

Specificity of LAMP for genetic diagnostics of Chilean needle grass and serrated tussock

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Summary Chilean needle grass (*Nassella neesiana* [Trin. & Rupr.] Barkworth) and serrated tussock (*Nassella trichotoma* [Nees] Hack. ex Arechav.) are related Weeds of National Significance (WoNS) undergoing rapid range expansion in Australia. They are difficult to distinguish from native tussock grasses and emergences of these weeds often remain undetected until after they have established as difficult to control populations. Genetic diagnostics using loop-mediated isothermal amplification (LAMP) may provide a simple cost-efficient means for rapid identification of these weeds, under laboratory and field conditions. Provision of LAMP diagnostics would assist in minimising the time-lag to initial recognition of tussock weed outbreaks in new areas, and subsequently improve the time to response for eradication of the weeds where they are recently emergent.

Here we report laboratory development and tests of seven LAMP assays incorporating novel primer suites matched to species-specific DNA barcode sequences of Chilean needle grass and serrated tussock, and three additional suites designed for general LAMP validation. LAMP assays conducted using a real-time PCR platform tested positive for replicates of the target weeds at several of the primer suites and in less than 20 min. Three of the suites are particularly promising (89–100% repeatability and specificity) and following optimisation to further minimise false positive or false negative test outcomes they are likely to be reliable for field use. As promising as these results are, the real challenge will be next-stage development of a simple but effective mobile LAMP platform for use in the field by officers engaged in weed surveillance efforts.

Keywords Genetic assay, *Nassella neesiana*, *Nassella trichotoma*, DNA barcodes, loop-mediated isothermal amplification.

INTRODUCTION

Nassella neesiana and *N. trichotoma* (commonly known as Chilean needle grass (CNG) and serrated tussock (ST) respectively) are introduced Weeds of National Significance (WoNS) in Australia, requiring co-ordinated national approaches to control their

spread and reduce their serious impacts on natural and agricultural ecosystems in the country (McLaren *et al.* 2002, CRC 2003a,b, Iaconis 2004). Both weeds are currently established in substantial portions of temperate south-eastern Australia, and if uncontrolled are likely to expand into broader areas across the country due to their high dispersal potential coupled with their high seed set and soil seedbank longevity.

Early detection and management to eradicate novel emergences of these two weeds, limiting their ability to establish into new locations, is preferable to the costly alternative of their perpetual control once they have established. Such early intervention is however reliant on fast and accurate identification of these two WoNS across all stages in their development. Descriptive morphology keys used for identification of CNG and ST require examination of subtle features, several of which are only apparent during later flowering stages. The issue is compounded by the close resemblance of these two species to a broad variety of tussock grasses native to Australia which overlap the distributions of the weeds. Subsequently field officers involved in weed surveillance may not recognise the two weeds until after they have increased their seed set and have established as difficult to control populations.

The potential development of in-field genetic diagnostics has received much focus in pathogen research, where uses of rapid genetic diagnostics have proven to be essential in the containment of emergent pathogens in host populations (Parida *et al.* 2008). Similar approaches could be adopted for diagnostics of weeds (Gopurenko *et al.* 2017). Loop-mediated isothermal amplification (LAMP, Notomi *et al.* 2000) in particular, has been developed extensively for use in pathogen field diagnostics, because of its increased sensitivity, specificity and portability over conventional PCR-based approaches (Parida *et al.* 2008). The feasibility of using LAMP diagnostics for in-field identification of weeds is appealing for multiple reasons, but mainly because of its potential to be based on growing DNA barcode sequence libraries useful for genetic based weed identifications, and by

its capacity to be compartmentalised as a modular and simple to use device (Gopurenko *et al.* 2017).

We believe a laboratory-based LAMP platform could be developed for targeted diagnostics of CNG and ST. Further, we propose the simplicity of the LAMP method allows it to be potentially developed as a mobile assay platform suitable for targeted diagnostics by non-specialist field officers. The primary laboratory development and proofing stages required for this proposed LAMP platform are reported below.

