10788 *Biochemistry* **2010,** *49,* 10788–10795
DOI: 10.1021/bi1016342

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Identification and Characterization of a Luciferase Isotype in the Japanese Firefly, *Luciola cruciata*, Involving in the Dim Glow of Firefly Eggs[†]

Yuichi Oba,*[,‡] Nanae Mori,[‡] Mayumi Yoshida,[‡] and Satoshi Inouye[§]

[‡]*Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan, and* [§]*Yokohama Research Center, Chisso Corporation, Yokohama 236-8605, Japan*

Received October 12, 2010; Revised Manuscript Received November 16, 2010

ABSTRACT: We isolated the cDNA of a luciferase isotype (*LcLuc2*) from the Japanese firefly, *Luciola cruciata* (Lampyridae, Coleoptera). The gene product of *LcLuc2* (LcLuc2) showed 59% amino acid identity with firefly luciferase LcLuc1, which was previously identified in *L. cruciata*. The recombinant LcLuc2 showed both luminescence activity and fatty acyl-CoA synthetic activity comparable to those of LcLuc1. The spectral maxima of the luminescence by LcLuc1 and LcLuc2 were 554 and 543 nm, respectively. Reverse transcription−PCR analysis showed that the transcripts of *LcLuc1* were abundant in the lanterns of larva, adult male, and adult female, whereas both *LcLuc1* and *LcLuc2* were expressed in eggs. The luminescence spectra of the lantern extracts from larva, adult male, and adult female were in good agreement with that of recombinant LcLuc1. On the other hand, the emission maximum of the extract from eggs was between those of LcLuc1 and LcLuc2. These results suggest that *L. cruciata* possesses two luciferases: LcLuc1 is responsible for the major luminescence in larva and adult, whereas LcLuc1 and LcLuc2 are responsible for the dim glow in firefly eggs.

In fireflies (Lampyridae, Coleoptera), approximately 2000 species are found around the world (*1*). The larvae reported in firefly species are all luminous, but the adult in some species are nonluminous (*2*). It has been reported that eggs and pupae in some lampyrid species, including the Japanese firefly *Luciola cruciata*, show dim light (*3*, *4*). One of the roles of their luminescence is sexual communication in adult, whereas the roles are unclear in the egg, larva, and pupal stages (those roles may involve aposematic defense (*5*)).

The luciferin−luciferase reaction in fireflies has been studied mainly in the North American firefly, *Photinus pyralis*, and in *L. cruciata* (*6*). The chemical structure of luciferin from *P. pyralis* and *L. cruciata* was determined to be (4*S*)-4,5-dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid (D-luciferin, beetle luciferin) (*7*, *8*). The luciferases (EC 1.13.12.7) purified from the lanterns of these fireflies showed similar enzymatic properties (*9*, *10*). The luminescence reaction of firefly is represented by the following two steps (*11*−*13*):

$$\text{D-luciferin} + \text{ATP} \leftrightarrows \text{D-luciferyl-AMP} + \text{PP}_\text{i} \quad (1)$$

$$\text{D-luciferyl-AMP} + \text{O}_2 \rightarrow \text{oxyluciferin} + \text{CO}_2 + \text{light} \quad (2)$$

Equation 1 is the formation of luciferyl adenylate and pyrophosphate from D-luciferin and ATP, and eq 2 is the light production process accompanying the formation of oxyluciferin and CO_2 from luciferase-bound luciferyl adenylate and molecular oxygen. When excess amounts of D-luciferin and ATP are present in the reaction mixture, a flash of light is produced, and then the luminescence decays rapidly due to product inhibition. This inhibition is most likely caused by dehydroluciferyl-AMP and is partially released by adding coenzyme A (CoA)[1] to the reaction mixture (*14*−*17*).

The primary structure of firefly luciferase was determined by cDNA cloning from *P. pyralis* (PpyLuc) (*18*, *19*) and then from *L. cruciata* (referred to as "LcLuc1" in this paper) (*20*). Up to now, the cDNAs of firefly luciferase have been identified in over 20 species of the family Lampyridae (*6*). In 1996, the crystal structure of PpyLuc was determined, and the result indicated that firefly luciferase consists of a large N-terminal domain and a small C-terminal domain (*21*). In 2006, the crystal structure of LcLuc1 in complex with a luciferyl-AMP analogue, 5′-*O*-[*N*-(dehydroluciferyl)sulfamoyl]adenosine (DLSA), was reported, showing that DLSA was bound between the N-terminal domain and the C-terminal domain (*22*).

