ENGINEERED DNA POLYMERASE IMPROVES PCR RESULTS FOR PLASTID DNA

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• Premise of the study: Secondary metabolites often inhibit PCR and sequencing reactions in extractions from plant material, especially from silica-dried and herbarium material. A DNA polymerase that is tolerant to inhibitors improves PCR results.

• Methods and Results: A novel DNA amplification system, including a DNA polymerase engineered via directed evolution for improved tolerance to common plant-derived PCR inhibitors, was evaluated and PCR parameters optimized for three species. An additional 31 species were then tested with the engineered enzyme and optimized protocol, as well as with regular Taq polymerase.

• Conclusions: PCR products and high-quality sequence data were obtained for 96% of samples for rbcL and 79% for matK, compared to 29% and 21% with regular Taq polymerase.

Key words: directed evolution; engineered KAPA3G DNA Polymerase; matK; PCR inhibition; rbcL.

Plants contain many secondary metabolites, including phenolics, polysaccharides, and glycoproteins, that can interfere with DNA extraction, PCR, and cycle sequencing. Multiple extraction protocols aimed at reducing or removing inhibitory compounds (e.g., Olmstead and Palmer, 1994; Setoguchi and Ohba, 1995; Hughey et al., 2001; Drábková et al., 2002; Malvick and Grunden, 2005), or attenuating their effects on PCR efficiency (Saunders, 1993; De Boer et al., 1995), have been developed, but these are often not effective. Certain taxa pose significant challenges to successful PCR and sequencing, even in cases where purified genomic DNA is used. While rbcL is generally considered an easy region to amplify and sequence with standard primers (Kress and Erickson, 2007; Hollingsworth et al., 2009), the first author had problems amplifying or sequencing the gene from multiple medicinal plants from Pakistan, including Amaranthus sp. (Chenopodiaceae), Anethum graveolens L. (Apiaceae), Butea monosperma (Lam.) Taub. (Fabaceae), Fagonia indica Burm. f. (Zygophyllaceae), Senna sp. (Fabaceae), and Trachyspermum ammi (L.) Sprague (Apiaceae). DNA was extracted from silica-dried or air-dried samples before PCR was attempted with regular Taq polymerase. An extraction of Anethum, prepared from fresh material, amplified and sequenced cleanly, suggesting that secondary metabolites in the dried material (which were not effectively removed with a commercial DNA purification kit), rather than suboptimal PCR parameters (identical for both samples), reduced amplification efficiency and inhibited sequencing. Inhibitors in dried material pose a serious challenge because fresh tissue is often not available.

The problem of poor PCR efficiency can be addressed at the polymerase level. Kapa Biosystems (Woburn, Massachusetts, USA) recently developed an enzyme with specific tolerance to common plant inhibitors, “KAPA3G” DNA Polymerase was derived from a previously engineered, more processive variant of Taq DNA polymerase (processivity reflects the average number of nucleotides added by a DNA polymerase per association/dissociation event with the template; processive enzymes synthesize DNA more quickly and are more efficient in the presence of inhibitors). In short, a randomized gene library of the parental “KAPA2G” DNA polymerase gene was generated and expressed in E. coli. Individual bacterial cells, each containing both the expressed, mutant DNA polymerase protein, as well as the gene encoding that variant, were compartmentalized in a water-in-oil emulsion (Griffiths and Tawfik, 2006). In this system, each mutant enzyme was required to amplify its own gene in the presence of secondary metabolites derived from different plant species. After several rounds of selection with increasing levels of inhibition pressure, gene variants coding for polymerases with improved tolerance to plant inhibitors were exponentially enriched over variants with no advantage. The KAPA3G DNA Polymerase that was evolved in this manner was blended with a small quantity of an engineered high-fidelity enzyme, to allow for the efficient amplification of DNA fragments >5 kb from plant samples. KAPA3G was effectively tested for PCR with purified plant DNA, crude plant extracts, and in direct PCR from leaf discs or seeds of a variety of crop plants (Appendix S1) before the KAPA3G Plant PCR Kit was released. This report constitutes the first study of the effectiveness of KAPA3G DNA Polymerase using dried
Fig. 1. Annealing temperature optimization by gradient PCR for the KAPA3G Plant PCR Kit, in the presence (+ PE) or absence (− PE) of the Plant Enhancer. Overall amplification was greater without Plant Enhancer. Numbers correspond to different annealing temperatures over a 20°C gradient: 1 = 50°C, 2 = 54°C, 3 = 58°C, 4 = 62°C, 5 = 66°C, 6 = 70°C. The highest temperature that resulted in successful product for all samples was 58°C. Marked PCR products (*) were submitted for sequencing with rbcL 1F, 636F, 724R, and 1460R primers.
TABLE 1. Comparison of rbcL sequencing data quality for Linum usitatissimum, Anethum graveolens, and Senna sp. using Taq polymerase and the KAPA3G PCR Kit with and without Plant Enhancer.

