# A SIMPLE, PCR-BASED METHOD FOR THE IDENTIFICATION OF *TRIODANIS* (CAMPANULACEAE) SPECIES AND THEIR HYBRIDS

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ABSTRACT

*Triodanis* Raf. is a genus of Campanulaceae with some cryptic species that are known to hybridize. Therefore, non-morphological identification methods are instrumental in differentiating species and their interspecific hybrids. Primers were developed from the ITS and ETS regions for 4 sympatric species present in North America to assess their utility in the identification of species, and natural and simulated hybrids by multiplex PCR amplification and gel electrophoresis. We found that these multiplexed PCRs were highly accurate in the identification of the 4 study species as well as natural hybrids. Simulated hybrids (in vitro combinations of total DNA extracts) to confirm co-amplification in multiplexed PCR also showed high rates of amplification but were sensitive to dosage effects of parental DNA. Overall, this study demonstrates a promising method to cheaply and quickly identify large numbers of individuals of most species of *Triodanis* and could be applied to more species, given sufficient differentiation in target DNA locus and species-specific constancy of targeted variation.

Key Words: Campanulaceae, genome skimming, interspecific hybridization, PCR, *Triodanis*

RESUMEN

*Triodanis* Raf. es un género de Campanulaceae con algunas especies crípticas que hibridan. Por lo tanto, los métodos de identificación no morfológicos son fundamentales para diferenciar las especies y sus híbridos interespecíficos. Se desarrollaron cebadores a partir de las regiones ITS y ETS para 4 especies simpátricas presentes en Norteamérica con el fin de evaluar su utilidad en la identificación de especies e híbridos naturales y simulados, mediante amplificación PCR multiplexada y electroforesis en gel. Se comprobo que estas PCR multiplexadas fueron muy precisas en la identificación de las 4 especies estudiadas, así como de los híbridos naturales. Los híbridos simulados (combinaciones in vitro de extractos de ADN total) para confirmar la co-amplificación en la PCR multiplexada también mostraron altas tasas de amplificación, pero fueron sensibles a los efectos de dosificación del ADN parental. En general, este estudio demuestra un método prometedor para identificar de forma economica y rápida un gran número de individuos de la mayoría de las especies de *Triodanis* y podría aplicarse a más especies, siempre que haya suficiente diferenciación en el locus de ADN objetivo y una constancia específica de la especie de la variación objetivo.

## INTRODUCTION

Simple and efficient methods for the identification of plants are critical for understanding the biodiversity in our environment. However, morphology-based identification may be problematic for addressing taxa with highly cryptic morphology. Moreover, natural hybridization between species introduces additional confounding variables to morphology-based identification because of the complex breeding patterns in hybrid zones, such as introgression (Gompert et al. 2016; Josić et al. 2024).

*Triodanis* Raf. is a small genus of Campanulaceae inhabiting North and South America, with multiple species occurring in sympatry, and several species in the genus known to readily hybridize in nature (Bradley 1975; Weakly 2010; Goodwillie & Stewart 2013; McConnell 2024), especially *T. biflora* (Ruiz & Pav.) Greene and *T. perfoliata* (L.) Nieuwl. Patterns of hybrid gene flow among other members of the genus are more poorly understood. However at least one flora suggests that while *T. perfoliata* and *T. biflora* may hybridize with several other species in the genus, and that these other taxa do not appear to hybridize among each other (Yatskievych 2006). Nonetheless, accurate identification between several species (and hybrids) of *Triodanis* can be difficult based on morphology alone (Goodwillie & Stewart 2013; McConnell 2024). For example, *T.*

