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Authors: Sullivan, Terrence J., Bultman, Thomas L., and Schoolcraft, Jennifer

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Applications in Plant Sciences

Primer Note

# Primers to amplify SNP markers in *Epichloë canadensis* (Clavicipitaceae)[1]

Terrence J. Sullivan[2,4], Thomas L. Bultman[3], and Jennifer Schoolcraft[2]

[2]School of Sciences, Indiana University Kokomo, Kokomo, Indiana 46904 USA; and [3]Department of Biology, Hope College, Holland, Michigan 49423 USA


- *Premise of the study:* Primers were designed to produce short amplicons containing single-nucleotide polymorphisms (SNPs) in β-tubulin (*tubB*) and translation elongation factor 1-α (*tefA*) in *Epichloë canadensis* (Clavicipitaceae), an endophytic fungus of *Elymus canadensis* (Poaceae).
- *Methods and Results:* Primers to amplify regions of *tubB* and *tefA* containing suspected SNPs were designed and tested on individuals from six populations. Two *tubB* alleles were identified that differed by a single SNP, and three *tefA* alleles were identified that differed by a combination of two SNPs. All six populations tested were polymorphic for the *tefA* marker, and three of the populations were also polymorphic for the *tubB* marker. These primers are also predicted to amplify these regions in 11 additional epichloid species.
- *Conclusions:* Primers for short amplicons within *tubB* and *tefA* genes can be used to successfully genotype *E. canadensis*, making them useful markers for population genetic or landscape genomic studies.


**Key words:** Clavicipitaceae; *Elymus canadensis*; endophyte; *Epichloë canadensis*; high-resolution melt analysis; single-nucleotide polymorphism (SNP).

Cool-season grasses often harbor symbiotic endophytic fungi from the genus *Epichloë* (Fr.) Tul. & C. Tul. (Clavicipitaceae) in their aboveground tissue. Some *Epichloë* species have strong effects on their hosts by deterring herbivores, increasing drought resistance, and increasing their host's competitive ability (Clay, 1990). This can lead to significant impacts on their hosts and on their surrounding community (Clay and Holah, 1999). However, there can be significant variation in the interaction based on specific combinations of host–endophyte genotypes (Faeth, 2002; Shymanovich et al., 2014).

Molecular markers, including microsatellites and sequences for the genes β-tubulin (*tubB*) and translation elongation factor 1-α (*tefA*), have proven useful for both identifying and genotyping epichloid endophytes (Moon et al., 1999; Sullivan and Faeth, 2004; Takach and Young, 2014; Young et al., 2014). In this paper, we describe primers producing small amplicons suitable for high-resolution melt (HRM) analysis that can be used for genotyping *tubB* and *tefA* single-nucleotide polymorphisms (SNPs) in *Epichloë canadensis* N. D. Charlton & C. A. Young, an endophyte of Canada wildrye (*Elymus canadensis* L., Poaceae), a native grass widespread in North America.

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[4]Author for correspondence: tjsull@iuk.edu

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## METHODS AND RESULTS

*Epichloë canadensis* is a haploid, hybrid species containing sequences from both *E. amarillans* White (typically found in *Agrostis perennans* (Walter) Tuck.) and *E. elymi* Schardl & Leuchtman (found in several *Elymus* L. species) (Charlton et al., 2012). Primers were designed to amplify SNPs in the *E. amarillans*–derived *tubB* and *tefA* genes based on sequences described in Charlton et al. (2012) (*tefA*: JN886775; *tubB*: JN886778), using Primer3 (Untergasser et al., 2012) as implemented in NCBI/Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were designed to produce an amplicon smaller than 300 bp, only amplify the *E. amarillans*–derived gene, and include the potential SNPs described by Charlton et al. (2012). Primer sequences and their characteristics are given in Table 1.

