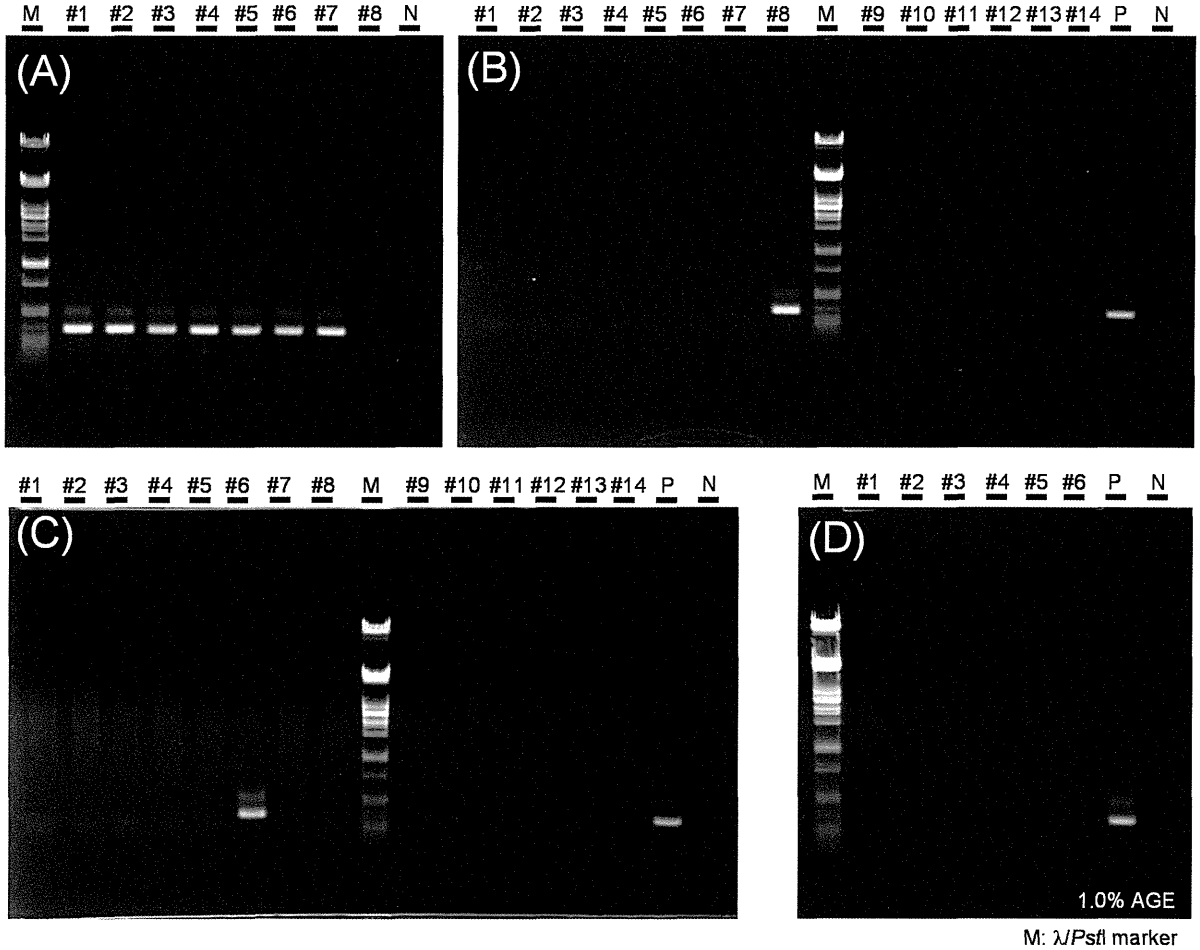
[RT reaction]			
Po(13-95) total RNA (tube #2)	8	μl	390 ng total RNA (DNase treated)
RACE32 primer (10 μM)	1	μl	
dNTP (@2.5 mM)	4	μl	TaKaRaBio
subtotal	13	μl	
↓ 65℃, 5 min then on ice			
5 x first strand buffer	4	μl	Invitrogen
DTT (0.1M)	1	μl	Invitrogen
RNaseOUT	1	μl	Invitrogen
Superscript III (200 U/ μl)	1	μl	Invitrogen
subtotal	20	μl	
↓ 50℃, 1 hr			
↓ 70℃, 15 min then on ice			
RNaseH	1	μl	Invitrogen
↓ 37℃, 20 min			
TE	29	μl	
ss-cDNA pool	50	μl	