In 2003, we demonstrated that LcLuc1 and PpyLuc catalyze fatty acyl-CoA synthesis (eqs 3 and 4) (*23*).

$$\text{fatty acid} + \text{ATP} \leftrightarrows \text{fatty acyl-AMP} + \text{PP}_\text{i} \quad (3)$$

$$\text{fatty acyl-AMP} + \text{CoA} \rightarrow \text{fatty acyl-CoA} + \text{AMP} \quad (4)$$

The most preferable fatty acid as a substrate for LcLuc1 and PpyLuc was lauric acid (C12:0) (*24*). Thus, firefly luciferase is a bifunctional enzyme possessing the catalytic functions of both luciferase and fatty acyl-CoA synthetase (EC 2.3.1.86).

To understand the genetic and functional origins of firefly luciferase, we have isolated the homologous cDNAs of luciferase from various insects (*25*−*29*). From *L. cruciata*, two homologous

[†]This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

*To whom correspondence should be addressed. E-mail: oba@agr.nagoya-u.ac.jp. Telephone: +81-52-789-4280. Fax: +81-52-789-4280.

[1]Abbreviations: RT-PCR, reverse transcription−PCR; DLSA, 5′-*O*-[*N*-(dehydroluciferyl)sulfamoyl]adenosine; CoA, coenzyme A; MES, 2-morpholinoethanesulfonic acid monohydrate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; IPTG, isopropyl β-thiogalactopyranoside; *p*-ABSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; RACE, rapid amplification of cDNA ends; PpyLuc, *Photinus pyralis* luciferase; LcLuc1, *Luciola cruciata* luciferase 1; LcLuc2, *Luciola cruciata* luciferase 2; rlu, relative light units.

 Published on Web 11/22/2010

cDNAs, *LcLL1* and *LcLL2*, were isolated. The gene product of *LcLL1* (LcLL1) showed a fatty acyl-CoA synthetic activity, and the gene product of *LcLL2* (LcLL2) showed neither luminescence nor fatty acyl-CoA synthetic activities (*26*). The gene product of *PaLL* (PaLL), isolated from the Panamanian luminous click beetle *Pyrophorus angustus*, showed a fatty acyl-CoA synthetic activity and very low luminescence activity with firefly luciferin (*29*). The gene products of the homologues from nonluminous insects, *Drosophila melanogaster* (CG6178) (*25*) and *Agrypnus binodulus* (AbLL) (*28*), showed a fatty acyl-CoA synthetic activity with various fatty acids and very low luminescence activity with firefly luciferin (*30*). The gene products of the homologues from nonluminous beetle *Tenebrio molitor* (TmLL-1, -2, -3) (*27*) showed a fatty acyl-CoA synthetic activity. On the basis of these findings, we have proposed that firefly luciferase originated from a fatty acyl-CoA synthetase by gene duplications and functional modifications (*6*, *26*).

In this study, we describe the isolation of a novel cDNA of firefly luciferase (referred to as "*LcLuc2*" in this paper) from a single adult specimen of *L. cruciata*.

MATERIALS AND METHODS

Chemicals. The sources of chemicals were as follows: isopropyl β-thiogalactopyranoside (IPTG), D-luciferin potassium salt, sodium acetate trihydrate, 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), coenzyme A trisodium salt (CoA), 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (*p*-ABSF), glycine, linoleic acid, and $MgCl_2$ (Wako Pure Chemical Industries, Osaka, Japan); leupeptin and pepstatin A (Peptide Institute, Osaka, Japan); 2-morpholinoethanesulfonic acid monohydrate (MES) (Dojindo, Kumamoto, Japan); ATP (Oriental Yeast, Osaka, Japan); α-linolenic acid (NOF, Tokyo, Japan); lauric acid sodium salt and myristic acid sodium salt (Tokyo Kasei Kogyo, Tokyo, Japan); octanoic acid sodium salt, decanoic acid sodium salt, oleic acid sodium salt, and arachidonic acid sodium salt (Sigma-Aldrich, St. Louis, MO); rhamnose (Nacalai Tesque, Kyoto, Japan); Benzonase nuclease (Novagen, Madison, WI).