MATERIALS AND METHODS

Whole mature plants of serrated tussock (ST, $n = 40$) and Chilean needle grass (CNG, $n = 40$) specimens were sampled from Canberra and three sites in New South Wales (Bungendore, Cooma and Goulburn). Specimens were catalogued and retained as vouchers at Wagga Wagga Agricultural Institute (NSW DPI). An additional 56 specimens of four other species of *Nassella* (*N. charruana* (Arechav.) Barkworth, *N. hyalina* (Nees) Barkworth, *N. leucotricha* (Trin. & Rupr.) Pohl and *N. tenuissima* (Trin.) Barkworth) and ten native Poaceae (*Austrostipa aristiglumis* (F.Muell.) S.W.L.Jacobs & J.Everet, *A. densiflora* (Hughes) S.W.L.Jacobs & J.Everett, *A. variabilis* (Hughes) S.W.L.Jacobs & J.Everett, three varieties of *Poa labillardieri* Steud., and four varieties of *P. sieberiana* Spreng.) similar in appearance to CNG and or ST were sampled as leaf cuttings, preserved in >90% ethanol from sites in east Australia, and included for LAMP testing (Table 1). Leaf tissue (<1 mg) was digested overnight at 55°C in 280 μ L of DXT tissue digest reagent (QIAGEN, Doncaster, Australia) with 1% added proteinase K (Sigma – Aldrich). Genomic DNA was isolated from digestions using a Corbett Research 1820 X-tractor Gene robot and associated DX buffers (QIAGEN) as reported in Gopurenko *et al.* (2013).

Species identities of voucher specimens were tested using DNA barcoding (Hebert *et al.* 2003). DNA barcode loci screened included two linked nuclear ribosomal DNA regions, (the external transcribed spacer [ETS], and the internal transcribed spacers [ITS] bounded by 18S and 28S genes). Both loci have utility for DNA barcode identifications of the two target *Nassella* species (Wang *et al.* 2017), and are potentially useful for LAMP assay development. Specimens were DNA barcoded at the *maturase* K (*matK*) chloroplast DNA gene to test for possible introgression among sympatric specimens of the two *Nassella* species. DNA barcoding methods and analysis used here follow that reported in Gopurenko *et al.* (2013), and specifically for grasses in Wang *et al.* (2017).

Seven independent primer suites specific for LAMP amplification of the two target *Nassella* species, were identified among nuclear encoded ribosomal *ETS*, *ITS* and chloroplast *matK* sequences reported earlier for a variety of invasive and native Stipeae grasses in Australia (Wang *et al.* 2017). As recommended for LAMP applications (Tomlinson *et al.* 2010), each suite included two external (F3 & B3), two internal (FIP & BIP) and two loop (LF & LB) primers. Primer suites were newly designed from DNA barcode sequences using PrimerExplorer V5 (<https://primerexplorer.jp/e/>) and synthesised at Sigma-Aldrich (Sydney, Australia). Suites were tested in LAMP assays for their specificity and repeatability among specimen replicates. Three additional primer suites were assayed for use as positive controls of Poaceae grass DNA, including suites for two mitochondrial cytochrome c oxidase subunit I (*COI*) targets (one novel, the other reported by Tomlinson *et al.* (2010) as LAMP control for various plants), and a chloroplast DNA ribulose-1,5-bisphosphate carboxylase oxygenase (*rbcL*) target in native Poacea (Wang *et al.* 2015). In total, ten LAMP primer suites were independently screened among replicates.