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*perfoliata*, *T. biflora*, and their hybrids are generally distinguished based on very subtle morphological differences such as capsule pore positioning (generally higher in *T. biflora*), relatively smaller plant size and the production of overall fewer chasmogamous (open) flowers in *T. biflora* (with intermediate chasmogamous production for hybrids) (Trent 1940; Gara & Meunchow 1990; Goodwillie & Stewart 2013; McConnell 2024). Likewise, *T. lamprosperma* McVaugh shares many key morphological characteristics with *T. biflora,* including capsule pore positioning and a narrower leaf shape than other members of the genus (Bradley 1975); notable differences include relatively larger capsule pores and seeds, as well as a generally larger plant size for *T. lamprosperma*. Other members of the genus exhibit some combination of morphological traits that facilitate easier identification, such as a very narrow capsule pores in *T. holzingeri* McVaugh, and long, wide capsule pores for *T. leptocarpa* (Nutt.) Nieuwl. and the narrowly endemic *T. coloradoensis* (Buckley) McVaugh. Overall, the generally cryptic morphology of several species in the genus combined with high sympatry (especially for *T. perfoliata*, *T. biflora*, *T. lamprosperma*, *T. leptocarpa*) and apparent hybridization, generate challenges in terms of morphological identification. Here we sought to develop a DNA-based method to differentiate species and their interspecific hybrids that was not based on morphology alone.

## MATERIALS AND METHODS

### Sampling & DNA Extraction

Ongoing phylogenetic and population-based studies on *Triodanis* have resulted in numerous collections of multiple species across their ranges (McConnell 2024; Simmonds et al. 2024; Simmonds 2025). These samples were leveraged for study of DNA-based identifications and have been extensively vetted: Those data used to design primers came from a study of deeply sampled populations across all known species of *Triodanis* that pair phylogenetic and morphological analyses (Simmonds 2025), while the primer efficacy was tested on a set of samples that were identified using those same morphological methods (McConnell 2024).

Total DNA was obtained from leaf material using a DNA extraction method. Leaf pieces of approximately 1 $cm^2$ were placed in a 2 mL screw-cap tube containing ~4 zirconia beads and 0.1 g of silica gel, then were pulverized in a homogenizer (Biospec Products, Bartlesville, OK) for 1 minute. DNA was isolated following a "modified CTAB approach" by incubating in a mixture of 10 µL Proteinase K (Thermo Fisher Scientific Inc., Waltham, MA) and 1.2 mL cetyltrimethylammonium bromide buffer (Doyle & Doyle 1987; Neubig et al. 2014), followed with a purification using 500 µL of a 24:1 chloroform and isoamyl alcohol solution. The supernatant (750 µL) was precipitated with 30 µL of 3 M sodium acetate and 510 µL of 100% isopropanol, incubated at –20°C for 1–12 hours, then centrifuged at 13,000 g for 20 minutes, resulting in a pellet that was washed with 1 mL of 70% ethanol, dried, and resuspended in 200 µL of 1× Tris-EDTA buffer. Gel electrophoresis in 1% agarose gel using 1× sodium boric acid (SBA) buffer (Brody & Kern 2004) was performed on total DNA to determine that high molecular weight DNA was present.

### Primer Design

Wild-collected material was acquired, DNA was extracted (as above), then samples were enriched with myBaits Expert Angiosperms-353 (Johnson et al. 2019; Arbor Biosciences, Ann Arbor, MI) to produce raw sequence data of 156 *Triodanis* samples as well as other genera of Campanulaceae (Simmonds 2025). Those resulting Illumina HiSeq data were assembled in HybPiper (Johnson et al. 2016) to "genome skim" from a custom target file of a single full-length nuclear ribosomal cistron from one *Triodanis* sample, which had been assembled manually in Geneious Prime v.2024.0. The resulting data matrix included 117 samples with clean nr cistrons sequences, representing all currently known species of *Triodanis*, which was aligned using MAFFT (Katoh & Standley 2013) as implemented in Geneious Prime. Using this data matrix, primers were designed using positions of unique nucleotide composition of the less conserved ITS and ETS regions for high-resolution identification at the species level (e.g., Michel et al. 2016) based on differential fragment lengths for four species of *Triodanis*. Primers are listed in Table 1.