PCR Cloning of a Luciferase Isotype, LcLuc2. An adult male of *L. cruciata* was collected at Mino (Gifu, Japan) on June 9, 2003. PCR cloning was performed as previously described (*26*). In brief, total RNA (24 μg) was obtained from a single body without lantern (wet weight, 15 mg) using the SV total RNA isolation system (Promega, Madison, WI) and was reverse-transcribed using the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA). The PCR reactions were carried out using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) with a Dice Gradient thermal cycler (Takara, Osaka, Japan) or a GeneAmp 9700 (Applied Biosystems). The first PCR reaction was performed using the degenerate primers LH1 and LH4 (Table S1 of the Supporting Information) (*26*), and the PCR product obtained was applied to the nested PCR using the degenerate primers LH2 and LH3 (Table S1 of the Supporting Information) (*26*). The amplified 0.7 kb fragment was cloned into pGEM-T Easy vector (Promega). The 5′- and 3′-RACEs (rapid amplification of cDNA ends) were carried out using a Smart RACE cDNA amplification kit (Clontech, Palo Alto, CA). The cDNA having a complete open reading frame (ORF) of *LcLuc2* was amplified using Phusion high-fidelity DNA polymerase (New England BioLabs, Ipswich, MA) with the gene-specific primers designed for 5′-end and 3′-end RACE products. The nucleotide sequence was determined using a BigDye terminator kit (Applied Biosystems) with an ABI Prism 3130 genetic analyzer (Applied Biosystems). The GenBank accession number of *LcLuc2* is AB490793 (Figure 1A).

Identification of Firefly Gene Introns. The nucleotide sequences of introns A and B (Figure 1B) were determined by PCR using genomic DNA and the gene-specific primers for *LcLuc1* and *LcLuc2* (Table S1 of the Supporting Information). In brief, genomic DNA was extracted from the legs of an adult male using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) and used as a template for genomic PCR. The 0.5 kb (for *LcLuc1*) and 1.0 kb (for *LcLuc2*) fragments amplified by PCR were subcloned into pGEM-T Easy vector, and the nucleotide sequence was determined using a BigDye terminator kit with an ABI Prism 3130.

Expression and Purification of L. cruciata Luciferases, LcLuc1 and LcLuc2. The ORFs for *LcLuc1* (GenBank accession number AB220162) and *LcLuc2* were cloned in-frame into the *Bam*HI/*Eco*RI and *Eco*RI/*Hin*dIII sites of pCold-ZZ-X (*31*) to give pCold-ZZX-LcLuc1-3 and pCold-ZZX-LcLuc2-1, respectively. The recombinant proteins, LcLuc1 and LcLuc2, were expressed in *Escherichia coli* KRX (Promega) and purified using nickel chelate affinity chromatography as previously described (*29*) with some modifications. In brief, the cells were cultured in Luria−Bertani broth (200 mL) at 37 °C for 2 h and then further incubated at 15 °C for 24 h in the presence of 0.2 mM IPTG and 0.1% rhamnose. The harvested cells were disrupted in 5 mL (for LcLuc1) or 10 mL (for LcLuc2) of buffer A (50 mM Tris-HCl, 0.4 M NaCl, pH 7.8, containing 2.5 units/mL benzonase) by sonication, and the resultant supernatant by centrifugation at 17400*g* for 15 min was then applied onto a nickel chelate Sepharose column (GE Healthcare, Buckinghamshire, U.K.). After the column was washed with 10 mL of buffer A, the bound proteins were eluted in a stepwise manner with 10 mL of buffer A containing 0, 30, 100, and 500 mM imidazole. The fractions eluted with the buffer containing 100 mM imidazole were concentrated using a Vivaspin 500 centrifugal concentrator (30000 MWCO PES; Sartorius Stedim Biotech, Goettingen, Germany). The protein concentration was determined using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin (Fraction V; Sigma-Aldrich) as a standard.