| Species     | rbcL Primer | Taq Phred KAPA3G + Enhancer | KAPA3G – Enhancer |
|-------------|-------------|-----------------------------|-------------------|
| Linum       | 1F          | 956                         | 464               |
| Linum       | 636F        | 866                         | 864               | 987               |
| Linum       | 724R        | 579                         | 777               |
| Linum       | 1460R       | 617                         | 1466              |
| Anethum     | 1F          | 0                           | 838               |
| Anethum     | 636F        | 0                           | 792               |
| Anethum     | 724R        | 0                           | 650               |
| Anethum     | 1460R       | 0                           | 870               |
| Senna       | 1F          | 12                          | 948               |
| Senna       | 636F        | 0                           | 797               |
| Senna       | 724R        | 837                         | 683               |
| Senna       | 1460R       | 871                         | 895               |
| 62°C        | 1F          | 598                         |                    |
| Senna       | 636F        | 909                         |                    |
| 62°C        | 724R        | 877                         |                    |
| 62°C        | 1460R       | 466                         |                    |

Note: An annealing temperature of 48°C was used with regular Taq, whereas the optimal annealing temperature with the KAPA3G chemistry was 58°C for all samples except Senna (62°C). Senna did not amplify with Taq polymerase. The highest Q20 values for each primer given the three different PCR master mixes are in bold.

TABLE 2. PCR and sequencing success of 31 species for rbcL and matK using Taq polymerase or the KAPA3G Plant PCR Kit.

| Species                | regular Taq | KAPA3G Plant PCR Kit | regular Taq | KAPA3G Plant PCR Kit |
|------------------------|-------------|----------------------|-------------|----------------------|
| Linum usitatissimum    | –           | –                    | –           | +                    |
| Anethum graveolens     | +           | +                    | +           | +                    |
| Senna sp.              | –           | –                    | –           | +                    |
| Euphorbia helioscopia  | +           | +                    | +           | +                    |
| Convolvulus arvensis   | –           | –                    | –           | +                    |
| Cucurbita maxima       | +           | +                    | +           | +                    |
| Datura stramonium      | –           | –                    | –           | +                    |
| Fumaria indica         | –           | –                    | –           | +                    |
| Justicia adhatoda      | +           | +                    | +           | +                    |
| Lathyrus aphaca        | –           | –                    | –           | +                    |
| Launaea nudicaulis     | –           | –                    | –           | +                    |
| Lavandula angustifolia | –           | –                    | –           | +                    |
| Lepidium didymum       | +           | +                    | +           | +                    |
| Matricaria chamomilla  | –           | –                    | –           | +                    |
| var. recutita (L.) Fiori | –       | –                    | –           | +                    |
| Mucuna pruriens (L.) DC. | +          | –                    | +           | +                    |
| Plumago auricula (L.)  | –           | –                    | –           | +                    |
| Schinus molle (L.)     | +           | +                    | +           | +                    |
| Solanum tuberosum (L.) | –           | –                    | –           | +                    |
| Taraxacum officinale   | –           | +                    | +           | +                    |
| Wigg.                  | –           | +                    | –           | +                    |
| Trichodesma indicum (L.) | –             | –                    | –           | +                    |
| Sm.                    | –           | –                    | –           | +                    |
| Urtica dioica (L.)     | +           | +                    | +           | +                    |
| Veronica polita Fr.    | –           | –                    | +           | +                    |
| Vicia faba (L.)        | +           | +                    | +           | +                    |
| Vitis negundo (L.)     | –           | –                    | –           | +                    |
| Withania somnifera (L.)| +           | +                    | +           | +                    |

