

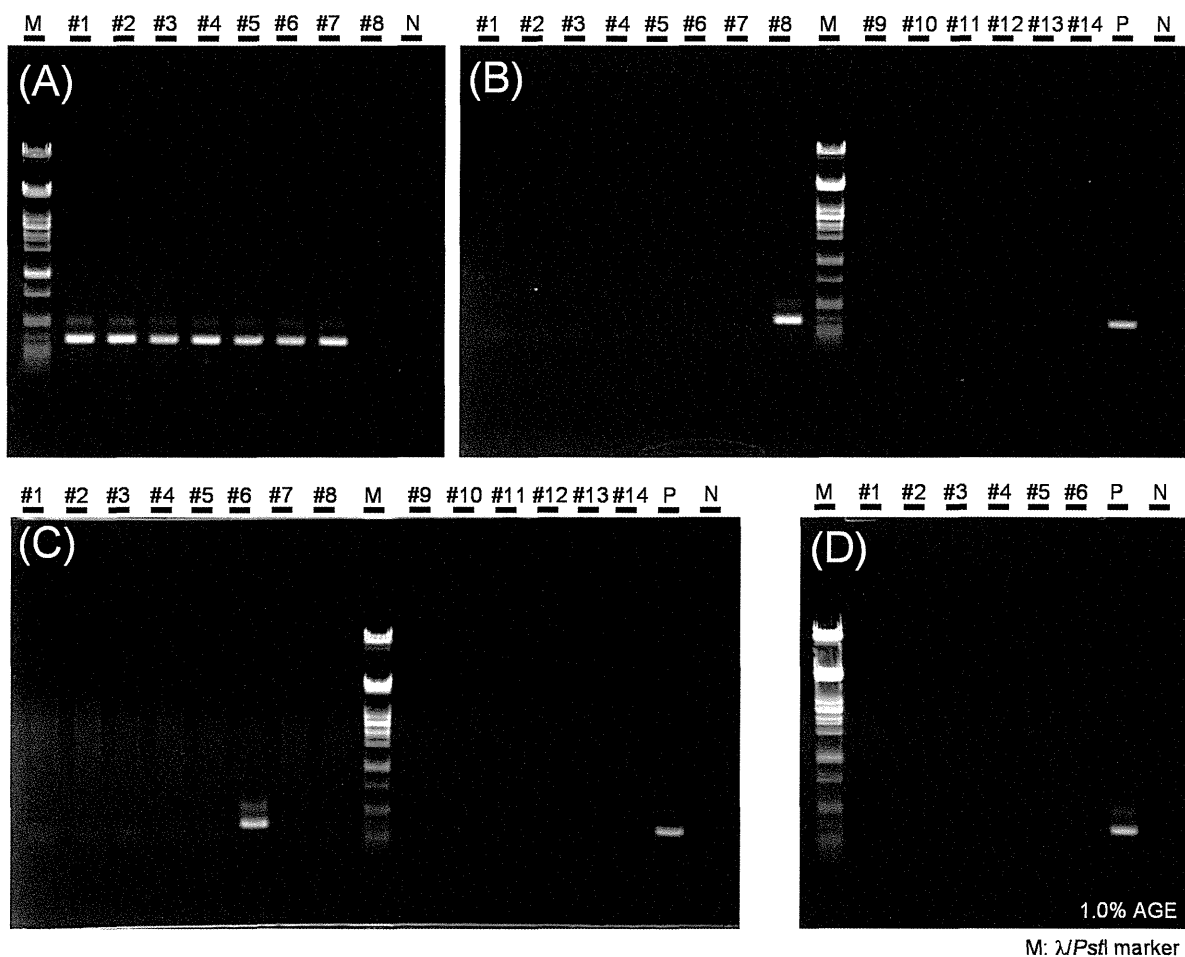
[RT reaction]		
Po(13-95) total RNA (tube #2)	8 μ l	390 ng total RNA (DNase treated)
RACE32 primer (10 μ M)	1 μ l	
dNTP (@2.5 mM)	4 μ l	TaKaRaBio
subtotal	13 μ l	
↓ 65°C, 5 min then on ice		
5 x first strand buffer	4 μ l	Invitrogen
DTT (0.1M)	1 μ l	Invitrogen
RNaseOUT	1 μ l	Invitrogen
Superscript III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

図3. オニゲシー本鎖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 μ l	390 ng total RNA (DNase treated)
1603-5'RACE-RT primer (2.5 μ M)	1 μ l	
dNTP (@2.5 mM)	4 μ l	TaKaRaBio
subtotal	13 μ l	
↓ 65°C, 5 min then on ice		
5 x first strand buffer	4 μ l	
DTT (0.1M)	1 μ l	
RNaseOUT	1 μ l	
Superscript III (200 U/ μ l)	1 μ l	
subtotal	20 μ l	
↓ 50°C, 1 hr		
↓ 70°C, 15 min then on ice		
↓ PCI, CIA treatment		
↓ NH ₄ OAc ppt., air dry ppt.		
1603-5'RACE RT-ss-cDNA pool	ppt.	