図3. オニゲシ一本鎖cDNAプール合成のための逆転写反応のプロトコル

[5'-RACE RT reaction]			
Po(13-95) total RNA (tube #2)	8	μl	390 ng total RNA (DNase treated)
1603-5'RACE-RT primer (2.5 μM)	1	μl	
dNTP (@2.5 mM)	4	μl	TaKaRaBio
subtotal	13	μl	
↓ 65℃, 5 min then on ice			
5 x first strand buffer	4	μl	
DTT (0.1M)	1	μl	
RNaseOUT	1	μl	
Superscript III (200 U/ μl)	1	μl	
subtotal	20	μl	
↓ 50℃, 1 hr			
↓ 70℃, 15 min then on ice			
↓ PCI, CIA treatment			
↓ NH_4OAc ppt., air dry ppt.			
1603-5'RACE RT-ss-cDNA pool	ppt.		

[5'-RACE poly(A) tailing]		
DEPC water (dissolve ppt.)	33	μl
5xTdT buffer	10	μl
0.1% BSA	5	μl
dATP (10 μM)	1	μl
TdT	1	μl
Rxn. Vol.	50	μl
↓ 37 ℃, 30 min		
↓ 65 ℃, 10 min		
used for PCR		

図4. オニゲシcontig #1603 5'-RACEの逆転写合成(poly(A)付加まで)



A	lane	1	2	3	4	5	6	7	8	N (nega)
	sample	#13-95個体#1	#13-95個体#2	#13-95個体#3	#13-95個体#4	#13-95個体#5	#13-95個体#6	#13-95個体#7	ナガミヒナゲシ	AE buffer
	amp.	○	○	○	○	○	○	○		

B	lane	1	2	3	4	5	6	7	8
	sample	#345-00#3	#345-00#4	#624-79#1	#624-79#2	#624-79#3	#307-00#2	#14-95#1	#14-95#2
	amp.								○
	lane	9	10	11	12	13	14	P (posi)	N (nega)
	sample	#86-00#2	#86-00#3	#89-00#1	#89-00#4	#89-00#6	#98-00#1	#13-95#1	AE buffer
	amp.							○	

C	lane	1	2	3	4	5	6	7	8
	sample	#99-00#1	#106-00#1	#106-00#2	#138-00#1	#138-00#2	#139-00#1	#188-00#1	#188-00#2
	amp.						○		
	lane	9	10	11	12	13	14	P (posi)	N (nega)
	sample	#188-00#3	#188-00#4	#188-00#5	#188-00#6	#117-00#5-1	#117-00#5-2	#13-95#1	AE buffer
	amp.							○	

D	lane	1	2	3	4	5	6	P (posi)	N (nega)	contig #1603
	sample	#873-09	#874-09	#875-09	0876-09	#14-95	#624-79	#13-95#1	AE buffer	PCR
	amp.							○		positive

図5. オニゲシ*ODDs*相同contig #1603について設計したプライマーの
ケシ属植物との交叉反応性試験結果(H27年度結果更新)

B, C, Dで供試した導入番号#13-95以外の試料はいずれもハカマオニゲシとして導入・維持されているものである.

