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Comparative aspects of a luciferase molecule from the Japanese luminous beetle, *Rhagophthalmus ohbai*

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イリオモテボタル（甲虫目：イリオモテボタル科）発光酵素による系統比較

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発光性甲虫はホタル科，ホタルモドキ科，ヒカリコメツキ科とイリオモテボタル科の４科よりなり，日本国内にはホタル科とイリオモテボタル科の発光甲虫が生息する。そのうち，イリオモテボタル科は西表島と石垣島に生息するイリオモテボタルのみである。既に十数種類の発光甲虫において，その発光酵素（ルシフェラーゼ）の構造や生化学的特性が明らかにされていることから，イリオモテボタルに含まれるルシフェラーゼのクローニング及び特性解析を行った。クローニングの結果，イリオモテボタルルシフェラーゼは543個のアミノ酸残基からなる分子量60132の単純タンパクであった。本酵素のアミノ酸配列を既知の発光性甲虫ルシフェラーゼ群と比較した結果，一次構造レベルの相同性は49～54%であり，とりわけホタルモドキ科のものと高い相同性を示した。さらに，マルチアライメント解析を行い，発光甲虫ルシフェラーゼの分子系統樹を作成したところ，発光甲虫始原型より，最初にヒカリコメツキ科が，次にホタルモドキ科・イリオモテボタル科，そしてホタル科が分岐し進化してきたことが示唆される。一方、イリオモテボタルルシフェラーゼの生化学的特性解析によりイリオモテボタル科がホタルモドキ科やヒカリコメツキ科とより近縁であることが示される。以上の結果より，４科の発光性甲虫ルシフェラーゼは大きく２つに分類でき，さらにイリオモテボタルがホタル科よりホタルモドキ科，そしてヒカリコメツキ科に近縁であることを明らかにした。

Introduction

Bioluminescent beetles produce light by a common mechanism in which the substrate luciferin (LH_2) is converted to the luciferyl adenylate in the presence of ATP, Mg^{2+}, and luciferase (E), and the luciferyl adenylate is then oxidized by molecular oxygen to yield light, oxyluciferin, CO_2 and AMP(McELROY et al., 1974; 1978).

$$E+LH_2+Mg\cdot ATP \rightarrow E\cdot LH_2-AMP+PPi$$
$$E\cdot LH_2-AMP+O_2 \rightarrow Light+Oxyluciferin+CO_2+AMP$$

The beetle luciferin is a substrate common to all luminous beetles, including the Lampyridae(true fireflies),Rhagophthalmidae, Phengodidae (railroad-worms) and Elateridae (click beetles). Luciferases are ca. 62 kDa enzymes (oxygenase), which catalyze the reaction leading to light emission ranging from green to red in luminous beetles. The color

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differences seen in bioluminescent beetles are due to the structural differences in luciferases (BIGGLEY *et al.*, 1967; McELROY *et al.*, 1969; KAJIYAMA and NAKANO, 1991; OHMIYA *et al.*, 1996). Many genes for beetle luciferases were cloned and analyzed. The clones of luminous beetles were derived from the fireflies *Luciola cruciata* (Lcr) (MASUDA *et al.*, 1989), *Luciola lateralis* (Lla) (TATSUMI *et al.*, 1992) *Pyrocoelia miyako* (Pmi) (OHMIYA *et al.*, 1995), and *Hotaria paravula* (Hpa) (OHMIYA *et al.*, 1995) from Japan, *Photinus pyralis* (Ppy) (DE WET *et al.*, 1987) and *Photuris pennsylvanica* (Ppe) (LI *et al.*, 1997) from the North America, *Luciola mingrelica* (Lmi) (DEVINE *et al.*, 1993), and *Lampyris noctiluca* (Lno) from Europe (SALA-NEWBY *et al.*, 1996), the click beetles *Pyrophorus plagiophthalamus* (Ppl) (WOOD *et al.*, 1989) and *Pyrearinus termitilluminans* (Pte) (VIVIANI *et al.*, 1999a), and the railroad-worms *Phrixothrix vivianii* (Pvi) and *P. hirtus* (Phi) (VIVIANI *et al.*, 1999b) from the South America. These luciferases showed sequence homologies ranging from ca. 46 to ca. 98 % each other. However, these sequences did not clarify the origin of color differences. On the other hand, the light-emitting colors of firefly luciferases were also changed from yellow to red by pH changes (BIGGLEY *et al.*, 1967; McELROY *et al.*, 1969). In general, the pH effect on color differences was explained by the different ionic structure of the excited oxyluciferin; the first excited monoanion state deprotonates to form a dianion which emits yellow light (DELUCA, 1969; WHITE *et al.*, 1971). Interestingly, the bioluminescent spectra of the Elateridae luciferases did not show the pH-dependent red shift although it is not clear for the reason of this lack of pH effect. Two major families of bioluminescent beetles are found in Japan; Lampyridae and Rhagophthalmidae. Only one genus, *Rhagophthalmus ohbai*(Roh), of the family Rhagophthalmidae was discovered in 1983 in Iriomote Island, which is a small island in the sea of East-China(WITTMER und OHBA, 1994). The locations of the family Phengodidae are limited to America, namely Neotropical region, and Southeast Asia (VIVIANI and BECHARA, 1997). The genus Roh could be a key species for the understanding of the evolution of the Rhagophthalmidae. Furthermore, there has been very little information for the characteristic aspects of the Rhagophthalmidae luciferases. This manuscript reports the cloning and characterization of a new luciferase from Roh.