[5'-RACE poly(A) tailing]	
DEPC water (dissolve ppt.)	33 μ l
5xTdT buffer	10 μ l
0.1% BSA	5 μ l
dATP (10 μ M)	1 μ l
TdT	1 μ l
Rxn. Vol.	50 μ l
↓ 37 °C, 30 min	
↓ 65 °C, 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)



M: λ /PstI marker

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 ₂ O	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 °C, 30 sec - 58 °C, 30 sec - 72 °C, 1 min) x 30
- ↓ 72 °C, 10 min - 4 °C, ∞
- GeneAmp PCR System 2400 (Perkin Elmer)
- ↓ 5 μl of 100 μl checked by AGE

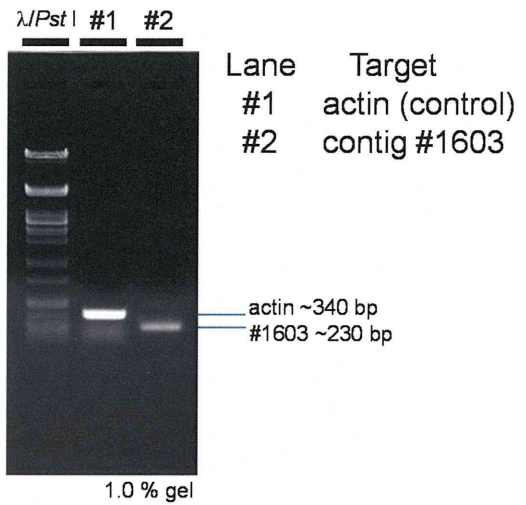


図6. オニゲシactin遺伝子, contig #1603(part)のcDNA増幅結果
両遺伝子の葉における発現を確認した

[#1603 CORE PCR (Hot start)]

dH ₂ O	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 °C, 5 min
- 1 x ExTaq soln. (Takarabio) 5 μl
- Rxn. Vol.** 100 μl
- ↓ (94 °C, 30 sec - 58 °C, 30 sec - 72 °C, 1 min) x 30
- ↓ 72 °C, 10 min - 4 °C, ∞
- GeneAmp PCR System 2400 (Perkin Elmer)
- ↓ 5 μl of 100 μl checked by AGE