[#1603 (part) PCR]

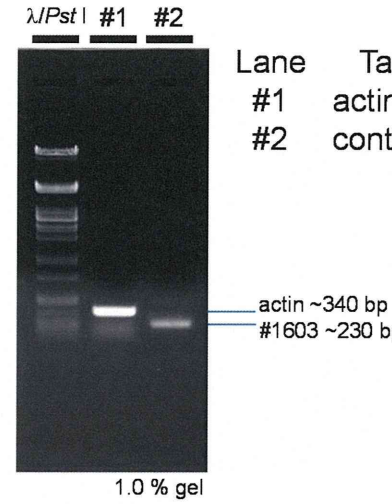
dH_2O	78.5	μl
10 x ExTaq Buffer (Takarabio)	10	μl
dNTP (@2.5 mM, Takarabio)	8	μl
Sense-primer (100 μM)	1	μl
Antisense-primer (100 μM)	1	μl
RACE RT ss-cDNA pool	1	μl
ExTaq (Takarabio)	0.5	μl
Rxn. Vol.	100	μl

↓ (94 ℃, 30 sec - 58 ℃, 30 sec - 72 ℃, 1 min) x 30

↓ 72 ℃, 10 min - 4 ℃, ∞

GeneAmp PCR System 2400 (Perkin Elmer)

↓ 5 μl of 100 μl checked by AGE



Lane	Target
#1	actin (control)
#2	contig #1603

図6. オニゲシactin遺伝子, contig #1603(part)のcDNA増幅結果

両遺伝子の葉における発現を確認した

[#1603 CORE PCR (Hot start)]

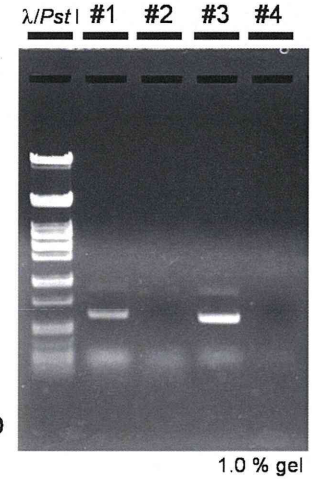
dH_2O	74	μl
10 x ExTaq Buffer (Takarabio)	10	μl
dNTP (@2.5 mM, Takarabio)	8	μl
Sense-primer (100 μM)	1	μl
Antisense-primer (100 μM)	1	μl
RACE RT ss-cDNA pool	1	μl
subtotal	95	μl
↓ 94 ℃, 5 min		
1 x ExTaq soln. (Takarabio)	5	μl
Rxn. Vol.	100	μl

↓ (94 ℃, 30 sec - 58 ℃, 30 sec - 72 ℃, 1 min) x 30

↓ 72 ℃, 10 min - 4 ℃, ∞

GeneAmp PCR System 2400 (Perkin Elmer)