Materials and Methods

Materials

The following reagents were obtained from commercial sources: Firefly d-luciferin-Na, Isopropyl-β-d-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indoyl-b-galactopyranoside (X-Gal), dithiothreitol (DTT), ampicillin (Wako Pure Chemicals, Osaka, Japan); coenzyme-A and adenosine triphosphate (ATP) (Oriental Yeast Co, Osaka, Japan); Isogen reagent, restriction enzymes and Taq polymerase (Nippon Gene, Toyama, Japan); Oligotex-dT30 and DNA ligation kit (Takara Shuzo, Kyoto, Japan); λ ZAP II vector, Gigapack III gold packaging kit, and *Escherichia coli*(*E.coli*) strains XL1-blue and SOLR (Stratagene, La Jolla, CA); cDNA synthesis kit (Amersham Pharmacia Biotech); ABI PRISM Dye terminator Cycle Sequencing kit (Perkin Elmer, Foster, CA).

Construction of cDNA library

The total RNA from four bodies of adult larviform females Roh, which were collected at Iriomote Island, was prepared by the guanidine isothiocyanate method (CHOMCZYNSKY und SACCHI, 1987). The yield of total RNA was 155 μg calculated from the absorbance at 260 nm. Poly(A)+RNA was

isolated using oligodT-labeled latex(Oligotex-dT30) and was employed to synthesize cDNA using a Pharmacia cDNA synthesis kit. Ligation of cDNA with λZAP II vector was carried out overnight at 14℃ and was packaged using Gigapack III Gold packaging kit. The packaged DNA was used to infect E.coli. XL1-blue, yielding 6.2×105 plaque-forming units (pfu). The ligation mixture was analyzed by polymerase chain reaction (PCR) using T3 and T7 primers and a model PC-700 ASTEC (Fukuoka, Japan) temperature controller run for 35 cycles (denaturation, 94℃ ×1 min; annealing, 55℃×2 min; elongation, 72℃×3 min), which showed that the insert sizes ranged from 200 to 10,000 bp. The cDNA library was converted to an expression library after the excision of the pBluescript phagemids according to the manufacturer instructions. The plasmid library was propagated in *E.coli* SOLR cells.

Screening and sequence determination of luciferase cDNA clone

Screening of the plasmid library was performed by photodetection (WOOD *et al.*, 1987) using a cooled-CCD camera system (ATTO, Japan), after spraying 1 mM d-luciferin (0.1 M citrate buffer pH 5.0) onto 1 mM IPTG induced colonies at 20℃ during 12 h. After screening of 2.5×10^5 clones, the positive colony was removed, screened and isolated by the same procedure. The resultant plasmid, pB-RmL, was digested by *Bam*HI and *Eco*RV, yielding three fragments, which were subcloned into the pUC18 vector. The nucleotide sequence of the purified plasmid DNA was determined using a Dye terminator sequencing kit(Applied Biosystems, USA).

