APPENDIX 1. Protocol for long PCR for amplification of 4–20-kb targets. Developed by the Tank Laboratory, University of Idaho; published January 2014.

Product	Contents	Catalog no.
QIAGEN *Taq* DNA Polymerase[1]	250 units *Taq* DNA Polymerase, 10× PCR Buffer,[†] 5× Q-Solution, 25 mM $MgCl_2$	201205
QIAGEN HotStar HiFidelity DNA Polymerase[2]	100 units HotStar HiFidelity DNA Polymerase,[2] 10× HotStar PCR Buffer, 5× Q-solution, 25 mM $MgSO_4$	202602

[1] Almost any high-quality *Taq* polymerase should work; however, cheap *Taq* polymerases (e.g., QIAGEN Top*Taq* or Promega Go*Taq*) do not work and result in large smears, rather than discrete bands.

[2] QIAGEN HotStar HiFidelity DNA Polymerase was the only high-fidelity polymerase used in this study.

[†] Q-solution does seem to be an important additive, thus the use of QIAGEN *Taq*. However, this does work using Q-solution with other high-quality *Taq* polymerases such as Promega's or New England Biolab's standard *Taq* (i.e., if you have a stock of Q-solution, but no QIAGEN *Taq*).

Genomic DNA must be high quality. Run a 0.8% or 1% gel to check. Standard CTAB extractions from silica gel–dried or herbarium material work well if they (1) are recent (extraction and tissue), and (2) contain high-molecular-weight DNA. Most important, we have found that recent DNA extractions that have not been through numerous freeze-thaw cycles work best. **For best results, long PCR should be done using new DNA extractions stored at 4°C while performing long PCR experiments**.

All preparations should be done on ice.

1. Number tubes or prepare plate. Make sure to include appropriate negative controls.

2. Prepare QIAGEN HotStar HiFidelity DNA polymerase dilution:

Reagents to prepare the HotStar *Taq* dilution	Volumes for 25 reactions (total 12.5 μL)	Volumes for 50 reactions (total 25 μL)	Volumes for 100 reactions (total 50 μL)
5× HotStar HiFidelity PCR buffer	2.5 μL	5.0 μL	10 μL
H_2O	9.0 μL	18 μL	36 μL
QIAGEN HotStar *Taq*	1.0 μL	2.0 μL	4.0 μL

3. Prepare cocktail:

Cocktail	×1 (25 μL reaction)
10× PCR buffer (QIAGEN CoralLoad PCR Buffer or colorless, 15 mM $MgCl_2$)	2.5 μL
$MgCl_2$ (25 mM)	1.0 μL (3 mM final conc.; adjustable)
dNTP (10 mM each)	0.75 μL (3 μL of 2.5 mM each)
Q solution (5×)	5.0 μL
5′ primer (5 μM)	2.5 μL (0.5 μM final conc.)
3′ primer (5 μM)	2.5 μL (0.5 μM final conc.)
Taq DNA polymerase (QIAGEN)	0.25 μL (1.25 units)[1]
QIAGEN HotStar DNA polymerase (diluted)	0.50 μL
H_2O	to 25 μL (9 μL if using 1.0 μL DNA)

[1] The success rate was lower when a smaller quantity was used, but the best DNAs work with ≥0.125 μL.

4. Add 1–2 μL of template to each of the tubes.

5. While the tubes/plate with template are on ice, add 24 μL of cocktail to each tube, being careful not to cross contaminate. Spin down to bring all liquid to the bottom of the tube.

6. Run appropriate long PCR profile. Generic temperatures and times are:
   i. 93°C infinity (important to go directly from ice to hot block)
   ii. 93°C for 3 min (initial denaturation)
   iii. 93°C for 15 s
   iv. 48–68°C for 30 s (T_a should be ~5°C below T_m of primers)
   v. 68°C for 5–20 min (1 min/kb of target)
   vi. go to step 3, 34×
   vii. 4°C infinity

7. Check reactions by running 2 μL on 1% agarose gel with appropriate size standards.

Primer combinations for long PCR amplification of the chloroplast genome.[1,2]