Genomic DNA used to test the primers came from Canada wildrye plants grown from seeds collected from six populations in August and September 2011. Population locations are provided in Appendix 1. A voucher specimen (PUL N17966), including seeds, from an individual from CC population was deposited in the Purdue University Kriebel Herbarium (West Lafayette, Indiana, USA). Seeds were germinated in potting soil at the Indiana University Kokomo campus until they developed multiple leaves. DNA was extracted from ~100 mg of leaf tissue using the FastDNA Spin Kit (MP Biomedicals, Santa Ana, California, USA) using their recommended protocol for plant material. Amplification of endophyte microsatellites from these samples found multiple alleles for at least one of the loci (data not shown), indicative of hybrid endophytes (Moon et al., 2004) like *E. canadensis*.

Initial HRM screening was done using a StepOne Real-Time PCR system (Applied Biosystems, Grand Island, New York, USA) and the MeltDoctor HRM Master Mix (Applied Biosystems), following the manufacturer's recommendations. Reactions were run in a total volume of 20 μL with primer concentrations of 0.3 μM. After an initial 10 min 95°C hot start, samples were cycled 40 times at 94°C for 15 s and 63°C for 60 s. The melt curve was generated immediately following amplification. After an initial denaturing stage of 95°C for 60 s, samples were annealed at 60°C and heated to 95°C using the continuous ramp mode with a 0.3% ramp rate. In this mode, the block warms at a constant rate throughout the melt curve step and readings are taken as quickly as possible. Melt curves were analyzed using High Resolution Melt software version 3.0.1 (Applied Biosystems). To ensure the consistency of the HRM genotyping, samples were run in duplicate to account for any potential variation

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Table 1. Characteristics of primers designed for SNP amplification in *Epichloë canadensis*.

| Locus | Primer sequences (5′–3′) | $T_a$ (°C) | Allele size (bp) | Based on sequences from | GenBank accession no. |
|---|---|---|---|---|---|
| *tefA* HRM | F: TCAACCCGTCACTGGTCTT | 58.5 | 205 | *tefA*-1 allele, JN886775 | KT214347–KT214349 |
| | R: GATGGTGATACCACGCTCAC | 58.4 | | | |
| *tubB* HRM | F: GAGCCCCTGATTTCGTACC | 57.6 | 242 | *tubB*-1 allele, JN886778 | KT214345–KT214346 |
| | R: TGCCCAAATGAATGTGAGTT | 55.8 | | | |

*Note*: $T_a$ = annealing temperature.

in amplification and only samples with threshold cycle ($C_t$) values less than 30 were used. Typical difference plots for *tubB* and *tefA* amplicons are shown in Fig. 1. To verify the genetic variation described by the software, PCR products from 10 individuals including representatives of each identified genotype were cloned into the pCR 4.0 plasmid vector using the TOPO-TA cloning kit (Invitrogen, Carlsbad, California, USA) for both the *tefA* and *tubB* PCR products. Sanger sequencing of the clones was performed by Functional Biosciences (Madison, Wisconsin, USA). Initial sequencing of seven to 10 clones confirmed only a single allele was being amplified for each individual, and subsequent sequencing was performed directly from PCR products by Functional Biosciences. In total, 85 individuals were sequenced to confirm *tefA* variation, and 52 individuals were sequenced to confirm *tubB* variation. Sequence alignments were constructed using the ClustalW algorithm in MEGA 5.2.2 (Tamura et al., 2011), and representatives for each genotype were used in a BLAST search to confirm their identity as *E. amarillans*–derived alleles in *E. canadensis*. No *E. elymi*–derived alleles were amplified by these primers.