Measurement of bioluminescence emission spectra

The transformed *E.coli* XL1-Blue cells with pB-RmL were grown overnight at 25℃ in 20 ml of LB medium, containing 1 mM IPTG and 50 μg/ml ampicillin. After centrifuging the culture medium at 4000 x g for 10 min at 4℃, the bacterial pellet was resuspended in lysis buffer (100 mM sodium phosphate, pH 8.0; 2 mM EDTA; 1 mg/mL of lysozyme), incubated on ice for 15 min and then placed in a freezer at -80℃. The frozen pellet was thawed at 25℃ and centrifuged at 15,000 rpm for 15 min at 4℃, and the supernatant was used for assays. To 50 μL of the luciferase solution in a quartz cell, 450 μL of substrate mixture consisting of 1 mM luciferin/100 mM phosphate buffer (pH 6.0 - 8.0), containing 2 mM ATP and 5 mM $MgSO_4$ was injected. The bioluminescence emission spectrum was measured with a Hitachi F-4010 Fluorescence Photometer (Tokyo, Japan) with the excitation lamp turned off after 3 min the mixing of reagent.

Sequence analysis of Roh luciferase

Multiple alignment of the amino acid sequences of Roh luciferase (RoL) and the other Japanese firefly luciferases was carried using a GENETYX-MAC ver 7.3 (Software Development Co., LTD., Tokyo). A phylogenetic tree was calculated based on the UPGMA method employing genetic distance.

Results and Discussion

Roh is the only one species of the Rhagophthalmidae in Japan. Roh larval and female's behavior and luminosity resembles those of South-American phengodidae.

Adult's females are larviform whereas adult males show characteristic beetle morphology with compound eyes and developed antennal sensillae. Adult male began to fly when females emitted continuous light from the 8th abdominal segment lanterns. During oviposition females emit weak continuous light and encircle their eggs like in the case of South-American Phengodidae species. A description of the lantern location in females would be useful.

The total RNA was prepared from 4 whole bodies (adult female) and the yield was 155 μg. The mRNA (1.2 μg) was purified by oligo dT-labeled latex beads and converted to the cDNA. The ligation of cDNA with λ ZAP II vector was packaged using a Gigapack III Gold packaging kit and the yield was 6.2×10^5 pfu. The packaged cDNAs were excised to give the pBluescript phagemid library using the EXASSIST helper phage. Luciferin solution was sprinkled over the colonies on LB plate and the photons on luciferin-luciferase reaction were detected using a cooled-CCD camera system. One positive clone was obtained from 2.5×10^5 clones of the cDNA library. The positive clone (pB-RmL) was isolated repeatedly by the same procedure. Fig.1 shows the cDNA sequence of RoL and its amino acid sequence, deduced from a sequence of 1968 nucleotides. An open reading frame of 1629 bp encoded a polypeptide of 543 amino acid residues. The calculated molecular mass was 60,132.

Multiple alignment of the amino acid sequences of RoL with those of Lcr, Lla, Hpa, and Pmi luciferases of Japanese Lampyridae yield sequence homologies of 52.8%, 50.6%, 49.2%, and 53.2%, respectively (Fig. 2). The Lcr luciferase probe within Japanese fireflies showed sequence homologies 93.6%, 81.0%, and 66.9% with Lla, Hpa, and Pmi luciferases, respectively. On the other hand, the RoL probe showed sequence homologies 66.4%, 56.4%, and 51.7% with Pvi, Phi, and Ppl luciferases of the Phengodidae and Elateridae (data not shown). The results suggest the primary structure of luciferases may reflect the evolutional aspect and/or the catalytic characteristics. Recently, the invariant residues Arg-218, His-245, Phe-247, Ala-348, and Lys-529 on Ppy luciferase appear to interact with luciferin in its binding site in according to the 3rd-structure model of the crystallized Ppy luciferase (CONTI *et al.*,

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                                                (EcoRI/NotI)TATCGAATTCAATTCGAAGTACCTATAATATAGAATCCAAA      -1

ATGCCTAATGAAATCATTTTACATGGGGCCAAACCTCGAGACCCGTTAGACCTGGGAACTGCAGGAATTCAATTGTATAGGGCTTTGACGAATTTTTCCTTTTTAAGGGAAGCCTTGATC   120
M  P  N  E  I  I  L  H  G  A  K  P  R  D  P  L  D  L  G  T  A  G  I  Q  L  Y  R  A  L  T  N  F  S  F  L  R  E  A  L  I    40