↓ 5 μl of 100 μl checked by AGE



Lane	Primer sets
#1	S1+A1(1051A)
#2	S1+A2
#3	S2+A1(1051A)
#4	S2+A2

図7. オニゲシcontig #1603 CORE領域のPCR増幅結果

アンチセンス側A1プライマーで増幅産物が得られた

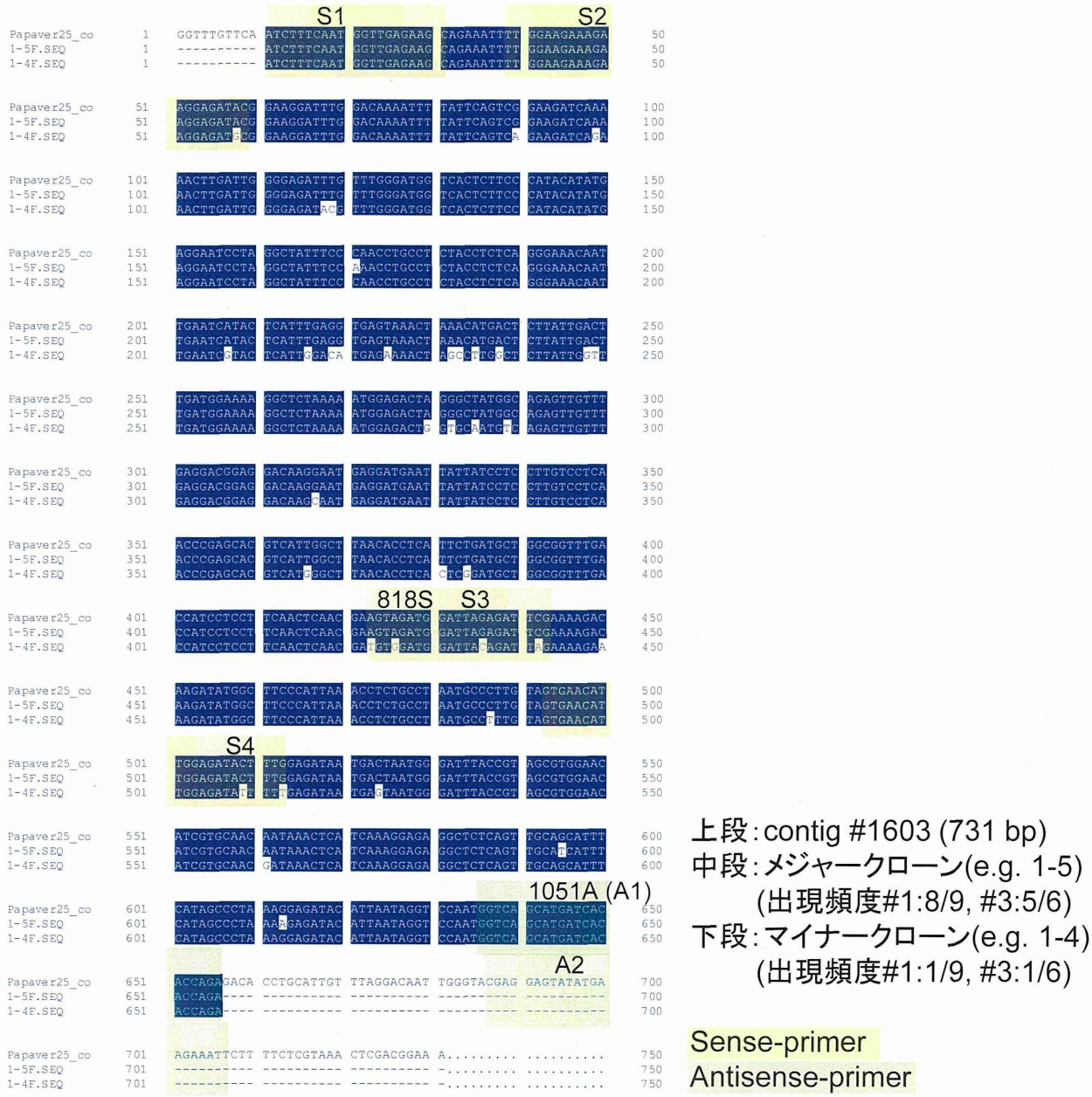


図8. オニゲシcontig #1603と，CORE領域のクローニング産物とのアラインメント

[1st PCR (Hot start)]

dH_2O	74	μl
10 x ExTaq Buffer (Takarabio)	10	μl
dNTP (@2.5 mM, Takarabio)	8	μl
#1603-818S(S3) (100 μM)	1	μl
RACE32, RACE17 (100 μM)	1	μl
RACE RT ss-cDNA pool	1	μl
subtotal	95	μl
↓ 94 ℃, 5 min		
1 x ExTaq soln. (Takarabio)	5	μl
Rxn. Vol.	100	μl

↓ (94 ℃, 1 min - 42 ℃, 2 min - 72 ℃, 3 min) x 30

↓ 72 ℃, 10 min - 4 ℃, ∞

GeneAmp PCR System 2400 (Perkin Elmer)