Note: Unless otherwise noted, a + indicates both successful PCR and sequencing. PCR for matK was not attempted with Taq for Euphorbia helioscopia, Galium aparine, or Taraxacum officinale. Gene regions that were successfully sequenced after PCR with Taq were generally not tried with the KAPA3G Plant PCR Kit.

METHODS AND RESULTS

Three DNA extracts that produced varying degrees of amplification and sequencing success with regular Taq polymerase were chosen for initial rbcL optimization (primers 1F [Fay et al., 1997] and 1460R [Fay et al., 1998; Cuénot et al., 2002]) with the KAPA3G Plant PCR Kit. See Appendix 1 for voucher information for all species included in the study. Wild collections were not georeferenced at the time of collection. Mini-extractions for all species were performed using a standard cetlytrimethylammonium bromide (CTAB) protocol (Doyle, 1991) and purified using the UltraClean 15 kit (MO BIO, Carlsbad, California, USA). PCR for rbcL, matK, and psbA-trnH had been attempted using ReadyMix PCR master mix with Taq polymerase (Sigma, St. Louis, Missouri, USA). The following thermal cycler program was used for rbcL and matK PCR with Taq polymerase: 94°C 5 min; 30 cycles: 94°C 1 min, 48°C 1 min, 72°C 1 min; 72°C 7 min. All three regions were successfully amplified and sequenced for Linum, but rbcL failed to sequence for Anethum and did not amplify for Senna, although the psbA-trnH spacer was sequenced for both. Linum was selected for the KAPA3G Plant PCR Kit evaluation as it had amplified and sequenced with Taq polymerase, while Anethum was chosen because it had amplified but failed to sequence, and Senna as it had not amplified at all. The KAPA3G Plant PCR Kit includes an optional Plant Enhancer, a reducing agent that improves amplification efficiency for some types of samples through an unknown mechanism. Two sets of reactions were run for each taxon, one with 0.5 μL (1×) Enhancer and one without. Each reaction contained the KAPA3G Plant Buffer (1× final concentration, includes dNTPs at 0.2 mM each), MgCl₂ (2 mM final concentration), 1 unit DNA polymerase, primers at a final concentration of 0.3 μM each, and Taq polymerase. An annealing temperature of 48°C was used with regular Taq, whereas the optimal annealing temperature with the KAPA3G chemistry was 58°C for all samples except Senna (62°C). Senna did not amplify with Taq polymerase. The highest Q20 values for each primer given the three different PCR master mixes are in bold.

PCR-grade water to bring the volume to 50 μL. An annealing temperature gradient PCR was performed, in increments of 4°C from 50°C to 70°C, using a Veriti Thermal Cycler (Applied Biosystems, Carlsbad, California, USA) and the following cycling parameters: 95°C 10 min; 40 cycles: 95°C 20 s, 50–70°C [gradient] 15 s, 72°C 90 s; 72°C 90 s. The gradient PCR identified the highest temperature at which amplification was successful for all samples (58°C). To test the amplification quality, six of the best PCR products (corresponding to the brightest bands in a 1% agarose gel) were selected for sequencing: Linum, Anethum, and Senna generated with an annealing temperature of 58°C, with and without Enhancer. The best overall PCR product (Senna without Enhancer, generated with an annealing temperature of 62°C) was also sequenced for