GACGCTCACACCGAGGAAGTAGTATCTTACGCGGACATTTTGGAAAACAGCTGTCGATTAGCAAAATGCTACGAAAACTATGGATTACGCCAAAACAGCGTCATATCGGTGTGCAGCGAA   240
D  A  H  T  E  E  V  V  S  Y  A  D  I  L  E  N  S  C  R  L  A  K  C  Y  E  N  Y  G  L  R  Q  N  S  V  I  S  V  C  S  E    80

AACAGCACGATCTTCTTCTACCCCGTAATTGCCGCTTTGTATATGGGAGTCATAACAGCAACCGTAAATGATAGTTATACCGAACGGGAATTATTGGAAACCTTAAATATATCAAAACCG   360
N  S  T  I  F  F  Y  P  V  I  A  A  L  Y  M  G  V  I  T  A  T  V  N  D  S  Y  T  E  R  E  L  L  E  T  L  N  I  S  K  P   120

GAATTAGTGTTCTGCTCGAAGAAAGCCATTAAAAATATGATGGCATTGAAAAGGAACGTCAATTTTATTAAAAAGGTAGTACTTTTGGATAGTAAGGAAGACATGGGCGAAGCCCAGTGT   480
E  L  V  F  C  S  K  K  A  I  K  N  M  M  A  L  K  R  N  V  N  F  I  K  K  V  V  L  L  D  S  K  E  D  M  G  E  A  Q  C   160

CTTAGCAACTTTATGGCACGCTATTCGGAACCCAATTTGGACGTAAGAAATTTTAAACCACGCGATTTTGATGCTAAAGAACAAGTCGCTTTGATCATGTCCTCATCGGGAACAACCGGG   600
L  S  N  F  M  A  R  Y  S  E  P  N  L  D  V  R  N  F  K  P  R  D  F  D  A  K  E  Q  V  A  L  I  M  S  S  S  G  T  T  G   200

CTGCCCAAAGGGGTCGTGTTAACCCATCGAAATTTAAGCGTTCGCTTCGTACACTGCAAGGATCCCTTATTCGGCACAAGAACTATTCCATCAACTTCGATTTATCTATCGTTCCCTTC   720
L  P  K  G  V  V  L  T  H  R  N  L  S  V  R  F  V  H  C  K  D  P  L  F  G  T  R  T  I  P  S  T  S  I  L  S  I  V  P  F   240

CATCATGCGTTTGGAATGTTTACAACGTTGTCTTATTTTATAGTAGGGCTTAGAGTTGTATTACTGAAAAGATTCGAAGAGAAGTTTTTCTTAAGCACCATTGAAAAGTACAGAATTCCA   840
H  H  A  F  G  M  F  T  T  L  S  Y  F  I  V  G  L  R  V  V  L  L  K  R  F  E  E  K  F  F  L  S  T  I  E  K  Y  R  I  P   280

ACTATCGTTCTTGCGCCGCCCGTAATGGTATTCCTAGCTAAGAGCCCCTTAGTTGATCAGTACGATTTGTCCAGTATTAGAGAAGTCGCTACCGGTGGCGCACCTGTTGGAACTGAAGTG   960
T  I  V  L  A  P  P  V  M  V  F  L  A  K  S  P  L  V  D  Q  Y  D  L  S  S  I  R  E  V  A  T  G  G  A  P  V  G  T  E  V   320

GCAGTGGCCGTTGCGAAACGGTTGAAAATTGGCGGAATCCTTCAGGGCTACGGATTGACCGAAACGTGTTGCGCCGTATTAATTACCCCTCATGACGACGTTAAAACAGGTTCTACCGGG  1080
A  V  A  V  A  K  R  L  K  I  G  G  I  L  Q  G  Y  G  L  T  E  T  C  C  A  V  L  I  T  P  H  D  D  V  K  T  G  S  T  G   360