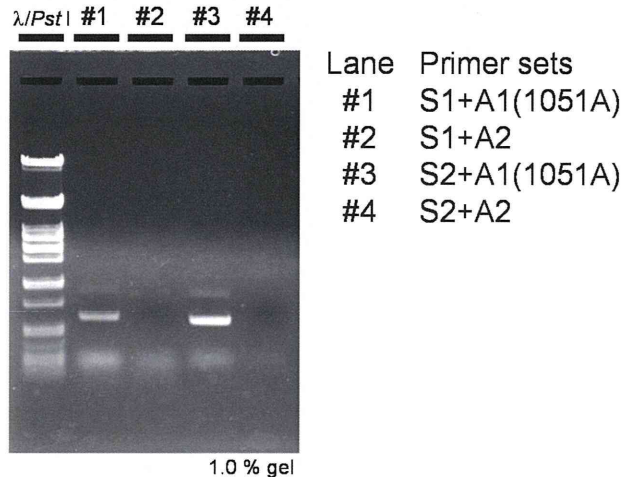


図7. オニゲシcontig #1603 CORE領域のPCR増幅結果
アンチセンス側A1プライマーで増幅産物が得られた

			S1		S2		
Papaver25_co	1	GGTTTGTTC	ATCTTTCAAT	GGTTGAGAAG	CAGAAATTT	GGAGAGAAAGA	50
1-5F.SEQ	1	-----	ATCTTTCAAT	GGTTGAGAAG	CAGAAATTT	GGAGAGAAAGA	50
1-4F.SEQ	1	-----	ATCTTTCAAT	GGTTGAGAAG	CAGAAATTT	GGAGAGAAAGA	50
Papaver25_co	51	AGGAGATACG	SAAGGATTTG	SACAAAATTT	TATTCACTCG	GAAGATCAAA	100
1-5F.SEQ	51	AGGAGATACG	SAAGGATTTG	SACAAAATTT	TATTCACTCG	GAAGATCAAA	100
1-4F.SEQ	51	AGGAGATACG	SAAGGATTTG	SACAAAATTT	TATTCACTCG	GAAGATCAAA	100
Papaver25_co	101	ARCTTGATTG	GGGAGATTTG	FTTGGGATGG	TCACCTCTCC	CATACATATG	150
1-5F.SEQ	101	ARCTTGATTG	GGGAGATTTG	FTTGGGATGG	TCACCTCTCC	CATACATATG	150
1-4F.SEQ	101	ARCTTGATTG	GGGAGATTTG	FTTGGGATGG	TCACCTCTCC	CATACATATG	150
Papaver25_co	151	AGGAATCCCTA	SGCTATTTCG	CAACCTGCCT	CTACCTCTCA	GGGAACAAT	200
1-5F.SEQ	151	AGGAATCCCTA	SGCTATTTCG	CAACCTGCCT	CTACCTCTCA	GGGAACAAT	200
1-4F.SEQ	151	AGGAATCCCTA	SGCTATTTCG	CAACCTGCCT	CTACCTCTCA	GGGAACAAT	200
Papaver25_co	201	TGAATCATACT	TCATTGAGG	TGAGTAAACT	AAACATGACT	CTTATTGACT	250
1-5F.SEQ	201	TGAATCATACT	TCATTGAGG	TGAGTAAACT	AAACATGACT	CTTATTGACT	250
1-4F.SEQ	201	TGAATCATACT	TCATTGAGG	TGAGTAAACT	AAACATGACT	CTTATTGACT	250
Papaver25_co	251	TGATGGAAAA	GGCTCTAAAA	ATGGAGACTA	GGGCTATGGC	AGAGTTGTTT	300
1-5F.SEQ	251	TGATGGAAAA	GGCTCTAAAA	ATGGAGACTA	GGGCTATGGC	AGAGTTGTTT	300
1-4F.SEQ	251	TGATGGAAAA	GGCTCTAAAA	ATGGAGACTA	GGGCTATGGC	AGAGTTGTTT	300
Papaver25_co	301	GAGGACGGAG	GACAAGGAAT	GAGGATGAAT	TATTATCCTC	CTTGTCCCTCA	350
1-5F.SEQ	301	GAGGACGGAG	GACAAGGAAT	GAGGATGAAT	TATTATCCTC	CTTGTCCCTCA	350
1-4F.SEQ	301	GAGGACGGAG	GACAAGGAAT	GAGGATGAAT	TATTATCCTC	CTTGTCCCTCA	350
Papaver25_co	351	ACCCGAGCAC	GTCAATGGCT	TAACACCTCA	TTCTGATGCT	GGCGTTTGA	400
1-5F.SEQ	351	ACCCGAGCAC	GTCAATGGCT	TAACACCTCA	TTCTGATGCT	GGCGTTTGA	400
1-4F.SEQ	351	ACCCGAGCAC	GTCAATGGCT	TAACACCTCA	TTCTGATGCT	GGCGTTTGA	400
Papaver25_co	401	CCATCCTCCT	TCAACTCAAC	SAAGTGGATG	GATTAGAGAT	TCGAAAAGAC	450
1-5F.SEQ	401	CCATCCTCCT	TCAACTCAAC	SAAGTGGATG	GATTAGAGAT	TCGAAAAGAC	450
1-4F.SEQ	401	CCATCCTCCT	TCAACTCAAC	SAAGTGGATG	GATTAGAGAT	TCGAAAAGAC	450
Papaver25_co	451	AAGATATGGC	TTCOCATTAA	ACCTCTGCCT	AATGCCCTTG	TAGTGAACAT	500
1-5F.SEQ	451	AAGATATGGC	TTCOCATTAA	ACCTCTGCCT	AATGCCCTTG	TAGTGAACAT	500
1-4F.SEQ	451	AAGATATGGC	TTCOCATTAA	ACCTCTGCCT	AATGCCCTTG	TAGTGAACAT	500
Papaver25_co	501	FGGAGATACT	FTGAGATAA	TGACTAATGG	GATTTACCGT	AGCGTGGAAC	550
1-5F.SEQ	501	FGGAGATACT	FTGAGATAA	TGACTAATGG	GATTTACCGT	AGCGTGGAAC	550
1-4F.SEQ	501	FGGAGATACT	FTGAGATAA	TGACTAATGG	GATTTACCGT	AGCGTGGAAC	550
Papaver25_co	551	ATCGTGCAAC	AATAAACTCA	TCAAAGGAGA	GGCTCTCAGT	TGCAGCATT	600
1-5F.SEQ	551	ATCGTGCAAC	AATAAACTCA	TCAAAGGAGA	GGCTCTCAGT	TGCAGCATT	600
1-4F.SEQ	551	ATCGTGCAAC	AATAAACTCA	TCAAAGGAGA	GGCTCTCAGT	TGCAGCATT	600
Papaver25_co	601	CATAGCCCTA	AAGGAGATAC	ATTAATAGGT	CCAATGGTCA	SCATGATCAC	650
1-5F.SEQ	601	CATAGCCCTA	AAGGAGATAC	ATTAATAGGT	CCAATGGTCA	SCATGATCAC	650
1-4F.SEQ	601	CATAGCCCTA	AAGGAGATAC	ATTAATAGGT	CCAATGGTCA	SCATGATCAC	650
Papaver25_co	651	ACCAGAGACA	CCTGCATTGT	TTAGGACAAT	TGGGTAGGAG	GAGTATATGA	700
1-5F.SEQ	651	ACCAGAGACA	CCTGCATTGT	TTAGGACAAT	TGGGTAGGAG	GAGTATATGA	700
1-4F.SEQ	651	ACCAGAGACA	CCTGCATTGT	TTAGGACAAT	TGGGTAGGAG	GAGTATATGA	700
Papaver25_co	701	AGAAATCTTT	TTCICGTRAA	CTCGACGGAA	A.....		750
1-5F.SEQ	701	-----	-----	-----	-----		750
1-4F.SEQ	701	-----	-----	-----	-----		750