LAMP reactions (25 μ L total) contained 5 μ L DNA (or nuclease free water for negative controls), 1 μ L (8 units μ L⁻¹) of *Bst* DNA polymerase (M0538L, New England Biolabs), 40 mM betaine (Sigma), 40 mM Tris-HCl (pH 8.8), 20 mM KCl, 10 mM (NH₄)₂SO₄, 12 mM MgSO₄, 4% Triton X-100, 8% DMSO, 400 μ M each dNTP, 5 μ L of a primer suite, and 1 μ L of SYBR Green I (1:32000, Invitrogen, Karlsruhe, Germany). Primer suites consisted of 5.0 μ M of each F3/B3 external primers, 15.0 μ M of each FIP/BIP internal primer, and 10.0 μ M of each loop F/B primer. Real-time isothermal LAMP assays of 48 reactions at a time (includes eight negative controls) were run using a Rotor-Gene Q (QIAGEN) real-time PCR cyclers set to run at a constant 65°C cycle for 30 min. with SYBR Green emission detection for intervals of 20 seconds every minute. Reactions were terminated at 80°C for 5 min. Fluorescent emission recordings were quantified and graphically represented using Rotor-Gene Q Series Software 1.7 (Build 94). For each primer suite, replicates of target CNG and ST weeds, other *Nassella* and native grass species were qualitatively scored at LAMP assays as positive/negative for a defined presence of LAMP amplification products. We defined a positive outcome at a replicate if its peak fluorescence intensity was >20% of that observed at the time during the cycle of the 1st instance of LAMP fluorescence saturation (indicative of high-yield LAMP product) among replicates in an assay run.

RESULTS

DNA barcodes of the sampled WoNS were correctly matched to existing sequence accessions of CNG and ST confirming their species identity. The DNA barcodes were reciprocally monophyletic for CNG and ST at each of three loci (data not shown) and minimum sequence differences between the two species varied as 3.31%, 2.77%, and 0.11% at *ETS*, *ITS* and *matK* respectively.

Saturation level LAMP assay products were detected by a real-time PCR platform after 15–20 min. of isothermal temperature cycling at 65°C. Contamination of LAMP master mix identified as presence of saturation level products in negative controls was observed at <5% of runs, and in all these instances, replicate scores were discarded and runs repeated using fresh reagents.

Specificity and repeatability of LAMP assays was highly variable among the ten primer suites (Table 1). Positive control (+) assays using primer suites 08 and 10 failed across all replicates; in contrast + assays using primer suite 09 targeted to mitochondrial *COI* (Tomlinson *et al.* 2010) were successful at detection of 83% of target CNG and ST replicates, but only 32% of other grass replicates.

Of the seven LAMP assays specific for the two target *Nassella* spp., the highest repeatability and specificity was observed using primer suite 05 which exclusively detected >97% of tested target CNG. Suite 02 detected 100% of target CNG, but also 15% of other *Nassella* spp. replicates. Suite 03 exclusively detected

>89% of target ST. Other suites had mixed propensity for partial detection across the two target WoNS (Suite 01) or across other tested grasses (suites 06 and 07). Suite 04 failed overall.

DISCUSSION

LAMP specificity testing of seven primer suites designed from DNA barcode sequences specific for Chilean needle grass (CNG) and serrated tussock (ST) demonstrated high accuracy and repeatability of two suites (05 and 02) for laboratory based diagnostic detection of CNG, and one suite (03) for ST. LAMP products generated using these three primer suites amplified to saturation levels of detection within 15–20 min. using a real-time PCR platform. In contrast, non-target specimens either failed to amplify using these suites or amplified at fewer replicates, at much later assay stages (>25 min.) and at sub-saturation levels of detection. Chimeric formation among primers during isothermal amplification may be inducing some of these observed false positive LAMP results. Attaining greater rates of repeatability and specificity (i.e. near 100%) of these LAMP assays for the target *Nassella* species is critical to the reliability of the proposed LAMP platform for in-field uses, where misdiagnosis would lead to an erroneous control response and potentially result in emergence of a new infestation and or loss of local native flora. Optimisation of LAMP reagent composition (Tomlinson *et al.* 2010) used with these primer suites and or primer modification will improve both specificity of primers to the targets

Table 1. Specificity and repeatability of LAMP primer suites (01–07) designed from loci (*ETS*, *ITS*, *matK*) for detection of target weeds Chilean needle grass (CNG, n = 40) or serrated tussock (ST n = 40), and primer suites (08–10) from loci (*COI* and *rbcL*) for use as positive (+) controls in Poaceae detection. Extra grasses tested include four other *Nassella* spp. (NS; n = 20) and ten native Poaceae (PO; n = 36) of similar appearance to the target weeds. All suites are novel except (09) Tomlinson *et al.* 2010.