AGGGTAGCTCCTTACGTCCAAGCGAAAATTGTAGATCTTACCACCGGAAAATCTCTGGGGCCAAATAAAAGAGGAGAGCTTTGTTTTAAAAGTGAGATCATTATGAAGGGCTATTTCAAC  1200
R  V  A  P  Y  V  Q  A  K  I  V  D  L  T  T  G  K  S  L  G  P  N  K  R  G  E  L  C  F  K  S  E  I  I  M  K  G  Y  F  N   400

AATAAACAAGCTACGGAAGAAGCCATCGATAAAGAAGGATGGTTACATTCTGGAGATGTTGGGTATTATGACGACGATGGTCATTTCTTCGTAGTCGATCGTTTAAAGGAACTTATCAAG  1320
N  K  Q  A  T  E  E  A  I  D  K  E  G  W  L  H  S  G  D  V  G  Y  Y  D  D  D  G  H  F  F  V  V  D  R  L  K  E  L  I  K   440

TACAAGGGATATCAAGTAGCACCGGCTGAACTGGAGTGGTTGCTTTTGCAACATCCATCTATTAAAGATGCCGGTGTTACTGGCGTTCCCGACGAAGCTGCTGGAGAACTACCAGGTGCT  1440
Y  K  G  Y  Q  V  A  P  A  E  L  E  W  L  L  L  Q  H  P  S  I  K  D  A  G  V  T  G  V  P  D  E  A  A  G  E  L  P  G  A   480

TGTATAGTTCTCCAAGAAGGAAAAAGTCTTACTGAACAAGAAATTATTGACTATATAGCCGAACGAGTTTCGCCAACTAAACGTATACGTGGTGGAGTGGTCTTCGTTGATGATATTCCT  1560
C  I  V  L  Q  E  G  K  S  L  T  E  Q  E  I  I  D  Y  I  A  E  R  V  S  P  T  K  R  I  R  G  G  V  V  F  V  D  D  I  P   520

AAAGGGGCGACTGGAAAACTGGTCAGAAGTGAATTACGAAAACTTCTTGCTCAGAAGAAATCGAAACTATAAATTAATGCACTAATGGTCAGTTGCGCCGACAATTGCCGAAAATTTAAG  1680
K  G  A  T  G  K  L  V  R  S  E  L  R  K  L  L  A  Q  K  K  S  K  L  Stop                                              543

TCTCGCTTAAGTTATTGATTTTCCTTAGATACGATTGTGTTTTCATGGATATATTCTATACTTAAGCGAGGCTTACATTTTTAGGTTACGTCGAGTCGACGGCGGTGCAACGGCCATAAA
TTTTTTGATCACCTGAAATAATATTAAAAGGTAAAGAAGAGTCACATTTTTCTGCGGTATTTCACTATGTATTTAAAATATTGGACGTAGTTTTAAATATATCAATTAAAGCAAAAAAAA
AAAAAA(NotI/EcoRI)                                                                                                  1926
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Fig. 1 Nucleotide sequence of the cDNA clone pB-RmL and deduced amino scid sequence of the respective luciferase.

1996; BRANCHINI *et al.*, 1999). The corresponding residues on Roh were homologous each other, suggesting these residues may be important for catalytic reaction. However,

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Roh   1:---M-PNEIILHGAKPRDPLDLGTAGIQLYRALTNFSFLREAL-IDAHTEEV-VSYADIL 54
Lcr   1:MENMENDENIVVGPKPFYPIEEGSAGTQLRKYMERYAKL-GAIAFTNAVTGVDYSYAEYL 59
Lla   1:MENMENDENIVYGPEPFYPIEEGSAGAQLRKYMDRYAKL-GAIAFTNALTGVDYTYAEYL 59
Hpa   1:ME-MEKEENVVYGPLPFYPIEEGSAGIQLHKYMQQYAKL-GAIAFSNALTGVDISYQEYF 58
Pmi   1:MED-D-SKHIMHGHRHSILWEDGTAGEQLHKAMKRYAQVPGTIAFTDAHAEVNITYSEYF 58

Roh  55:ENSCRLAKCYENYGLRQNSVISVCSENSTIFFYPVIAALYMGVITATVNDSYTERELLET 114
Lcr  60:EKSCCLGKALQNYGLVVDGRIALCSENCEEFFIPVIAGLFIGVGVAPTNEIYTLRELVHS 119
Lla  60:EKSCCLGEALKNYGLVVDGRIALCSENCEEFFIPVLAGLFIGVGVAPTNEIYTLRELVHS 119
Hpa  59:DITCRLAEAMKNYGMKQEGTIALCSENCEEFFIPVLAGLYIGVAVAPTNEIYTLRELNHS 118
Pmi  59:EMSCRLAETMKRYGLGLQHHIAVCSETSLQFFMPVCGALFIGVGVAPTNDIYNERELYNS 118