上段: contig #1603 (731 bp)
 中段: メジークローン(e.g. 1-5)
 (出現頻度#1:8/9, #3:5/6)
 下段: マイナークローン(e.g. 1-4)
 (出現頻度#1:1/9, #3:1/6)

Sense-primer
 Antisense-primer

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 ₂ O	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 °C, 5 min

1 x ExTaq soln. (Takarabio)	5 μl
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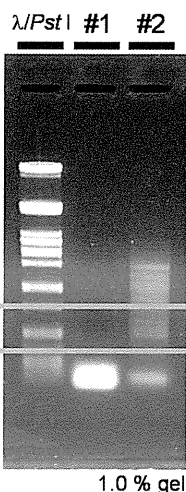
Rxn. Vol.	100 μl
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↓ (94 °C, 1 min - 42 °C, 2 min - 72 °C, 3 min) x 30

↓ 72 °C, 10 min - 4 °C, ∞

GeneAmp PCR System 2400 (Perkin Elmer)

↓ 5 μl of 100 μl checked by AGE



Lane Primer sets
 #1 S3(818S) + RACE32
 #2 S3(818S) + RACE17

切り出し精製 (340-800 bp)

2nd PCRに使用

図9. オニゲシcontig #1603の3'RACE 1st PCR結果

[2nd PCR]

dH ₂ O	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

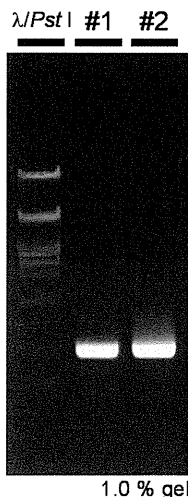
↓ 94 °C, 5 min

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

↓ 72 °C, 10 min - 4 °C, ∞

GeneAmp PCR System 2400 (Perkin Elmer)

↓ 5 μl of 100 μl checked by AGE



Lane Template
 #1 1st PCR #1
 #2 1st PCR #2

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

[1st PCR (Hot start)]

dH ₂ O	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 °C, 5 min

1 x ExTaq soln. (Takarabio)	5 μl
Rxn. Vol.	100 μl

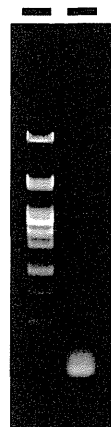
↓ (94 °C, 1 min - 42 °C, 2 min - 72 °C, 3 min) x 30

↓ 72 °C, 10 min - 4 °C, ∞

GeneAmp PCR System 2400 (Perkin Elmer)

↓ 5 μl of 100 μl checked by AGE

λ/PstI rxn.



