Protocolo de PCR para sequenciamento Sanger usando *primers* universais

Versão 1.0 (12/11/2020)

Para uma reação de volume final (V_f) de 50 µl:

Reagentes	C_i	C_f	V_i
DNA	10 - 100ng/µl	20 - 200ng	2
Tampão da Taq	10×	1	5
TBT	5×	1	10
dNTP	2,5mM	0,2	4
MgCl	50 mM	3	3
Primer Forward	10 µM	0,1	0,5
Primer Reverse	10 µM	0,1	0,5
Taq caseira		0,0125	0,125
H_2O			24,875

C_i= concentração inicial; C_f = concentração final; V_i= volume inicial

Obs. 1: A amplificação de cada região de interesse deve ser inicialmente testada num volume total de 25 µl.

Obs. 2: Para amplificação de várias amostras simultaneamente, uma Master Mix deve ser preparada de acordo com o número de amostras com um excesso de 10% para todos os reagentes.

Obs. 3: A quantidade ideal de DNA na reação pode ser testada em série de diluição.

Obs. 4: Para preparar a solução de 5× TBT pH 8,0, segundoSAMARAKOON et al, 2013.

Reagente	C_f
BSA (Soro de albumina bovina)	1 g/L
Tris- HCL ph 8,0	8.5 mM
Tween-20	1% (v/v)
Trehalose	750 mM

Programa do termociclador

5 min – 95 °C ---------- Desnaturação do DNA dupla fita
1 min – 95°C
1 min – 48 - 65°C } 35 Ciclos ------ Desnaturação, Anelamento e Extensão
1 min – 72°C
10 min – 72°C ---------- Extensão final
∞ 10°C ---------- Temperatura de conservação do produto

Obs. 4: Tempos mais curtos podem ser testados nas diferentes etapas para diferentes regiões. O tempo de extensão varia de acordo com o tamanho do produto final.

Obs. 5: A temperatura de anelamento depende dos *primers* usados (ver tabela abaixo) e pode ser ajustada para cada espécie por gradiente, se necessário.

Tabela 1. *Primers* universais disponíveis para análises filogenéticas e filogeográficas

Nome da região	Sequência do primer *forward*	Nº estoque	Sequência do primer *reverse*	N° estoque	Temperatura de anelamento	Tamanho de fragmento	Referência
atpβ-rbcL	atpβF (GTGGAAACCCCGGGACGAGAAGTAGT)	192	atpβR (ACTTGCTTTAGTTTCTGTTTGTGGTGA)	193	53ºC (original) 56ºC (otimizada)	~580-850pb	Hodges e Arnold 1994
ITS1-5.8S-ITS2	ITS5 (GGAAGTAAAAGTCGTAACAAGG)	475, 870	ITS4 (TCCTCCGCTTATTGATATGC)	576	50-60ºC	~600-850pb	White *et al*, 1990
ITS1	ITS5 (GGAAGTAAAAGTCGTAACAAGG	475	ITS2 (GCTGCGTTCTTCATCGATGC)	76	50°C	1500 pb	White *et al*, 1990
ITS2	ITS3 (GCATCGATGAAGAACGCAGC)	69	ITS4 (TCCTCCGCTTATTGATATGC)	576	55°C	300 pb	White *et al*, 1990
*mat*K	matK3F (AAGATGCCTCTTCTTTGCAT)	638	matK1R (GAACTAGTCGGATGGAGTAG)	637	52ºC	~1500pb	Sange*t al*, 1997
*ndh*F	1318F (GGATTAACYGCATTTTATATGTTTCG)	868	2110R (CCCCCTAYATATTTGATACCTTCTCC)	869	50ºC	~800-2000pb	Olmstead e Sweere, 1994