Roh 115:LNISKPELVFCSKKAIKNMMALKRNVNFIKKVVLLDSKEDMGEAQCLSNFMARYSEPNLD 174
Lcr 120:LGISKPTIVFSSKKGLDKVITVQKTVTTIKTIVILDSKVDYRGYQCLDTFIKRNTPPGFQ 179
Lla 120:LGISKPTIVFSSKKGLDKVITVQKTVTAIKTIVILDSKVDYRGYQSMDNFIKKNTPQGFK 179
Hpa 119:LGIAQPTIVFSSRKGLPKVLEVQKTVTCIKTIVILDSKVNFGGHDCMETFIKKHVELGFP 178
Pmi 119:LFISQPTIVFCSKRALQKILGVQKKLPVIQKIVILDSREDYMGKQSMYSFIESHLPAGFN 178

Roh 175:VRNFKPRDFDAK-EQVALIMSSSGTTGLPKGVVLTHRNLSVRFVHCKDPLFGTRTIPSTS 233
Lcr 180:ASSFKTVEV-DRKEQVALIMNSSGSTGLPKGVQLTHENTVTRFSHARDPIYGNQVSPGTA 238
Lla 180:GSSFKTVEV-NRKEQVALIMNSSGSTGLPKGVQLTHENAVTRFSHARDPIYGNQVSPGTA 238
Hpa 179:PTSFVPLDVKNRKQHVALLMNSSGSTGLPKGVRITHEGAVTRFSHAKDPIYGNQVSPGTA 238
Pmi 179:EYDYIPDSF-DRETATALIMNSSGSTGLPKGVDLTHMNVCVRFSHCRDPVFGNQIIPDTA 237

Roh 234:ILSIVPFHHAFGMFTTLSYFIVGLRVVLLKRFEEKFFLSTIEKYRIPTIVLAPPVMVFLA 293
Lcr 239:VLTVVPFHHGFGMFTTLGYLICGFRVVMLTKFDEETFLKTLQDYKCTSVILVPTLFAILN 298
Lla 239:ILTVVPFHHGFGMFTTLGYLTCGFRIVMLTKFDEETFLKTLQDYKCSSVILVPTLFAILN 298
Hpa 239:ILTVVPFHHGFGMFTTLGYFACGYRVVMLTKFDEELFLRTLQDYKCTSVILVPTLFAILN 298
Pmi 238:ILTVIPFHHVFQMFTTLGYLTCGFRIVLMYRFEEELFLRSLQDYKIQSALLVPTLFSFFA 297

Roh 294:KSPLVDQYDLSSIREVATGGAPVGTEVAVAVAKRLKIGGILQGYGLTETCCAVLITPHDD 353
Lcr 299:KSELLNKYDLSNLVEIASGGAPLSKEVGEAVARRFNLPGVRQGYGLTETTSAIIITPEGD 358
Lla 299:RSELLDKYDLSNLVEIASGGAPLSKEIGEAVARRFNLPGVRQGYGLTETTSAIIITPEGD 358
Hpa 299:KSELIDKFDLSNLTEIASGGAPLAKEVGEAVARRFNLPGVRQGYGLTETTSAFIITPEGD 358
Pmi 298:KSTLVDKYDLSNLHEIASGGAPLAKEVGEAVAKRFKLPGIRQGYGLTETTSAIIITPEGD 357

Roh 354:VKTGSTGRVAPYVQAKIVDLTTGKSLGPNKRGELCFKSEIIMKGYFNNKQATEEAIDKEG 413
Lcr 359:DKPGASGKVVPLFKAKVIDLDTKKSLGPNRRGEVCVKGPMLMKGYVNNPEATKELIDEEG 418
Lla 359:DKPGASGKVVPLFKAKVIDLDTKKTLGPNRRGEVCVKGPMLMKGYVDNPEATREIIDEEG 418
Hpa 359:DKPGASGKVVPLFKVKVIDLDTKKTLGVNRRGEICVKGPSLMLGYSNNPEATKETIDEEG 418
Pmi 358:DKPGACGKVVPFFTAKIVDLDTGKTLGVNQRGELCVKGPMIMKGYVNNPEATNALIDKDG 417