1.0 % gel

fr.#1 (1.0 - 1.7 kbp)
fr.#2 (0.8 - 1.0 kbp)
fr.#3 (0.5 - 0.8 kbp)
fr.#4 (0.3 - 0.5 kbp)

切り出し精製

2nd PCR

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

[2nd PCR] (1)

dH ₂ O	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 °C, 5 min

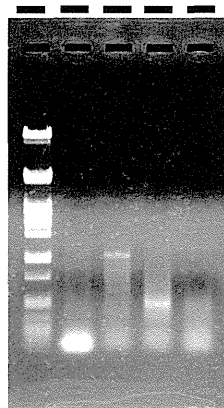
↓ (94 °C, 1 min - 48 °C, 2 min - 72 °C, 3 min) x 30

↓ 72 °C, 10 min - 4 °C, ∞

GeneAmp PCR System 2400 (Perkin Elmer)

↓ 5 μl of 100 μl checked by AGE

λ/PstI #1 #2 #3 #4



1.0 % gel

Lane Templates
#1 1st PCR fraction #1
#2 1st PCR fraction #2
#3 1st PCR fraction #3
#4 1st PCR fraction #4

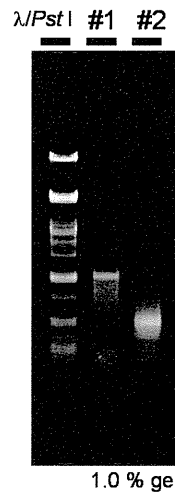
Specific(?) PCR products

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

[2nd PCR] (2)

dH ₂ O	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 °C, 5 min
- ↓ (94 °C, 30 sec - 58 °C, 30 sec - 72 °C, 1 min) x 30
- ↓ 72 °C, 10 min - 4 °C, ∞
- iCycler (BioRad)
- ↓ 5 μl of 100 μl checked by AGE



Lane Templates
 #1 1st PCR fraction #2
 #2 1st PCR fraction #3

Specific(?) PCR products

切り出し精製

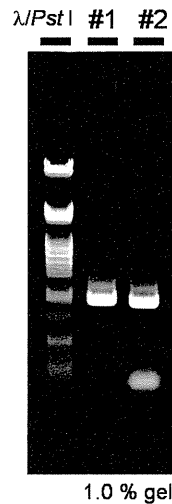
Cloning & sequencing

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 °C, 2 min
- ↓ (94 °C, 15 sec - 58 °C, 30 sec - 68 °C, 90 sec) x 35
- ↓ 4 °C, ∞
- iCycler (BioRad)
- ↓ 3 μl of 100 μl checked by AGE