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comparison. PCR products were cleaned with the Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, Wisconsin, USA). DNA sequences were generated at Ohio University’s Genomics Facility and analyzed using an ABI 3130xL Genetic Analyzer (Applied Biosystems), 0.5 μL dimethyl sulfoxide (DMSO; Sigma), 0.5 μL BigDye (Applied Biosystems), 0.1 μL Thermofidela (Fidelity Systems, Gaithersburg, Maryland, USA), 10–40 ng template DNA, and PCR-grade water for a total volume of 8 μL. Cycle sequencing products were cleaned with the BigDye XTerminator Purification Kit (Applied Biosystems). Phred Q20 values (Ewing et al., 1998) were used as an initial indication of sequence quality. External primers 1F and 1460R, and internal primers 636F and 724R (Fay et al., 1997), were used for sequencing.

Results of the PCR optimization are shown in Fig. 1. Amplification was successful at annealing temperatures from 50–62°C, although amplification at 62°C was reduced or failed when Enhancer was present. More product was produced without Enhancer, but more nonspecific amplification occurred. Senna, which did not amplify for Taq polymerase, amplified strongly using the KAPA3G enzyme. Sequencing results for rbcL primers 1F, 636F, 724R, and 1460R are presented in Table 1, with partial rbcL 1F chromatograms in Appendices S2 and S3. Sequence data for Lumin 1F and 636F were of higher quality from samples without Enhancer. This suggested that residual Enhancer (carried through PCR clean-up) may have inhibited the cycle sequencing reaction. However, the results of a second optimization did not support this conclusion. A second round of optimization for rbcL was performed with the Lumin, Anethum, and Senna extracts to reduce non-specific amplification, although no significant improvements were observed for these particular species. See Appendices S4 and S5 for the protocol and results, which tested the effects of different thermal cycling programs, MgCl₂ concentrations, and the presence/absence of Enhancer.

Extracts of an additional 31 species from 23 different families, prepared with the same methods outlined above, were tested for rbcL (Table 2), first with Taq polymerase, and then with the KAPA3G enzyme using the optimized cycling program with an annealing temperature of 58°C. Nine out of 31 samples (29%) amplified and sequenced for rbcL with Taq, whereas 21 out of 22 samples (95%) that failed with Taq amplified and sequenced with the KAPA3G enzyme (1.5 mM MgCl₂, no Enhancer). This success rate is much higher than the best rbcL PCR rate (26%) reported by Särkkinen et al. (2012) for several different DNA polymerase enzymes, although extracts from much older herbarium specimens were used in their study. The same initial optimization outlined above was performed for matK 390F/1360R (Cuénoud et al., 2002), and an annealing temperature of 54°C was selected for this assay. Three out of 26 samples (12%) amplified and sequenced with Taq polymerase for matK while 21 out of 28 samples (75%) amplified and sequenced with the KAPA3G enzyme (Table 2). A higher concentration (2 mM) of MgCl₂ was required for successful PCR of these samples. Effective amplification of Glycyphila, Urtica (e.g., Hygrophiila, Urtica) did not amplify for one or both gene regions with the KAPA3G enzyme. These samples were characterized by abundant mucilage during the extraction process, and purifying the genomic DNA did not remove all the mucilage. A non-nucleic acid extract prepared from seeds (market sample) of Hygrophiila did not amplify successfully for matK (but not rbcL) with the KAPA3G enzyme after PCR with Taq polymerase failed. An Urtica extract prepared with the PowerPlant Pro DNA Isolation Kit (MO BIO) amplified readily for rbcL (but not matK) at 1.5 mM MgCl₂ with the KAPA3G enzyme (Table 2). For certain species of Lamiaceae (Ajuga, Mentha, Ocimum), results not shown here), successful amplification of rbcL and matK was achieved with the KAPA3G enzyme from dirty pellets (not purified after CTAB extraction), while other species (Lycopus, Nepeta, Origanum) failed to amplify until genomic DNA had been purified or extracted with the PowerPlant Pro DNA Isolation Kit. Taken together, these results suggest that while the KAPA3G enzyme offers much higher success rates than Taq polymerase, PCR from plant samples remains challenging in the presence of high levels of inhibitors, particularly when primers are not perfectly matched to target sequences.