Table 1. Primers for species-level DNA identification in *Triodanis* using nrDNA.

| Target species | Primer pairs | Nucleotide sequence (5′–3′) | Melting temp. (°C) | Amplicon length (bps) |
|---|---|---|---|---|
| *T. lamprosperma* | Forward: Tla_ETS_258F | TGC TGG GTT TGC TGT GC | 56.7°C | ~98 |
| | Reverse: Tla_ETS_339R | TTC ACC ATG CAT CAC GC | 53.4°C | |
| *T. biflora* | Forward: TB_ITS_2933F | TCC AAG TGC TTG CGT GA | 54.6°C | ~196 |
| | Reverse: TB_ITS_3113R | ACC GTA GAG TCA TGT CG | 50.8°C | |
| *T. perfoliata* | Forward: TP_ITS_2641F | GCC AAG GAA AAC TTT AC | 45.5°C | ~488 |
| | Reverse: TP_ITS_3113R | ACA GTA GAG TCA TGT CA | 46.2°C | |
| *T. leptocarpa* | Forward: TL_ETS_100F | GGGTGTGGGTTGTTCTC | 53.1°C | ~304 |
| | Reverse: TL_ETS_400R | GCC AAA TTA ATT GGC CG | 49.4°C | |

### Multiplex PCR Assay

To optimize PCR amplification, individual primer pair combinations were used against a test set of samples of known species. These tests yielded a range of annealing temperatures (45–50°C) that gave clean PCR bands for all independent combinations of primers. Multiplexing of all primers was then pursued.

All primers were used in the Multiplex PCR (MPCR) assay (e.g., Ali et al. 2024). Templates included samples of parental species, natural hybrids, as well as simulated hybrids (*in vitro*, total DNA combinations to simulate every possible combination of these 4 species) of known species for the confirmation of the successful amplification of putative hybrids (Table 2). To effectively demonstrate co-amplification of multiplexed markers in the simulated hybrids, the relative amount of contributed total DNA was adjusted through iterative PCRs. For natural hybrids, only those of *T. biflora* and *T. perfoliata* were available for this study. Comparisons by gel electrophoresis were used to confirm estimates of amplicon length prior to use in multiplex PCR.

Amplifications were performed using the SimpliAmp Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, MA) in 25 µL volumes with the following reaction components: 1.0 µL template DNA, 7.5 µL water, 12.5 µL of Gotaq Green Master Mix (Thermo Fisher Scientific Inc., Waltham, MA), 0.5 µL of all forward primers, and 0.5 µL of all reverse primers (Table 1). The MPCR amplification protocol parameters were as such: 98°C, 2 min; 35× (94°C, 15 s; 47°C, 15 s; 72°C, 1 min); 72°C, 2 min; holding at 8°C. Manufacturer's guidelines suggested 30 s denaturation and annealing steps, but strong amplifications occurred with only 15 s during PCR optimizations, so those steps were shortened accordingly.

### Identification by Gel Electrophoresis

PCR sample identification was accomplished using electrophoresis. Gels were made using 1.2 g of agarose in 75 mL of 1× SBA buffer (Brody & Kern 2004) with 7.5 µL of SYBRsafe gel stain (Lyfe tech corp., Thermo Fisher, Carlsbad, CA). PCR products were compared against a 100 bp GeneRuler ladder (Thermo Scientific, Carlsbad, CA). Gel images were captured using a TruBlu 2 Transilluminator (Edvotek, Washington, DC). Species identifications were confirmed based on presence/absence and length of bands.

## RESULTS

Amplification was successful across all species and natural hybrids (Fig. 1A). Simulated hybrids (*in vitro* combinations of the total DNA of different species) also successfully amplified but showed some inconsistency for those that included *T. leptocarpa*, which amplified less reliably (Fig. 1B). As a result, the simulated hybrids had differing band fluorescence due to the varying parent species contribution.