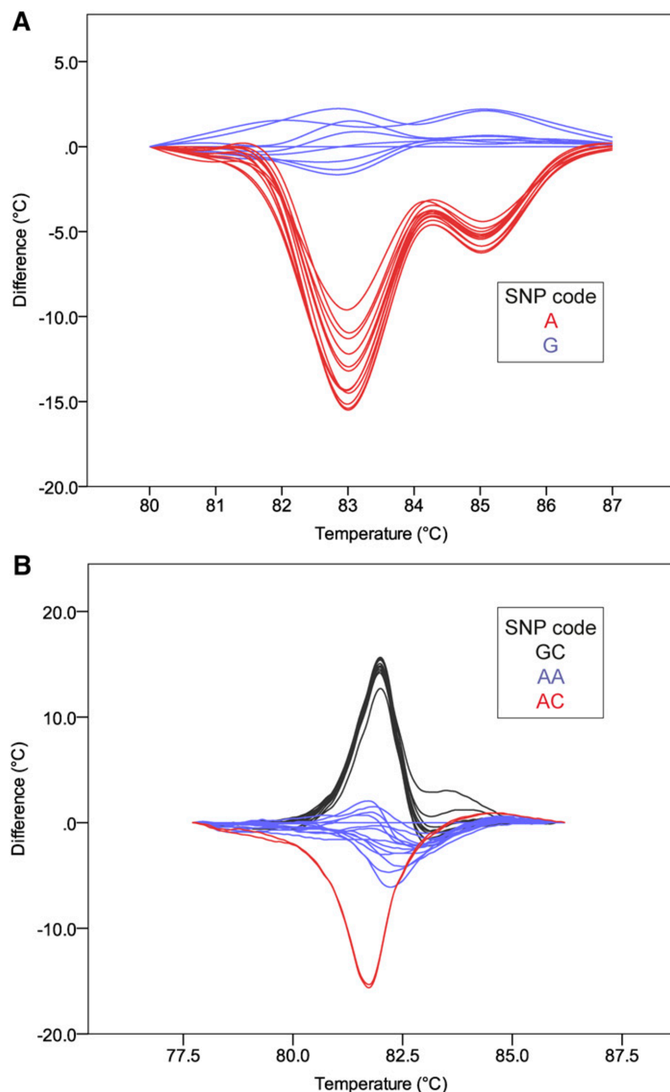


Fig. 1. Representative difference plots for the *tubB* (A) and *tefA* (B) SNP alleles of the *Epichloë amarillans*–derived gene in *E. canadensis*.

An A/G SNP was found in the *tubB* amplicon. Direct sequencing found that both amplicons were most similar to the *E. canadensis* isolate CWR5 *tubB*-1 allele (JN886778), with one allele being identical and the other only differing by the SNP. Both alleles had 99% identity with the *E. amarillans tubB* gene (L78272). Representative sequences for the two alleles have been deposited in GenBank (KT214345–KT214346). Two SNPs were found in the *tefA* amplicon, an A/G SNP and an A/C SNP. From these two SNPs, three *E. amarillans*–derived alleles were found containing the SNP combinations AA (identical to *tefA* in *E. amarillans* strain E4668 [KP689563] in *Agrostis hyemalis* (Walter) Britton, Sterns & Poggenb.), GC (identical to *E. amarillans* E57 [KP689562] and *E. canadensis* CWR 34 *tefA*-1 [KF719188]), and AC (identical to *E. canadensis* isolate CWR 5 *tefA*-1 [JN886775]). Representative sequences for each allele have been deposited in GenBank (KT214347–KT214349). The *tefA* SNP was polymorphic in all six populations, and the *tubB* SNP was polymorphic in three of the populations. As *E. canadensis* is haploid, gene diversity ($\hat{H}$) for each marker in each population was calculated using Arlequin (Excoffier et al., 2005) instead of observed and expected heterozygosity. $\hat{H}$ is equivalent to expected heterozygosity in diploid organisms, but can be used with any organism regardless of ploidy (Nei, 1987). The results are given in Table 2.

The ability of the primers to amplify other epichloid species was tested in silico using Primer-BLAST (Ye et al., 2012). The primer pairs were tested using sequences with the *Epichloë* taxon ID (taxid:5112) in the National Center for Biotechnology Information (NCBI) nr database. Eleven species, not including *E. amarillans* and *E. canadensis*, were identified where both primer sets are expected to match the target sequence exactly and produce an amplicon (Appendix 2).

## CONCLUSIONS

*Epichloë* endophytes are important plant symbionts, and in this paper, we describe primers that can be used to describe genetic diversity within *E. canadensis* using SNPs in the *tubB* and *tefA* genes. Both markers are polymorphic and we expect them to be valuable for future population genetic and landscape genomics studies of epichloid endophytes of cool-season grasses.