↓ 5 μl of 100 μl checked by AGE

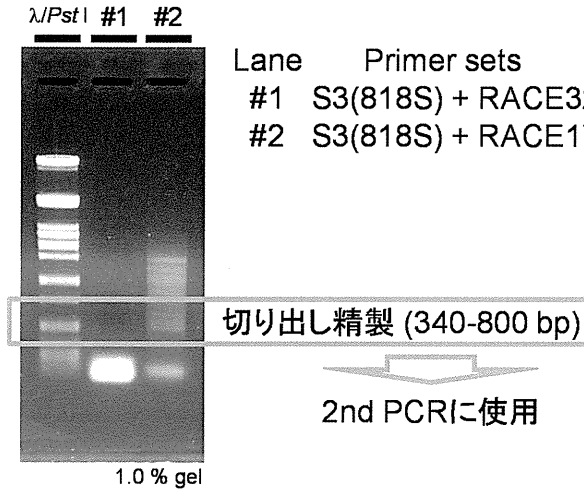


図9. オニゲシcontig #1603の3'RACE 1st PCR結果

[2nd PCR]

dH_2O	78.5	μl
10 x ExTaq Buffer (Takarabio)	10	μl
dNTP (@2.5 mM, Takarabio)	8	μl
S4 (100 μM)	1	μl
RACE17 (100 pmole/ μl)	1	μl
1st PCR product (340-800 bp)	1	μl
ExTaq (takarabio)	0.5	μl
Rxn. Vol.	100	μl

↓ 94 ℃, 5 min

↓ (94 ℃, 1 min - 48 ℃, 2 min - 72 ℃, 3 min) x 30

↓ 72 ℃, 10 min - 4 ℃, ∞

GeneAmp PCR System 2400 (Perkin Elmer)

↓ 5 μl of 100 μl checked by AGE

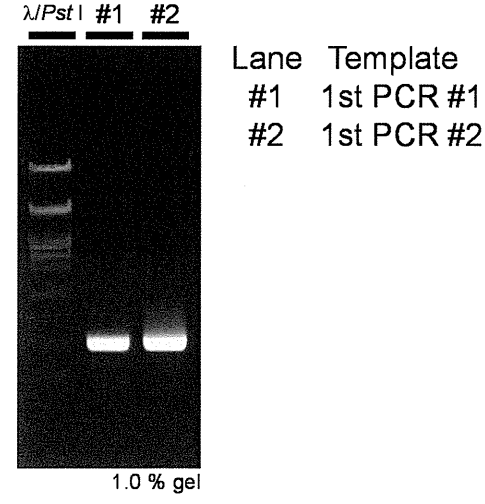


図10. オニゲシcontig #1603の3'RACE 2nd PCR結果

[1st PCR (Hot start)]

dH_2O	74	μl
10 x ExTaq Buffer (Takarabio)	10	μl
dNTP (@2.5 mM, Takarabio)	8	μl
RACE32 (100 μM)	1	μl
1603-5'RACE-A1 (100 μM)	1	μl
RACE RT ss-cDNA pool	1	μl
subtotal	95	μl
↓ 94 ℃, 5 min		
1 x ExTaq soln. (Takarabio)	5	μl
Rxn. Vol.	100	μl

↓ (94 ℃, 1 min - 42 ℃, 2 min - 72 ℃, 3 min) x 30

↓ 72 ℃, 10 min - 4 ℃, ∞

GeneAmp PCR System 2400 (Perkin Elmer)