Roh 414:WLHSGDVGYYDDDGHFFVVDRLKELIKYKGYQVAPAELEWLLLQHPSIKDAGVTGVPDEA 473
Lcr 419:WLHTGDIGYYDEEKHFFIVDRLKSLIKYKGYQVPPAELESVLLQHPSIFDAGVAGVPDPV 478
Lla 419:WLHTGDIGYYDEEKHFFIVDRLKSLIKYKGYQVPPAELESVLLQHPNIFDAGVAGVPDPI 478
Hpa 419:WLHTGDIGYYDEDEHFFIVDRLKSLIKYKGYQVPPAELESVLLQHPNIFDAGVAGVPDPQ 478
Pmi 418:WLHSGDIAYYDKDGHFFIVDRLKSLIKYKGYQVPPAELESILLQHPFIFDAGVAGIPDPD 477

Roh 474:AGELPGACIVLQEGKSLTEQEIIDYIAERVSPTKRIRGGVVFVDDIPKGATGKLVRSELR 533
Lcr 479:AGELPGAVVVLESGKNMTEKEVMDYVASQVSNAKRLRGGVRFVDEVPKGLTGKIDGRAIR 538
Lla 479:AGELPGAVVVLEKGKSMTEKEVMDYVASQVSNAKRLRGGVRFVDEVPKGLTGKIDGKAIR 538
Hpa 479:AGELPGAVVVMEKGKTMTEKEIVDYVNSQVVNHKRLRGGVRFVDEVPKGLTGKIDAKVIR 538
Pmi 478:AGELPAAVVVLEEGKMMTEQEVMDYVAGQVTASKRLRGGVKFVDEVPKGLTGKIDSRKIR 537

Roh 534:KLLAQ-KKSKL                                                  543
Lcr 539:EILKK-PVAKM                                                  548
Lla 539:EILKK-PVAKM                                                  548
Hpa 539:EILKK-PQAKM                                                  548
Pmi 538:EILTMGQKSKL                                                  548
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Fig. 2 Multiple alignment of the amino acid sequence of the luciferases of *Luciola cruciata*(Lcr), *L.lateralis* (Lla),*Pyrocoelia miyako*(Pmi), *Hotaria paravula*(Hpa), and *Rhagophthalmus ohbai* (Roh),. Amino acids are abbreviated using standard single letter codes. A dash indicates the gapsite and red boxes showed the homologous regions deduced from the relative of the alignment of these regions.

the region from Asn-400 to Ala-500 in RoL is the most homologous part with Lampyridae luciferases, whereas the region from Met-1 to Gly-200 is less homologous than the other parts. The most homologous region does not always correspond to the active site of the bioluminescent reaction.

The evolutionary tree based on the multiple alignment of the bioluminescent beetles, including the Lampyridae, Elateridae, Rhagophthalmidae, and Phengodidae, was constructed and shown in Fig. 3. As expected, Phi and Pvi luciferases for the South-America were found to be close related to the RoL, although the habitat of them are separated by a vast geographic distant. Inspection of the tree shows that the bioluminescent beetles may be divided into three groups in accord with the biological classification. However, the bioluminescent spectra of the bioluminescent beetles will indicate another classification based on functional properties. Fig. 4 showed the bioluminescence emission spectrum of this recombinant luciferase when reacted with luciferin and ATP at pH 6.0-8.0. The *in vitro* bioluminescence spectra showed a peak