CONCLUSIONS

This study demonstrated that the KAPA3G Plant PCR Kit successfully amplified DNA from extracts that failed with Taq. Quality sequence data were obtained from species from 24 different families. The variable results obtained with Taq polymerase and the KAPA3G Plant PCR Kit indicate that PCR success and sequence quality may be as much a function of the taxon as the methodologies used. Differences in secondary metabolites presumably account for some of this variation. Although the KAPA3G Plant PCR Kit did not always lead to high-quality sequence data, it effectively amplified DNA that failed to amplify with Taq polymerase. The KAPA3G Plant PCR Kit can therefore be a very useful tool for plant biologists working with difficult taxa that have failed to amplify with Taq polymerase. We recommend using the optimization protocol (Appendix 2) to select the best annealing temperature for a specific assay, and then performing the PCR with 1.5 mM MgCl₂ and no Enhancer. If the PCR fails, increasing the MgCl₂ concentration (2 mM) and/or adding Enhancer should be tried as these proved to be critical for successful PCR for certain taxa.

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## Appendix 1. Voucher specimens of medicinal plant species from Pakistan used in this study.

| Species                        | Voucher specimen accession no. | Collection locality | Geographic coordinates |
|--------------------------------|--------------------------------|---------------------|------------------------|
| Acacia nilotica               | M. N. Badshah MSAE-1           | Islamabad, Pakistan | 33°53′50″N, 72°51′43″E  |
| Achyranthes aspera            | M. N. Badshah MSAE-4           | Islamabad, Pakistan |                        |
| Anethum graveolens            | M. N. Badshah MSAE-10          | Islamabad, Pakistan |                        |
| Argemone mexicana             | M. N. Badshah 206              | Islamabad, Pakistan |                        |
| Artemisia absinthium          | Farooq 9(1)-1.absinthium       | Qarshi Herb Garden, Hattar, Pakistan | 33°53′50″N, 72°51′43″E  |
| Asparagus racemosus           | Nazir 49(3)-3.racemosus        | Qarshi Herb Garden, Hattar, Pakistan | 33°53′50″N, 72°51′43″E  |
| Buxus papillosa               | M. N. Badshah 216              | Islamabad, Pakistan |                        |
| Convolvulus arvensis          | M. N. Badshah 218              | Islamabad, Pakistan |                        |
| Crocus sativus                | Nazir 45(2)-1.sativus          | Qarshi Herb Garden, Hattar, Pakistan | 33°53′50″N, 72°51′43″E  |
| Cuminum cyminum               | Khan 89(2)-1.cymimum          | Qarshi Herb Garden, Hattar, Pakistan | 33°53′50″N, 72°51′43″E  |
| Euphorbia helioscopia         | M. N. Badshah 211              | Islamabad, Pakistan |                        |
| Fumaria indica                | M. N. Badshah 203              | Islamabad, Pakistan |                        |
| Fumaria indica                | Farooq 37(1)-1.indica          | Qarshi Herb Garden, Hattar, Pakistan | 33°53′50″N, 72°51′43″E  |
| Galium aparine                | M. N. Badshah 210              | Islamabad, Pakistan |                        |
| Hygrophila auriculata         | Farooq 4(2)-1.longifolia       | Qarshi Herb Garden, Hattar, Pakistan | 33°53′50″N, 72°51′43″E  |
| Justicia adhatoda             | M. N. Badshah 202              | Islamabad, Pakistan |                        |
| Lathyrus aphaica              | M. N. Badshah 217              | Islamabad, Pakistan |                        |
| Laurus philippica             | M. N. Badshah 209              | Rawalpindi, Pakistan |                        |
| Lawsonia inermis              | Nazir 4(7)-1.inermis           | Qarshi Herb Garden, Hattar, Pakistan | 33°53′50″N, 72°51′43″E  |
| Lepidium didymum              | M. N. Badshah 207              | Islamabad, Pakistan |                        |
| Linum usitatissimum           | N. Allam MSAE-8                | Islamabad, Pakistan |                        |
| Matricaria chamomilla var. recutita | Farooq 9(14)-1.recutita         | Qarshi Herb Garden, Hattar, Pakistan | 33°53′50″N, 72°51′43″E  |
| Mucuna pruriens               | Nazir 9(14)-1.recutita         | Qarshi Herb Garden, Hattar, Pakistan | 33°53′50″N, 72°51′43″E  |
| Plumbago auriculata           | Farooq 73(2)-2.auriculata      | Qarshi Herb Garden, Hattar, Pakistan | 33°53′50″N, 72°51′43″E  |
| Schinus molle                 | M. N. Badshah 215              | Islamabad, Pakistan |                        |
| *Senna* sp. (cf. *auriculata*) | 50945                        | Market sample from Sawat Pansar Store and Dawakhana, Aabpara Market, Islamabad | |