*ndh*F–*rpl*32	ndhF-rpl32 nestedF (TTTTTCTGATTCACCTGC)	464	ndhF-rpl32 nestedR (CATCTATTGTTCAAAACG)	465	50-55°C	~700pb	Steele*et* al, 2010
*psb*A-*trn*H	psbAF (GTTATGCATGAACGTAATGCTC)	470	trnHR (CGCGCATGGTGGATTCACAAATC)	471	50ºC		Sang*et* al, 1997
	psbA (CGAAGCTCCATCTACAAATGG)	641	trnH (GUG) (ACTGCCTTGATCCACTTGGC)	642	53ºC	~495pb	Hamilton *et* al, 1999
*psb*J-*pet*A	psbJ (ATAGGTACTGTARCYGGTATT)	162	petA (AACARTTYGARAAGGTTCAATT)	163	50ºC	~700-1300pb	Shaw *et al*, 2007
*psb*E-*pet*L	psbE-petL F (TGCTATGAATGACCCAGTATCG)	436	psbE-petL R (CAGACCGATAAATAGAGCTGAGG)	437	50-55°C	~1200 pb	Steele*et* al, 2010
*rbc*L	rbcL N (a F NY1151) (ATGTCACCACAAACAGAAACTAAAGC)	536	a R (NY1152) (TCACAAGCAGCAGCTAGTTCAGGACT)	537	53°C	~500-1400 pb	Käss and Wink, 1996/ Knopf et al, 2012
*rps*16-*trn*Q	*rps*16-*trn*Q F (GTCATTGGTTTAGTTGGTCC)	438	*rps*16-*trn*Q R (GCCAAGTGGTAAGGCAACG)	439	50-59°C	~1200 pb	Steele*et* al, 2010
*trn*L-*trn*F	c (CGAAATCGGTAGACGCTACG)	636	f (ATTTGAACTGGTGACACGAG)	635	50-56ºC	~900 pb	Taberlet*et al*, 1991
*trn*L-*trn*T	a (CATTACAAATGCGATGCTCT)	81	b (TCTACCGATTTCGCCATATC)	82	50-57ºC	~250-830 pb	Taberlet*et al*, 1991
*trns*S-*trns*G	trnG^{UUC} (GTAGCGGGAATCGAACCCGCATC)	191	trnS^{GCU}(AGATAGGGATTCGAACCCTCGGT)	190	50-66ºC	~600-1000pb	Shaw *et al*, 2005
*ycf*1	ycf1bF (TCTCGACGAAAATCAGATTGTTGTGAAT)	645	ycf1bR (ATACATGTCAAAGTGATGGAAAA)	646	52-53°C	900 pb	Dong*et al,* 2015

Referências

DONG, W.; XU, C.; LI, C.; SUN, J.; ZUO, Y.; SHI, S.; CHENG, T.; GUO, J; ZHOU, S. ycf1, the most promising plastid DNAbarcode of land plants. **Scientific Reports** v.5, n. 8348, 2015.http://doi.org/10.1038/srep08348

HAMILTON, M.B.Four primer pairs for the amplification ofchloroplast intergenic regions with intraspecific variation.**Molecular Ecology**v. 8, p. 521–523, 1999.

HODGES, S.A.; ARNOLD, M.L. Columbines, a geographically spread species flock. **Proceedings of the National Academy of Sciences of the USA**, v.91, p.5129–5132, 1994.https://doi.org/10.1073/pnas.91.11.5129

KÄSS, E.; WINK, Michael. Molecular evolution of the Leguminosae: phylogeny of the three subfamilies based on rbcL-sequences. **Biochemical Systematics and Ecology**, v. 24, p. 365-378, 1996.https://doi.org/10.1016/0305-1978(96)00032-4

KNOPF, P.; Schulz, C.; Little, D.P.;Stützel, T.; Stevenson, D.W. Relationships within Podocarpaceae based on DNA sequence, anatomical, morphological, and biogeographical data. **Cladistics**, v. 28, p. 271-299, 2012. https://doi.org/10.1111/j.1096-0031.2011.00381.x

OLMSTEAD, G.R.; SWEERE, J.A. Combining data in phylogenetic systematics: An empirical approach using three molecular data sets in the Solanaceae. **Systematic Biology**, v.43, n.4, p.467-481, 1994. http://doi.org/10.2307/2413546

SAMARAKOON, T.; WANG, S.Y.; ALFORD, M.H. Enhancing PCR amplification of DNA from recalcitrant plant specimens using a trehalose-based additive.**Applications in Plant Sciences**, v. 1, n. 1, p. 1200236, 2013. http://doi.org/10.3732/apps.1200236

SANG, T.; CRAWFORD, D.J.; STUESSY, T.F. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). **American Journal of Botany**, v.84, p.1120–1136, 1997. http:/doi.org/10.2307/2446155

SHAW, J.; LICKEY, E.B.; BECK, J.T.; FARMER, S.B.; LIU, W.; MILLER, J.; SIRIPUN, K.C.; WINDER, C.T.; SCHILLING, E.E.; SMALL, R.L. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis.**American Journal of Botany**, v. 92, n. 1, p. 142-166, 2005. http://doi.org/10.3732/ajb.92.1.142

SHAW, J.; LICKEY, E.B.; SCHILLING, E.E.; SMALL, R.L. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. **American Journal of Botany** v.94, n. 3, p.275–288, 2007. http://doi.org/10.3732/ajb.94.3.275

STEELE, P. R.;FRIAR, L.M.; GILBERT, L.E.; JANSEN, R.K. Molecular systematics of the neotropical genus*Psiguria* (Cucurbitaceae): implications for phylogeny and species identification. **American Journal of Botany**v.97,n.1, p.156–173, 2010. http://doi.org/10.3732/ajb.0900192

TABERLET, P.; GIELLY, L.; PAUTOU, G.; BOUVET, J. Universal primers for amplification of three non-coding regions of chloroplast DNA. **Plant Molecular Biology**, v.17, p.1105-1109, 1991.https://doi.org/10.1007/BF00037152

WHITE, T.J. - BRUNS, T. - LEE, S. - TAYLOR, J.W. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: **PCR Protocols:** A Guide to Methods and Applications. San Diego, CA, USA: Academic Press. ISBN:01-23721-81-4.p.315–22, 1990.