↓ 5 μl of 100 μl checked by AGE

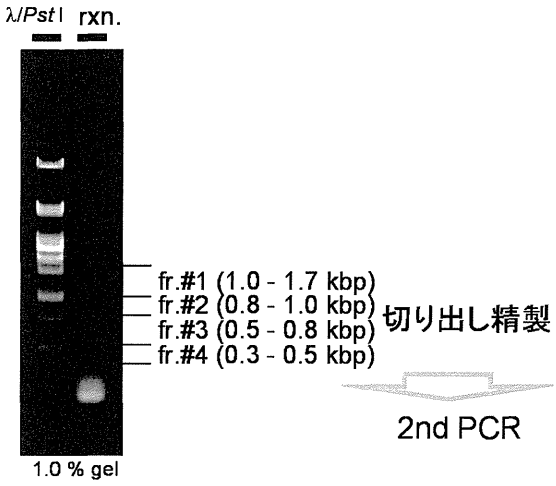


図11. オニゲシcontig #1603の5'RACE 1st PCR結果

[2nd PCR] (1)

dH_2O	78.5	μl
10 x ExTaq Buffer (Takarabio)	10	μl
dNTP (@2.5 mM, Takarabio)	8	μl
RACE17 (100 μM)	1	μl
1603-5'RACE-A2 (100 μM)	1	μl
1st PCR product (fraction)	1	μl
ExTaq (takarabio)	0.5	μl
Rxn. Vol.	100	μl

↓ 94 ℃, 5 min

↓ (94 ℃, 1 min - 48 ℃, 2 min - 72 ℃, 3 min) x 30

↓ 72 ℃, 10 min - 4 ℃, ∞

GeneAmp PCR System 2400 (Perkin Elmer)

↓ 5 μl of 100 μl checked by AGE

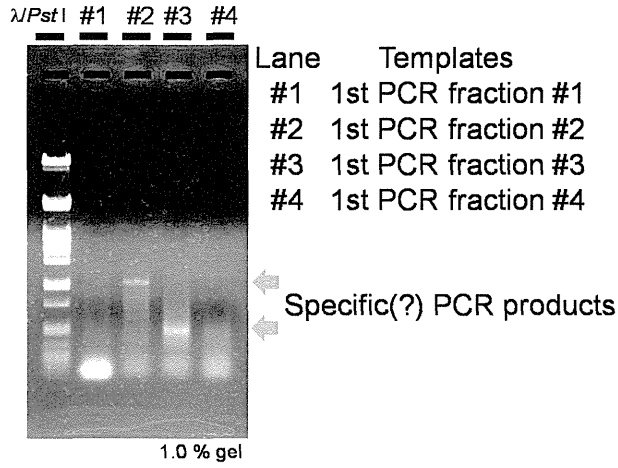


図12. オニゲシcontig #1603の5'RACE 2nd PCR結果(その1)

[2nd PCR] (2)

dH_2O	78.5	μl
10 x ExTaq Buffer (Takarabio)	10	μl
dNTP (@2.5 mM, Takarabio)	8	μl
RACE17 (100 μM)	1	μl
1603-5'RACE-A2 (100 μM)	1	μl
1st PCR product (fraction)	1	μl
ExTaq (takarabio)	0.5	μl
Rxn. Vol.	100	μl

↓ 94 ℃, 5 min

↓ (94 ℃, 30 sec - 58 ℃, 30 sec - 72 ℃, 1 min) x 30

↓ 72 ℃, 10 min - 4 ℃, ∞

iCylcer (BioRad)

↓ 5 μl of 100 μl checked by AGE

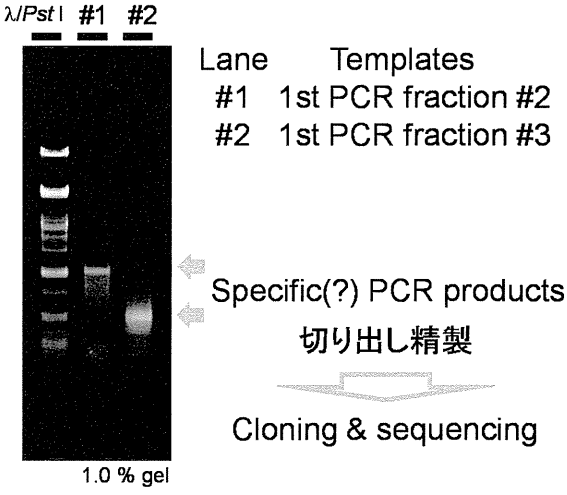


図13. オニゲシcontig #1603の5'RACE 2nd PCR結果(その2)