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Table 2. Genetic properties of the newly developed SNP primers for six populations of *Epichloë canadensis*.[a]

| | BF (*tubB* SNP $n$ = 6; *tefA* SNP $n$ = 11) | | CC (*tubB* SNP $n$ = 17; *tefA* SNP $n$ = 16) | | HP (*tubB* SNP $n$ = 9; *tefA* SNP $n$ = 20) | | OFL (*tubB* SNP $n$ = 2; *tefA* SNP $n$ = 2) | | TLI (*tubB* SNP $n$ = 10; *tefA* SNP $n$ = 8) | | VNWR (*tubB* SNP $n$ = 24; *tefA* SNP $n$ = 9) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $A$ | $\hat{H}$ | $A$ | $\hat{H}$ | $A$ | $\hat{H}$ | $A$ | $\hat{H}$ | $A$ | $\hat{H}$ | $A$ | $\hat{H}$ |
| *tubB* SNP | 1 | 0.00 | 1 | 0.00 | 2 | 0.39 | 1 | 0.00 | 2 | 0.36 | 2 | 0.29 |
| *tefA* SNP | 3 | 0.58 | 2 | 0.50 | 2 | 0.39 | 2 | 1 | 2 | 0.25 | 3 | 0.42 |

*Note*: $A$ = number of haplotypes found (total individuals sampled); $\hat{H}$ = gene diversity (probability of two randomly drawn haplotypes being different).
[a] Full population names and locations are given in Appendix 1.

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Appendix 1. Locations of sampled *Epichloë canadensis* populations.

| Population | Abbreviation | Geographic coordinates |
|---|---|---|
| Boone Forks Wildlife Management Area, Iowa | BF | 42°20′48.42″N, 93°50′39.78″W |
| Carleton College Cowling Arboretum, Minnesota | CC | 44°28′4.79″N, 93°8′27.59″W |
| Hayden Prairie State Preserve, Iowa | HP | 43°26′36.31″N, 92°23′0.04″W |
| Ottawa State Fishing Lake, Kansas | OFL | 39°6′52.05″N, 97°34′16.85″W |
| Salina, Kansas | TLI | 38°40′54.08″N, 97°35′28.11″W |
| Valentine National Wildlife Refuge, Nebraska | VNWR | 42°31′51.45″N, 100°39′18.34″W |

Appendix 2. *Epichloë* species[a] identified by Primer-BLAST to produce amplicons with the described primers.

| Species | *tubB* | *tefA* |
|---|---|---|
| *Epichloë australiense* (C. D. Moon & Schardl) Leuchtm. | AF323379.1 | AF323400.1 |
| *Epichloë baconii* White | KF042062.1 | KF811547.1 |
| *Epichloë cabralii* Iannone, M. S. Rossi & Schardl | JX679132.1 | KJ934942.1 |
| *Epichloë chisosa* (J. F. White & Morgan-Jones) Schardl | AF457470.1 | AF457510.1 |
| *Epichloë coenophiala* (Morgan-Jones & W. Gams) C. W. Bacon & Schardl | KF811599.1 | KF811568.1 |
| *Epichloë festucae* Leuchtm., Schardl & M. R. Siegel | KF042045.1 | KF042045.1 |
| *Epichloë festucae* var. *lolii* (Latch & Samuels) C. W. Bacon & Schardl | AY865628.1 | AF457540.1 |
| *Epichloë melicicola* (C. D. Moon & Schardl) Schardl | AF323387.1 | AF323404.1 |
| *Epichloë occultans* (C. D. Moon, B. Scott & M. J. Chr.) Schardl | AF176270.1 | AF457541.1 |
| *Epichloë siegelii* (K. D. Craven, Leuchtm. & Schardl) Leuchtm. | AF308139.1 | AF308133.1 |
| *Epichloë tembladerae* (Cabral & J. F. White) Iannone & Schardl | AF457496.1 | AF457545.1 |

[a] Species names follow classifications proposed in Leuchtmann et al. (2014).



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