Suite	Target		n detected / n tested			
	loci	grass	CNG	ST	NS	PO
01	<i>ETS</i>	ST	10/40	30/38	0/20	0/36
02	<i>ETS</i>	CNG	40/40	0/40	6/20	0/36
03	<i>ETS</i>	ST	0/40	34/38	0/20	0/36
04	<i>ITS</i>	ST	0/10	0/10	0/5	0/9
05	<i>ITS</i>	CNG	39/40	0/38	0/20	0/36
06	<i>matK</i>	ST	39/40	31/38	3/20	1/36
07	<i>matK</i>	CNG	28/40	28/38	6/20	5/36
08	<i>COI</i>	+	0/10	0/10	0/5	0/9
09	<i>COI</i>	+	35/40	30/38	4/20	14/36
10	<i>rbcL</i>	+	0/10	0/10	0/5	0/9

(minimising false positives) and repeatability of the assay among target replicates (minimising false negatives). Unexpected partial success in detection of both target weeds (to the exclusion of all other assayed grasses) using suite 01 may allow for the possibility of a single LAMP assay useful for diagnostics of either weed, however modifications of the constituent primers in the suite are required to improve detection success (currently 51% detection across the two target *Nassella* species)

Integral to our future proposed use of LAMP for in-field detection of the *Nassella* WoNS is the need for a secondary LAMP test, ancillary to the primary assay for use as a positive control of Poaceae grasses in general. This secondary test would allow accurate interpretation of negative test outcomes observed in the primary assay as resulting either from absence of the target organism (true negative) or due to corruption of the LAMP reaction itself (false negative). Of the three suites of primers tested for this purpose as a general LAMP positive control, only one (suite 09) showed some measure of success across replicates of the assayed taxa. The suite was originally designed as a control in detection of Rhododendrons but shown to have broader specificity across a variety of plants (Tomlinson *et al.* 2010). The suite provided high levels of positive repeatability (83%) for detection of the two target weeds but less repeatability among the remaining grasses (32%). Modifications to constituent primers in this suite to better anneal to Poaceae will likely improve its reliability as a positive diagnostic of that group of grasses.

Ultimately, preparation of all these diagnostic LAMP assays for in-field identification of CNG and ST will require: 1) identification of a rapid and crude DNA release method that is non-inhibitive to LAMP reactivity; and 2) development of a simple low-cost isothermal platform incorporating inexpensive LAMP product visualisation capacity. As discussed earlier (Gopurenko *et al.* 2017) existing methods for crude DNA extraction, isothermal heating and visual detection of LAMP products using inexpensive lateral flow devices have been developed for in-field diagnostics of pathogens in a range of host tissues. Similar approaches may be modified and applicable here for diagnostics of CNG and ST and will be the focus of the next stage of our research.

ACKNOWLEDGMENTS

Funding for this project (CT-17) was provided through the Agricultural Competitiveness White Paper (Commonwealth of Australia) Control Tools and Technologies for Established Pest Animals and Weeds Programme, administered by the Department

of Agriculture and Water Resources. We gratefully thank Brett Jones (Snowy Monaro Regional Council), Neville Plumb (Queanbeyan-Palerang Regional Council), Malcolm Ross (Goulburn Mulwaree Council), Steve Taylor and Jenny Conolly (Australian Capital Territory Parks and Conservation Service) for invaluable assistances in field sampling of weeds examined here. We acknowledge edits by Gretchen Kay Foster and anonymous reviewers to improve our manuscript.

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