We also tested the efficacy of this MPCR technique on a set of samples collected that directly studied natural hybrid zones of *T. perfoliata* and *T. biflora* (see McConnell 2024). In this case, the 275 samples that were tested resulted in the identification of 61 *T. perfoliata*, 144 *T. biflora*, and 70 *T. biflora* × *T. perfoliata* interspecific hybrids. The methods developed here specifically improved identification of hybrid individuals; approximately 20% of the overall samples were discovered to in fact be hybrid in origin but had been

Table 2. Specimens used in multiplex PCR to represent parental species and natural hybrids and used to create simulated hybrids. Samples marked with "*" were used to create the simulated hybrids.

| Sample Name | Scientific Name | Voucher |
|---|---|---|
| TL_MO_709 | *T. leptocarpa* | *T. Simmonds 142* (SIU) |
| TL_KS_728* | *T. leptocarpa* | *T. Simmonds 194* (SIU) |
| TL_OK_746 | *T. leptocarpa* | Unvouchered |
| TLa_AR_682* | *T. lamprosperma* | *T. Simmonds 224* (SIU) |
| TLa_MO_884 | *T. lamprosperma* | *T. Simmonds 191* (SIU) |
| Hyb_TB_TN_71 | *T. biflora* | Unvouchered |
| Hyb_TB_TN_72* | *T. biflora* | Unvouchered |
| Hyb_Hy_TN_138 | *T. hybrid* | Unvouchered |
| Hyb_Hy_TN_212 | *T. hybrid* | Unvouchered |
| Hyb_TP_TN_256* | *T. perfoliata* | Unvouchered |
| Hyb_TP_TN_254 | *T. perfoliata* | Unvouchered |

morphologically identified as strictly parental individuals. Morphological identification of all individuals of both parental taxa were consistent with our molecular analyses for the McConnell study.

## DISCUSSION

For objective identification of the species of *Triodanis*, we designed a DNA-based technique because of the complex intermediacy in morphological features among hybrids and some species. The four *Triodanis* species selected for primer development are the most widely distributed species throughout North America. Further, *Triodanis perfoliata*, *T. biflora*, and *T. lamprosperma* occur sympatrically, are morphologically cryptic, and are known to hybridize readily. *Triodanis leptocarpa* is not known to hybridize with other members of the genus but is widely distributed and sympatric with the other three species included in this study; the poor amplification of *T. leptocarpa* when combined in simulated hybrids could not be resolved through PCR optimization, but the problem may be moot because of the lack of known real-world instances of natural hybrids. Primers were not developed for *Triodanis texana* and *T. coloradoensis* for this study due to being endemic to central Texas, nor for *T. holzingeri* which also has a relatively narrow distribution; overall, relatively few samples of these excluded species were available for analyses. Though future studies could develop DNA-based identification of these taxa, both *T. coloradoensis* and *T. holzingeri* are readily identifiable by key morphological features; and neither is known to frequently hybridize with other taxa in the genus *Triodanis*. This study provides a pragmatic framework for accurate identification of the most widespread, morphologically cryptic and most commonly hybridizing species of *Triodanis*.

The highly variable ITS and ETS regions are useful for discerning species-level differences in *Triodanis*. Primers developed using these regions are effective in identifying species, natural hybrids, and simulated hybrids. Identification by PCR and gel electrophoresis confirmed prior morphological identification of the species as evidenced by the McConnell (2024) study of hybrid zones. The clear demarcation of band presence in this process allows for an objective discernment of taxon-specific haplotypes within a study sample, making for a powerful tool for morphologically independent identification.

This study also demonstrates how current phylogenomic datasets can be leveraged to produce additional useful products, outside of the normal targeted DNA sequence data captured (i.e., Johnson et al. 2019). These nr cistron data are a common serendipitous portion of data produced during probe-based sequence enrichment, although not targeted by those probes. By "genome skimming" these data (e.g., Reginato et al. 2016), an otherwise ignored dataset can enrich the wealth of nr DNA data already in existence in GenBank, bridging the data used over the past decades with the genomic era.