Assay for Luminescence Activity. The reaction mixture (100 μL) contained firefly luciferin (50 μM), ATP (250 μM), $MgCl_2$ (2.5 mM), and the purified enzyme in 50 mM Tris-HCl (pH 7.8). The reaction was initiated by injecting buffer solution containing firefly luciferin, ATP, and $MgCl_2$ (50 μL) into the solution of enzyme (50 μL) at 25 °C. The light intensity was measured by using a Centro LB960 luminometer (Berthold, Bad Wildbad, Germany) for 1 min in 0.5 s intervals (for normal luminescence assay) or 0.1 s intervals (for kinetic analysis). For kinetic analyses, the maximum light intensities (I_{max}) were measured using 0.1 pmol of purified enzyme and various concentrations of firefly luciferin (0.5−8 μM, with 2 mM ATP) and ATP (1−30 μM, with 1 mM firefly luciferin). Under these conditions, the times to reach I_{max} by the reaction of LcLuc1 and LcLuc2 were 0.8−1.1 and 2−5 s, respectively. No correction was made for the differences in rise times to I_{max}.

Measurement of Bioluminescence Spectrum. The adult male and female specimens were collected at Okazaki (Aichi, Japan) on June 7, 2008, June 14, 2008, June 9, 2010, and June 24, 2010. Fertilized eggs were spawned by females, and the larvae were raised from eggs in our laboratory. The firefly lanterns of larva (located at the abdominal segment VIII) and of adult (located at the true abdominal segment VI in female and VI + VII in male)