Region no.	Approx. size (kb)	Primers (F/R)[3]	Primer sequence (5′–3′)
1	8	trnH.GUG.6R	CCTTRATCCACTTGGCTACAT
		psbK.195R	ACTTACAGCAGCTTGCCAAAC
2	10.3	trnQ.UUG.50R	GGACGGAAGGATTCGAACC
		rpoC2.4805F	GYCGTATYGATTGGTTRAAAGG
2a[4]	6.3	trnQ.UUG.50R	GGACGGAAGGATTCGAACC
		atpH.17F	CTGCYGCTTCYGTTATTGCT
2b[4]	4	atpF.65R	CGGTATTAAACCCGAAACTCC
		rpoC2.4805F	GYCGTATYGATTGGTTRAAAGG
3	7	atpI.705R	CRGCTAAAGTTGCAAAAATAAGAGCT
		rpoC1.1670F	GRGATCAAATGGCTGTTCAT
4	9	rpoC2.520R	GTTCGTACAGCAGTATCYACAAC
		petN.3R	GCCCAAGCRAGACTTACTATATCC
5	10.5	trnC.GCA.47F	CCCAGTTCAAATCCGGGT
		psaB.2170F	GCRGCTTTCTTGATTGCYTC
6	10	trnfM.CAU.21R	GGTTATGAGCCTTGCGAGCTA
		trnT.UGU.17F	GGTTAGAGCATCGCATTTGTAATG
7	10.3	rps4.380R	GGTTTGCARCGATAACTTGGKATATC
		rbcL.178R	GTCCATGTACCAGTAGARGATTC
8	9.2	rbcL.2F	TGTCACCACAAACAGARACTAAAG
		psbJ.3F	GGCYGATACTACTGGAAGRAT
9	9.8	petA.920F	CTTCAAGAYCCATTACGTGTHCAAG
		psbB.160R	TRCCYTGTCTCCACATTGGAT
10	10.9	psbB.3F	GGGTTTRCCTTGGTATCGTGT
		rps3.17F.new	ATCCACTTGGTTTYMGACTTGG
11	8.7	rpl16.3R	AACCAACGAGTCACACACTAAGC
		ycf2.5100R	CAGATCATGAATGTTTGGAATCCAT
12	10	ycf2.2300F	TCGGGATCCTRATGCATATAGATAC
		rps12.190F	GTTGCCAGAGTACGMTTAACCT
13[5]	11	rps12.360R	CCCTTGTTGACGATCCTTTACTC
		ycf1.59R	CCGACCACAACGACCGAAT
14	11.2	trnN.GUU.7R	CCGCTCTACCACTGAGCTAC
		ndhA.535F	GCTGCTCAATCDATTAGTTATGAA
14′[6]	7	trnR.ACG.15F	GAGGATTAGAGCACGTGG
		ccsA.890R	TCCAAGTAATAAANGCCCAAGTTTC
15	10.5	ndhI.194R	CGAACRCATACTTCACAAGCAA
		trnN.GUU.7R	CCGCTCTACCACTGAGCTAC
16	8.2	psbA.640F	GCTATGCATGGTTCYTTGGTAAC
		ycf2.5100R	CAGATCATGAATGTTTGGAATCCAT

[1] Universal primers designed by M.J.M.; compiled and tested by D.C.T. and S.U.C.

[2] T_a should be ~5°C below T_m of primers; however, temperatures of 55°C have worked for all primer combinations.

[3] The name of each primer consists of three parts: (1) the gene in which the primer is anchored in, (2) the approximate position of the primer within that gene (based on all-angiosperm alignment per Moore et al., 2007), and (3) either an "F" or an "R." The F and R designations do not indicate that the primer should be used as a forward or reverse primer; rather, they indicate the 5′ to 3′ orientation of the primer with respect to the gene. In other words, a primer that is designated as an F primer has its 5′ to 3′ orientation in the same orientation as the gene (i.e., on the forward strand, or from start to stop), whereas an R primer is oriented in the direction opposite to the 5′ to 3′ orientation of the gene (i.e., on the reverse strand).

[4] Regions 2a and 2b can be used to amplify region 2 in two pieces.

[5] Regions 11, 12, and 13 represent a large portion of the inverted repeat (IR), thus, one amplification for both IRa and IRb.

[6] Region 14′ amplifies ca. 2/3 of region 14.