[full-length PCR]

DEPC water	35	μl
10 x KOD-plus Buffer (Toyobo)	5	μl
dNTP	5	μl
$MgSO_4$	2	μl
primer N-end (100 μM)	0.5	μl
primer C-end (100 μM)	0.5	μl
ss-cDNA (6/2RT)	1	μl
KOD-plus (Toyobo)	1	μl
Rxn. Vol.	50	μl

↓ 94 ℃, 2 min

↓ (94 ℃, 15 sec - 58 ℃, 30 sec - 68 ℃, 90 sec) x 35

↓ 4 ℃, ∞

iCylcer (BioRad)

↓ 3 μl of 100 μl checked by AGE

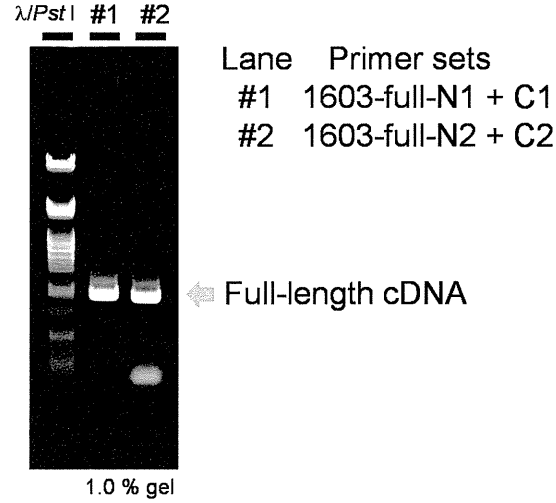


図14. オニゲシcontig #1603の全長cDNA PCR結果

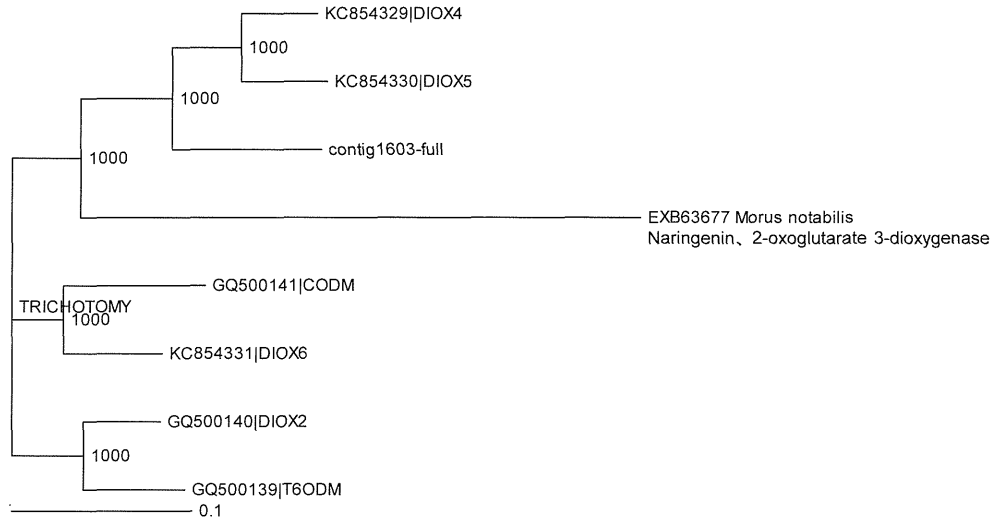


図15. オニゲシ**contig #1603**の全長**cDNA**配列を用いた分子系統樹

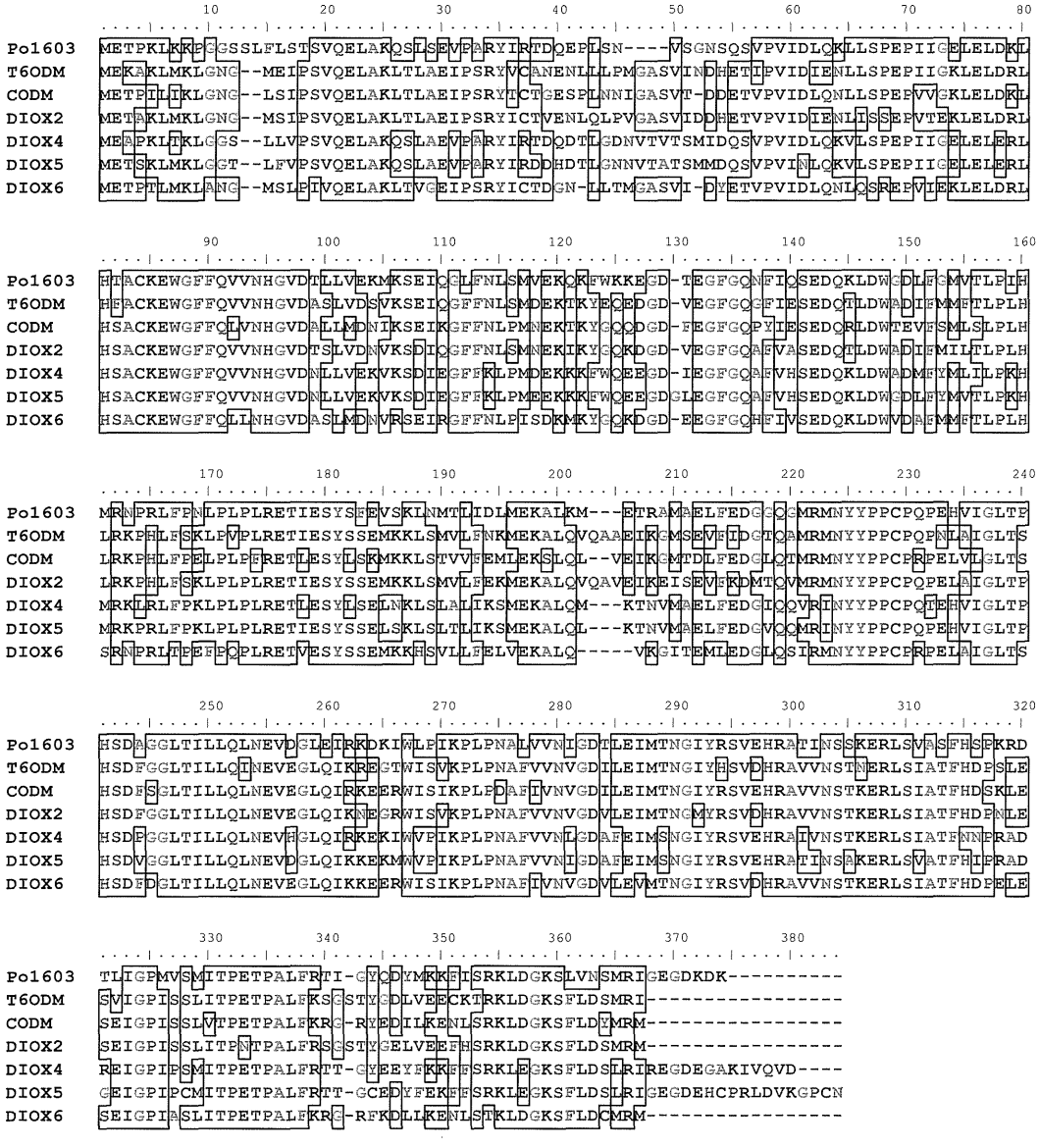


図16. オニゲシ**contig #1603**の推定アミノ酸配列を用いたケシ由来**ODDs**とのアラインメント