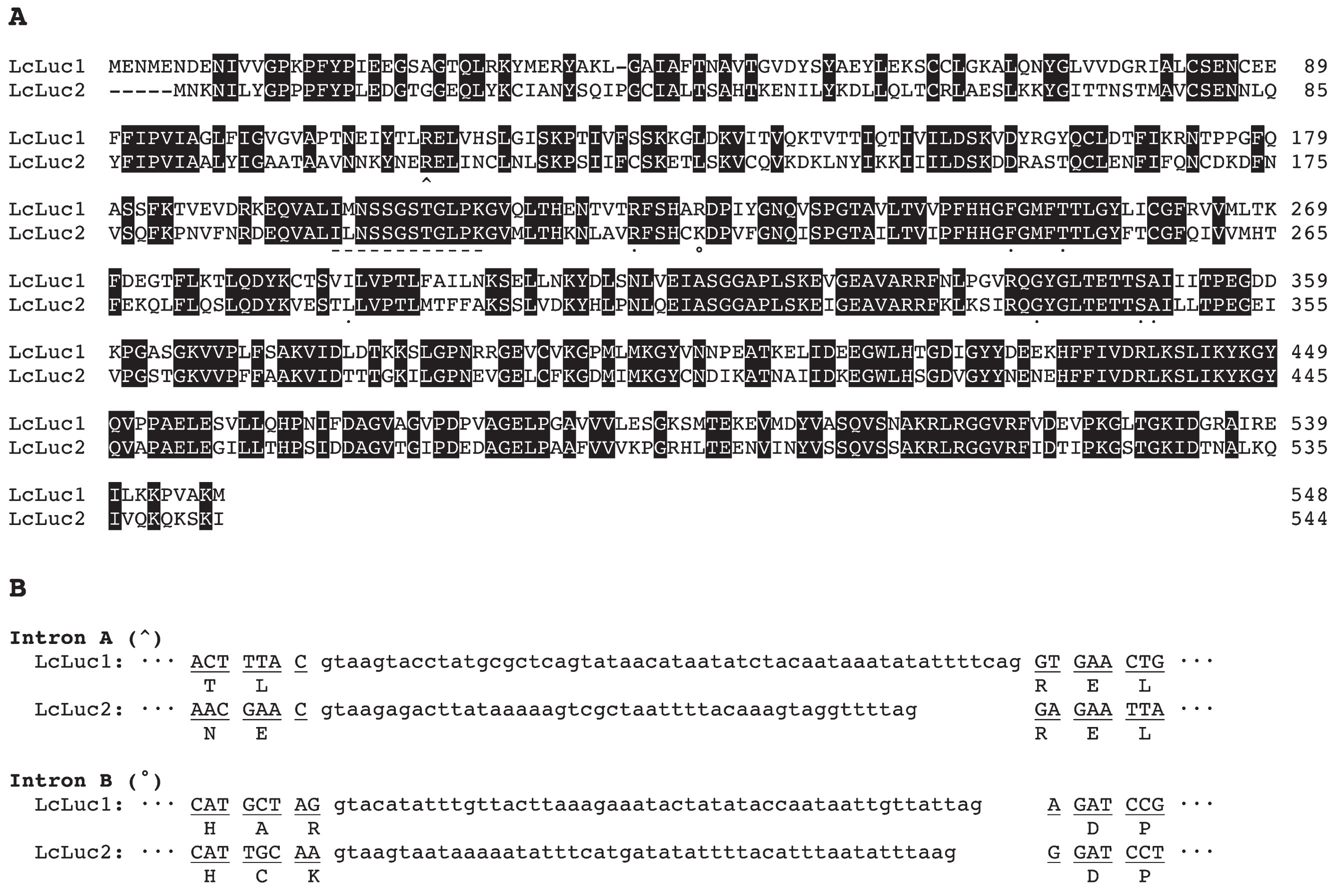
A
LcLuc1 MENMENDENIVVGPKPFYPIEEGSAGTQLRKYMERYAKL-GAIAFTNAVTGVDYSYAEYLEKSCCLGKALQNYGLVVDGRIALCSENCEE 89
LcLuc2 -----MNKNILYGPPPFYPLEDGTGGEQLYKCIANYSQIPGCIALTSAHTKENILYKDLLQLTCRLAESLKKYGITTNSTMAVCSENNLQ 85
LcLuc1 FFIPVIAGLFIGVGVAPTNEIYTLRELVHSLGISKPTIVFSSKKGLDKVITVQKTVTTIQTIVILDSKVDYRGYQCLDTFIKRNTPPGFQ 179
LcLuc2 YFIPVIAALYIGAATAAVNNKYNERELINCLNLSKPSIIFCSKETLSKVCQVKDKLNYIKKIIILDSKDDRASTQCLENFIFQNCDKDFN 175
LcLuc1 ASSFKTVEVDRKEQVALIMNSSGSTGLPKGVQLTHENTVTRFSHARDPIYGNQVSPGTAVLTVVPFHHGFGMFTTLGYLICGFRVVMLTK 269
LcLuc2 VSQFKPNVFNRDEQVALILNSSGSTGLPKGVMLTHKNLAVRFSHCKDPVFGNQISPGTAILTVIPFHHGFGMFTTLGYFTCGFQIVVMHT 265
LcLuc1 FDEGTFLKTLQDYKCTSVILVPTLFAILNKSELLNKYDLSNLVEIASGGAPLSKEVGEAVARRFNLPGVRQGYGLTETTSAIIITPEGDD 359
LcLuc2 FEKQLFLQSLQDYKVESTLLVPTLMTFFAKSSLVDKYHLPNLQEIASGGAPLSKEIGEAVARRFKLKSIRQGYGLTETTSAILLTPEGEI 355
LcLuc1 KPGASGKVVPLFSAKVIDLDTKKSLGPNRRGEVCVKGPMLMKGYVNNPEATKELIDEEGWLHTGDIGYYDEEKHFFIVDRLKSLIKYKGY 449
LcLuc2 VPGSTGKVVPFFAAKVIDTTTGKILGPNEVGELCFKGDMIMKGYCNDIKATNAIIDKEGWLHSGDVGYYNENEHFFIVDRLKSLIKYKGY 445
LcLuc1 QVPPAELESVLLQHPNIFDAGVAGVPDPVAGELPGAVVVLESGKSMTEKEVMDYVASQVSNAKRLRGGVRFVDEVPKGLTGKIDGRAIRE 539
LcLuc2 QVAPAELEGILLTHPSIDDAGVTGIPDEDAGELPAAFVVVKPGRHLTEENVINYVSSQVSSAKRLRGGVRFIDTIPKGSTGKIDTNALKQ 535
LcLuc1 ILKKPVAKM 548
LcLuc2 IVQKQKSKI 544
B
Intron A (^)
LcLuc1: ··· ACT TTA C gtaagtacctatgcgctcagtataacataatatctacaataaatatattttcag GT GAA CTG ···
T L R E L
LcLuc2: ··· AAC GAA C gtaagagacttataaaaagtcgctaattttacaaagtaggttttag GA GAA TTA ···
N E R E L
Intron B (°)
LcLuc1: ··· CAT GCT AG gtacatatttgttacttaaagaaatactatataccaataattgttattag A GAT CCG ···
H A R D P
LcLuc2: ··· CAT TGC AA gtaagtaataaaaatatttcatgatatattttacatttaatatttaag G GAT CCT ···
H C K D P