*Badshah and Allam collections are at ISL; Qarshi collections are privately held at the company herbarium in Hattar.*

*bQarshi collections are from cultivated plants in a demonstration herb garden at the company headquarters in Hattar.*

*cMarket sample from Sawat Pansar Store and Dawakhana, Aabpara Market, Islamabad.*
APPENDIX 2. KAPA3G optimization protocol.

This optimization procedure should be performed for each new primer set to select the best (highest effective) annealing temperature with the KAPA3G Plant PCR Kit (Kapa Biosystems, Woburn, Massachusetts, USA). Once the temperature is selected, it may be necessary to increase the final MgCl₂ concentration (from 1.5 mM at 1× in the KAPA3G Plant PCR Buffer) and/or add Plant Enhancer to PCR reactions for successful amplification of specific taxa. Four different PCR reaction mixtures (A–D) are recommended for the initial optimization. Certain taxa (e.g., some Clusiaceae) may fail to amplify without Enhancer. See Note 1 for more details.

Gradient PCR program: 95°C 10 min; 40 cycles: 95°C 20 s, 50–70°C (gradient) 15 s, 72°C 90 s; 72°C 90 s; 10°C hold. The gradient was programmed at 4°C intervals on a Veriti 96-well, 0.2 mL Thermal Cycler (Applied Biosystems, Carlsbad, California, USA).

| Mix A | 1.5 mM MgCl₂, with Enhancer (50 μL reaction volume) |
|-------|
| PCR-grade water | 20.1 μL |
| KAPA3G Plant PCR Buffer (2×) | 25 μL |
| Forward primer (10 μM)* | 1.5 μL |
| Reverse primer (10 μM)* | 1.5 μL |
| KAPA3G Plant DNA Polymerase (2.5 U/μL) | 0.4 μL |
| KAPA Plant PCR Enhancer (100×) | 0.5 μL |
| Template | 1.0 μL |

| Mix B | 1.5 mM MgCl₂, without Enhancer (50 μL reaction volume) |
|-------|
| PCR-grade water | 20.6 μL |
| KAPA3G Plant PCR Buffer (2×) | 25 μL |
| Forward primer (10 μM)* | 1.5 μL |
| Reverse primer (10 μM)* | 1.5 μL |
| KAPA3G Plant DNA Polymerase (2.5 U/μL) | 0.4 μL |
| Template | 1.0 μL |