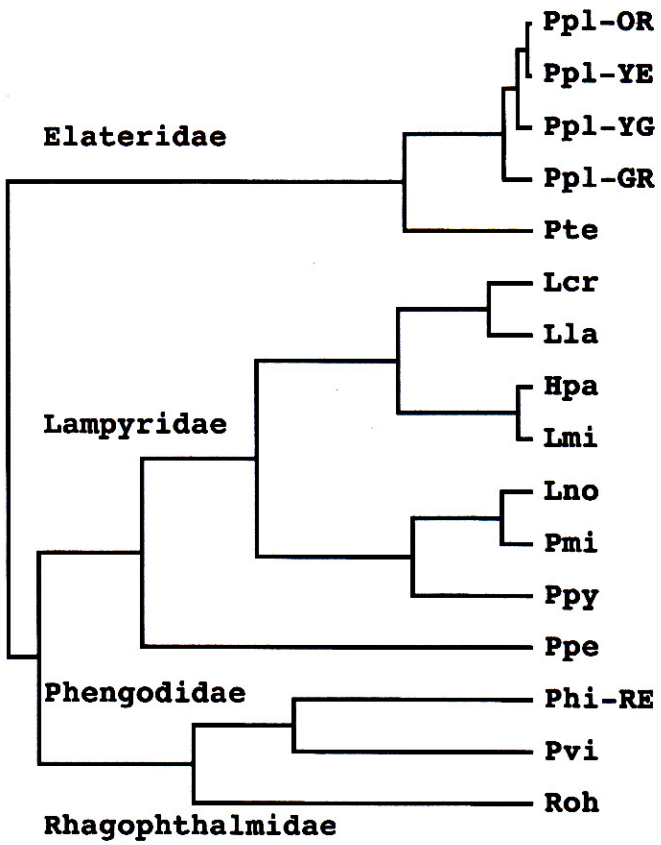

Fig. 3 Phylogenetic tree of the luciferases of *Luciola cruciata*(Lcr), *L.lateralis*(Lla), *Pyrocoelia miyako*(Pmi), *Hotaria paravula*(Hpa), *Photinus pyralis*(Ppy), *Photinus pennsylvanica* (Ppe), *Luciora mingrelica*(Lmi), *Lampyris noctiluca*(Lno), *Pyrophorus plagiophthalamus* (Ppl-OR(orange light emission), Ppl-YE(yellow light emission), Ppl-YG(yellow green light emission), and Ppl-GR(green light emission)), *Pyrearinus termitilluminans*(Pte), *Phrixothrix vivianii*(Pvi), *P.hirtus*(Phi-RE(red light emission)),and *Rhagophthalmus ohbai* (Roh), constructed according to the UPGMA method. A qutative root was introduced at the point where the average branch length to the cluster of the Lampyridae, Phengodidae, and Rhagophthalmidae was the same as the lenbth to the Elateridae enzymes.

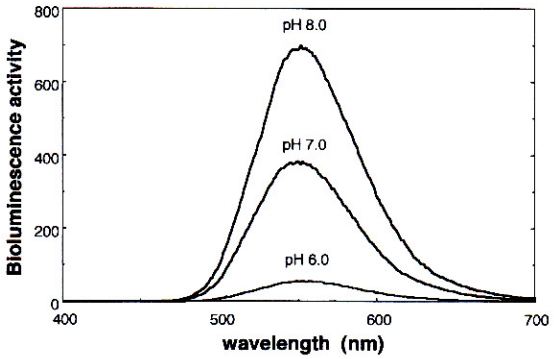

Fig. 4 Bioluminescence emission spectra of recombinant RoL st pH 6.0, 7.0, and8.0. To 50 μl of the luciferase solution, 450 μL of substrate mixture consisting of 1 mM LUCIFERIN/ 100 mM phosphate buffer (pH 6.0-8.0),containing 2 mM ATP and 5 mM $MgSO_4$ was injected. The spectrum was measured with a Hitachi F-4010 Fluorescence Photometer and corrected for photoresponse of equipment.

of 554 nm, which did not shift from pH 6.0 - 8.0, although the luminescent activities were different. In the case of firefly luciferase, it exhibits the large spectral shift to red bioluminescence under the acid pH, whereas the click beetle and the Phengodidae luciferases do not show such spectral shift under the same conditions (WOOD *et al.*, 1989; DELUCA, 1969; VIVIANI *et al.*, 1993). These results indicate spectral characteristics of RoL resemble more closely that of Elateridae luciferases than those of the Lampyridae. The functional domain for the pH resistance in luciferases may be kept between Elateridae and Phengodidae. Then, the luciferase structures could be divided into two groups, one comprising the families of true fireflies (Lampyridae) and another including the Elateridae, Rhagophthalmidae, and Phengodidae.

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