We recommend that these primers be used for future investigations in parental and hybrid identification

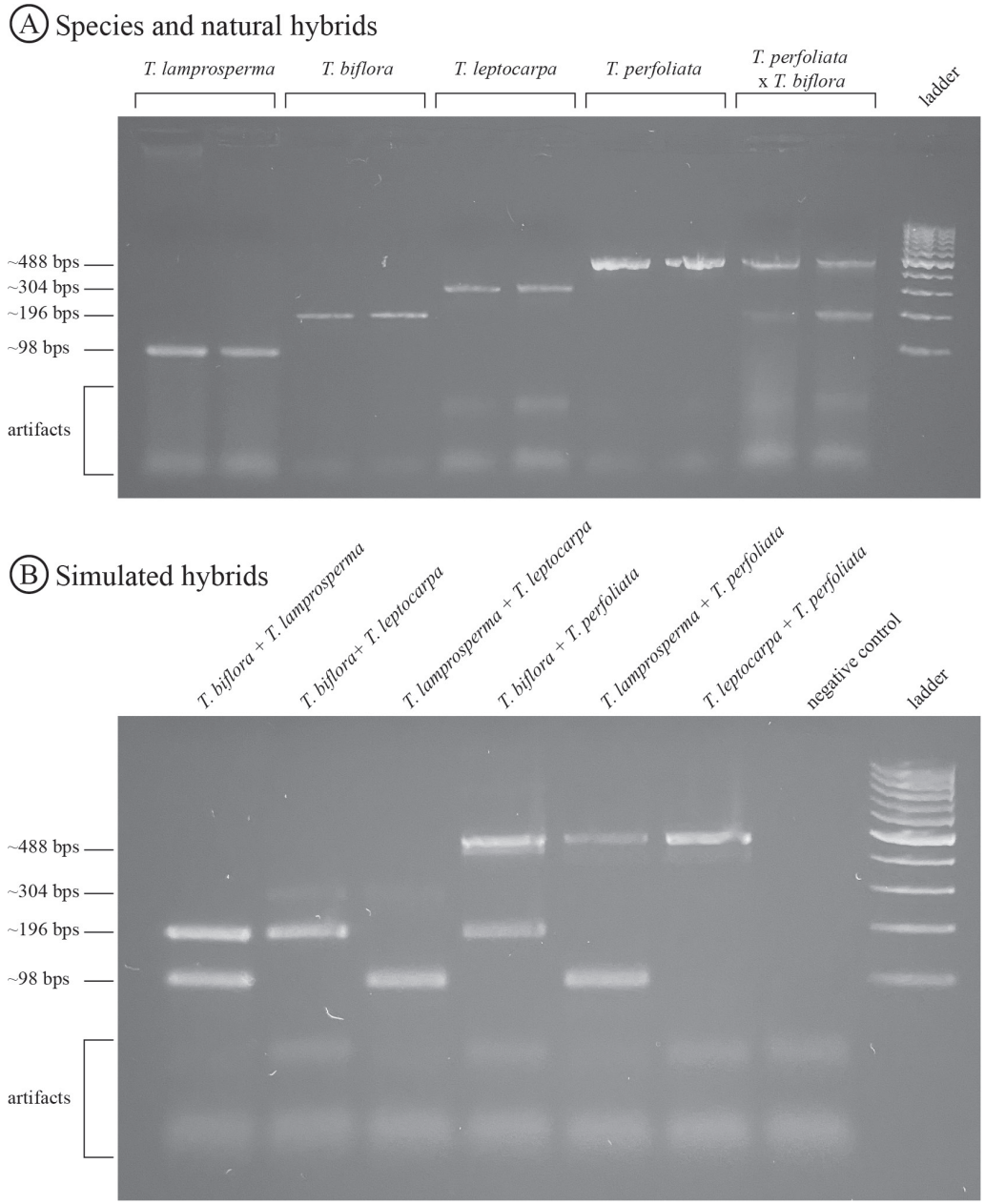


**Fig. 1. Agarose gel electrophoresis images. Lengths on left indicate the approximate lengths of the four different species-specific markers. A. Samples of each of the four species used in this study and natural hybrids, including replicate individuals to demonstrate infraspecific consistency. B. Simulated hybrids created by mixing total DNAs of different species combinations. Note that it was difficult to amplify *T. leptocarpa* in combination with other taxa, thus simulated combinations of DNA that included *T. leptocarpa* did not yield robust amplification.**

among *Triodanis* species. Further, this technique could be applied to other taxa, especially when large numbers of individuals need identification in morphologically cryptic taxa and when there is DNA differentiation among the focal taxa.

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