| Mix C | 2.0 mM MgCl₂, with Enhancer (50 μL reaction volume) |
|-------|
| PCR-grade water | 19.1 μL |
| KAPA3G Plant PCR Buffer (2×) | 25 μL |
| Forward primer (10 μM)* | 1.5 μL |
| Reverse primer (10 μM)* | 1.5 μL |
| KAPA3G Plant DNA Polymerase (2.5 U/μL) | 0.4 μL |
| MgCl₂ (25 mM) | 1.0 μL |
| KAPA Plant PCR Enhancer (100×) | 0.5 μL |
| Template | 1.0 μL |

| Mix D | 2.0 mM MgCl₂, without Enhancer (50 μL reaction volume) |
|-------|
| PCR-grade water | 19.6 μL |
| KAPA3G Plant PCR Buffer (2×) | 25 μL |
| Forward primer (10 μM)* | 1.5 μL |
| Reverse primer (10 μM)* | 1.5 μL |
| KAPA3G Plant DNA Polymerase (2.5 U/μL) | 0.4 μL |
| MgCl₂ (25 mM) | 1.0 μL |
| Template | 1.0 μL |

† Includes dNTPs at a final concentration of 0.2 mM each.
* If primer stocks are at a different concentration than 10 μM, include the appropriate volume of each primer for a final concentration of 0.3 μM each, and adjust the volume of water accordingly (for a reaction volume of 50 μL).

NOTES

1. For initial optimization, the following PCR schedule is recommended: first use Mix B (1.5 mM MgCl₂, no Enhancer). Use Mix D (2 mM MgCl₂, no Enhancer) for samples that did not amplify well with Mix B, then try Mixes A and C at the same time. Systematicists working on one particular group of plants may be able to select an optimal mix for that group, while those working with a broader range of genera or families may need to identify the optimal mix for each taxon. PCR efficiency tends to be lower if Enhancer is added, so if Enhancer is required with any samples, the highest temperature that is effective with Enhancer should be selected.

2. A reaction volume of 50 μL is necessary for the appropriate PCR chemistry. We do not recommend reducing the volume to 25 μL or increasing it to 100 μL as this may adversely affect PCR efficiency.

3. Addition of Enhancer results in PCR products that appear cloudy. This is normal and does not affect the product or sequencing.

4. Clean PCR products with a spin-filter system to ensure removal of all dNTPs, MgCl₂, and enzyme prior to sequencing.
TROUBLESHOOTING

Non-specific amplification—Non-specific amplification may occur and appear as discrete bands or a smear above or below the targeted amplification product. The KAPA3G DNA polymerase is very active and will amplify fragments of DNA from spurious annealing events to a greater extent than Taq polymerase. Post-PCR clean-up sometimes reduces higher-molecular-weight smears that initially appear to be non-specific amplification products, but could be an artifact from the electrophoretic analysis. Non-specific amplification can be reduced by further optimization of annealing temperature, reducing extension time per cycle, or adding Plant Enhancer. The addition of polyvinylpyrrolidone (PVP), dimethyl sulfoxide (DMSO), or 2-mercaptoethanol (BME) (not used in this study) may also increase the yield of specific product. Try different strategies or combinations of the strategies outlined above for taxa that prove to be particularly problematic (see Appendix S4). Plant Enhancer generally reduces the overall amount of both specific and non-specific amplification products. If all of the above strategies fail, primers may have to be redesigned.

No bands/faint bands on gel—PCR may fail for a variety of reasons. Check the quality of genomic DNA by running 10 μL in a 1% agarose gel. Older extractions that were eluted in water may have degraded over time—always elute, store, and dilute DNA (and primers) in 10 mM Tris-HCl, pH 8.5. Primer mismatches, especially at the 3’ end, will reduce yield and specificity, and can affect the quality of sequence data. Note that “universal” primers may have one or more mismatches for particular taxa. For some species, the final MgCl₂ concentration in the reaction could affect the yield of the specific product significantly. If the PCR produced a “clean” band (i.e., non-specific amplification is not an issue), the yield of specific product may be increased by extending the PCR with five or 10 more cycles.