ($F_{ST}=0.3490$), consistent with the observed dominance of clonal reproduction [59]. On the other hand, gene flow was inferred to be high in asexual *E. festucae* infecting *Festuca rubra* ($F_{ST}=0.197$ in Garcia et al. [58] and $F_{ST}=0.0814$ in Wäli et al. [60]), where genetic exchange may be attributed to the presence of migrants. Despite this contrasting evidence, results from a study on the endophytes of *Brachypodium sylvaticum* has shown that sexual stromata-forming and asexual asymptomatic isolates form genetically clearly differentiated subpopulations [61]. While evidence is accumulating on the genetic structure of populations from asexually reproducing *Epichloë* species, to our knowledge no study has addressed the genotypic delimitation of sympatric sexually reproducing taxa.

This study showed high genetic differentiation among populations with pairwise F_{ST} values of 0.446 between *E. typhina* and *E. poae*, 0.453 between *E. clarkii* and *E. poae*, and 0.280 between *E. typhina* and *E. clarkii*. The higher F_{ST} value of *E. poae* in pairwise comparisons with the other two species indicates that *E. poae* is a more genetically isolated taxon, supporting previous results [22]. While this result can be caused by the geographic isolation of a great proportion of the *E. poae* samples external to the Aubonne site, comparisons of the population statistics among the three species reveal peculiar characteristics for this taxon. We found similar levels of allelic richness for *E. typhina* and *E. clarkii*, but these values were more than two times higher than for *E. poae* (Table 3). Furthermore, *E. poae* showed lower percentage of polymorphic loci and lowest gene diversity (Table 3), despite the fact that it was represented by samples from three different locations. These characteristics of *E. poae* may be explained by its different transmission mode. In contrast to *E. typhina* and *E. clarkii* that are only horizontally transmitted by sexual ascospores, *E. poae* can transmit both horizontally and vertically by seed. This may lead more frequently to clonal endophytes in plants growing from seeds. Accordingly, only 16 genotypes out of 22 were unique in *E. poae*, whereas in *E. typhina* and *E. clarkii* all genotypes were found to be unique.

Reproductive Isolation of *Epichloë* Species

Hypotheses on the origin of reproductive barriers should include both intrinsic and extrinsic mechanisms, and our results provide preliminary insights to evaluate alternative

scenarios. An obvious mechanism of extrinsic prezygotic isolation includes the preferential transfer of spermatia by *Botanophila* flies that actively contribute to exchange of gametes. Yet, given that *Botanophila* flies are largely unspecific in their visiting behavior, spermatia from different hosts are collected and actively transported in mixtures among the stromata [27]; thus, a preference by fly vectors is unlikely to constitute an efficient barrier. Alternatively, gene flow between *Epichloë* species could be restricted via prezygotic assortative mating on the stromata, if individuals were able to discriminate between conspecific and heterospecific spores. Preliminary data in *E. typhina* and *E. clarkii* showed that the outcome of matings depends on the concentrations of conspecific and heterospecific spores applied in mixtures on the stroma (Schirrmann, unpublished data). For example, if conspecific and heterospecific spores were applied in equal concentrations, only progeny of intraspecific matings were found, thus indicating a preference for spores from the same species. These results are consistent with previous evidence for effective interference between matings of different *Epichloë* species on different parts of the stroma surface, depending upon the fungal strains involved [62]. The observed interferences suggested that signals generated after positive mating could be transported throughout the stroma and thus prevent or abort other mating interactions on the same stroma.

In contrast, intrinsic postzygotic isolation is often associated with hybrid sterility or inviability as shown by in vitro crosses that are initiated and then later aborted [2]. In *Neurospora* species, interspecific compared to intraspecific crosses resulted in fewer viable ascospores, and *Microbotryum* species produced fewer viable mycelia in interspecific crosses [9, 63]. Furthermore, in *Saccharomyces* species, the incompatibility between genes of the nuclear and mitochondrial genomes causes hybrid sterility, which might have evolved as a by-product of ecological adaptation to different nutrient sources [64]. In our system, the viability of experimentally generated hybrids between species of the *E. typhina* complex was not reduced, and their radial growth rates in vitro were not significantly different, suggesting a lack of obvious intrinsic postzygotic isolation barriers between the parental strains (Schirrmann, unpublished data). Therefore, postzygotic barriers may depend on extrinsic factors as for example a decreased fitness of the hybrids due to a likely maladaptation to host grasses, preventing infection or persistence of hybrids. Host specificity was shown in a study by Chung et al. [65], where the seedling infection frequency and stability of *E. typhina* isolates and their progeny were tested. A reduced infectivity of interspecific progeny in parental hosts was demonstrated previously also for *E. typhina* and *E. clarkii* [66] and was confirmed by reciprocal inoculation experiments for these species and *E. poae* (Schirrmann, unpublished data).

Overall, the evidence collected so far indicates that natural populations of sympatric *Epichloë* species may be genetically differentiated due to assortative mating on the stroma and host specificity of parental strains. In the future, further investigations will hopefully test these hypotheses thoroughly.

Primer Design and Testing

In this study, we developed new microsatellite markers to study population structure at the interspecific level within the *E. typhina* complex. Of the 38 successfully amplifying microsatellites tested on the investigated lab strains, i.e., *E. typhina*, *E. clarkii*, *E. poae*, and *E. bromicola*, and the four progeny from inter- and intraspecific crosses, 82–100 % yielded a PCR product. This suggests that our set of loci has a high probability of cross-species transferability within *Epichloë* and that hybrids could be detected if they would be present in nature (Table 1). Additionally, the large set of markers identified in *E. poae* and successfully in silico verified in *E. festucae* provides a valuable resource of candidate primers applicable to a broad taxonomic range of *Epichloë*. However, careful testing should always be performed to optimize amplification protocols and multiplex design, as well as to rule out unspecific amplification products. Overall, the loci detected and tested here constitute a substantial genetic resource that complements previous microsatellite markers developed for *Epichloë*/*Neotyphodium* species [28–31].

Conclusions

Using a new set of 15 microsatellites, we assessed the population structure of sympatric species of the *E. typhina* complex, i.e., *E. typhina*, *E. clarkii*, and *E. poae*, at a natural field site in Aubonne, Switzerland. Our results showed that high genetic differentiation and very limited gene flow exist among these three species, indicating that mechanisms of reproductive isolation keep natural *Epichloë* populations genetically distinct. Preliminary evidence suggests that host specificity and maladaptation of hybrids to host grasses may act as reproductive isolation barriers in *Epichloë* and therefore promote their speciation. However, further studies are needed to disentangle the complex interplay of isolation barriers that may be responsible to maintain species boundaries in natural populations.

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