Lane Primer sets
 #1 1603-full-N1 + C1
 #2 1603-full-N2 + C2

Full-length cDNA

図14. オニゲシcontig #1603の全長cDNA PCR結果

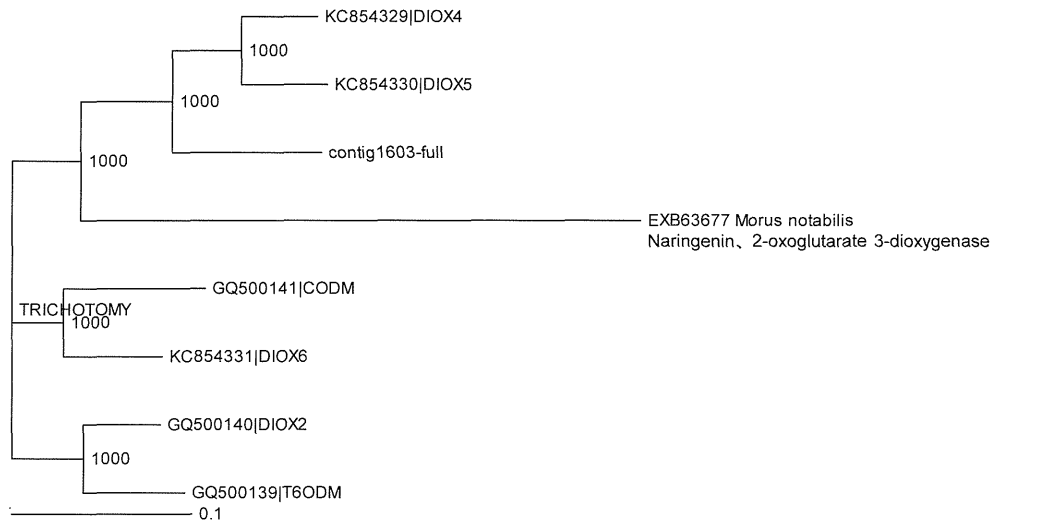


図15. オニゲシcontig #1603の全長cDNA配列を用いた分子系統樹

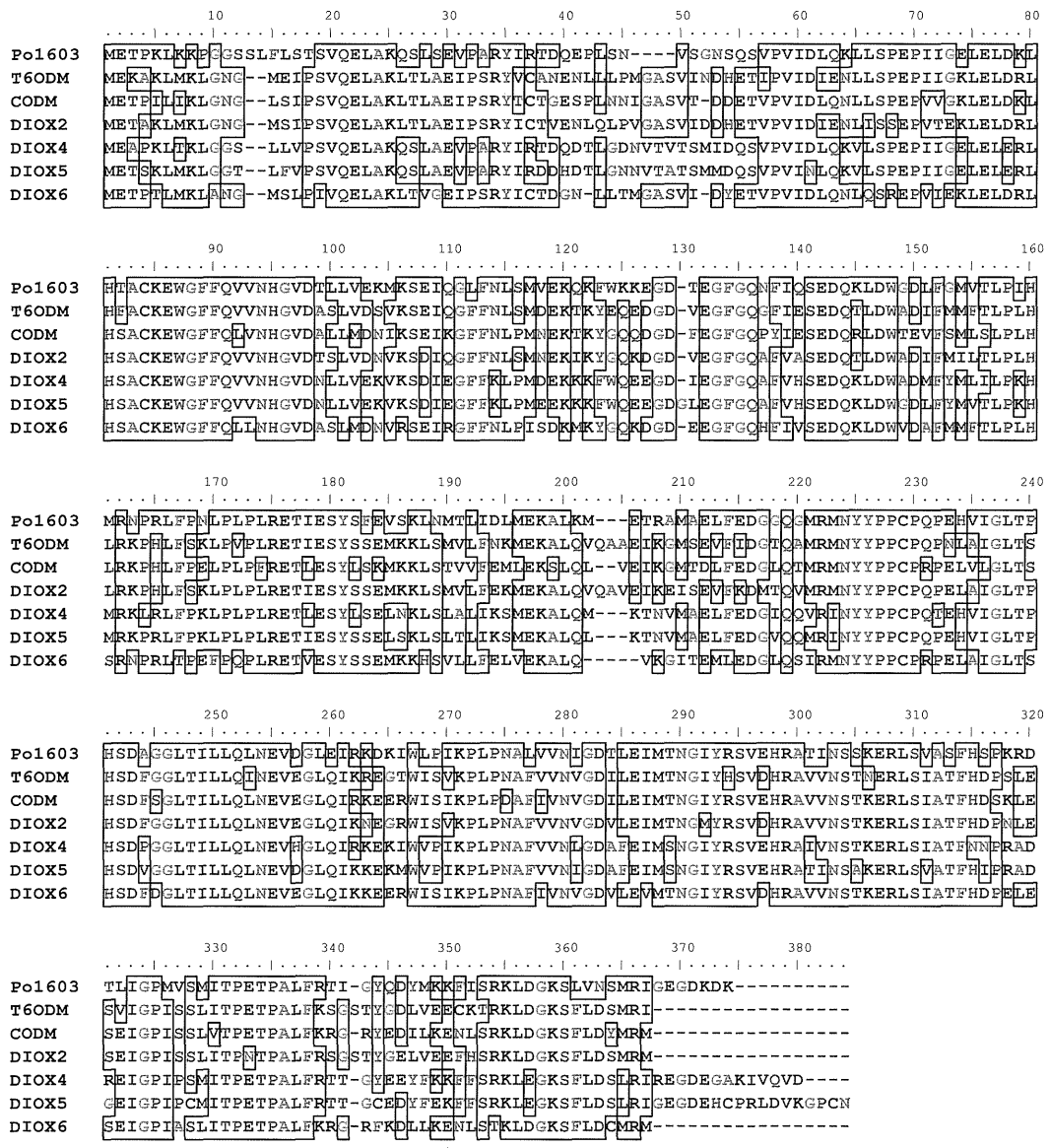


図16. オニゲシcontig #1603の推定アミノ酸配列を用いたケン由来ODDsとのアラインメント