FIGURE 1: (A) Amino acid sequence alignment of LcLuc1 and LcLuc2. The numbers in the right margin refer to the positions of amino acid residues. Identical amino acid residues are shown with black shading. Dashed underlining indicates a putative AMP-binding domain predicted by Prosite (http://www.expasy.ch/prosite/). Seven dots represent amino acid residues for the possible interaction with firefly luciferin (*22*, *35*). (B) Intron structures for *LcLuc1* and *LcLuc2*. The positions of intron A (∧) and B (°) are shown in panel A.

were obtained by cutting the frozen specimen at the abdominal segment VII (in larva) and the true abdominal segment V (in adult). Eggs were collected within 3 days after spawning using microforceps. The crude extract from eggs (161 mg, ∼1000 eggs) and a single frozen lantern (larva, 59 mg; adult male, 10 mg; adult female, 9 mg) were prepared by homogenizing in 500 μL of 0.8 M NaCl−water solution containing protease inhibitors (5 μg/mL leupeptin, 0.2 mM *p*-ABSF, and 20 μM pepstatin A). After centrifugation at 17400*g* for 2 min, the supernatant was immediately used to measure the bioluminescence spectra. The reaction mixture (600 μL) contained firefly luciferin (1 mM), ATP (2 mM), CoA (0.5 mM), $MgCl_2$ (4 mM), NaCl (0.4 M), and the purified enzyme (18.5−55.3 pmol) or the crude lantern extract (150 μL) in 500 mM Tris-HCl (pH 7.8), MES−NaOH (pH 5.7), or glycine−NaOH (pH 10.2). The bioluminescence spectra were measured on a FP-777W fluorescence spectrophotometer (Jasco, Tokyo, Japan) at 22 °C with the excitation light source turned off (emission bandwidth, 20 nm; scan speed, 500 nm/min). When the luminescence intensity was low, the spectral data were recorded repeatedly (1−50 times) and integrated to reduce the noise. After the spectra were measured, the pH value of the spent mixture was measured with a Twin pH B-212 compact pH meter (Horiba, Kyoto, Japan).

Assay for Fatty Acyl-CoA Synthetic Activity. The reaction mixture (20 μL) contained 10 μM [1-^{14}C]lauric acid (12 nCi; GE Healthcare), 250 μM ATP, 250 μM CoA, 5 mM $MgCl_2$, and the purified enzyme (1 pmol) in 100 mM Tris-HCl (pH 7.8). After incubation for 30 min at 25 °C, the reaction was terminated by adding 20 μL of ethanol, and 3 μL was developed on a TLC plate (silica gel 60 F_{254}; Merck, Darmstadt, Germany) with 1,4-dioxane/ammonium hydroxide/water (3:0.5:2). The TLC plate was dried and exposed to a BAS-MS imaging plate (Fuji Film, Tokyo, Japan) for 14 h, and the radioactivities of [^{14}C]lauroyl-CoA were detected by a BAS 2500 imaging analyzer (Fuji Film).

Assay for Substrate Specificity. The reaction mixture (20 μL) contained [α-^{32}P]ATP (0.33 μCi = 5.6 nM; PerkinElmer, MA), 100 μM ATP, 250 μM CoA, 5 mM $MgCl_2$, 10 μM fatty acid, and purified protein (1 pmol) in 100 mM Tris-HCl buffer (pH 7.8). After incubation for 30 min at 25 °C, the reaction was terminated by adding 20 μL of ethanol, and 2 μL was applied onto a silica gel TLC plate. The first development was carried out using 1,4-dioxane/50 mM acetic acid (4:1), followed by the second development using 1,4-dioxane/ammonium hydroxide/water (6:1:5). The TLC plate dried was exposed to a BAS-MS imaging plate for 2 h, and the radioactivities of [^{32}P]AMP were measured on a BAS 2500 imaging analyzer.

Analysis of the Transcripts of LcLuc1 and LcLuc2. Adult males and females of *L. cruciata* were collected at Minokamo (Gifu, Japan) on June 21, 2009. Total RNAs were prepared from eggs (10 mg, ∼50 eggs) and single frozen lantern (larva, 124 mg; male, 13 mg; female, 43 mg). The mRNAs were then purified using an Oligotex-dT30 Super mRNA purification kit (Takara). The mRNA was reverse-transcribed by SuperScript III and used for the PCR template. The RT-PCR was carried out using AmpliTaq Gold DNA polymerase (Applied Biosystems) with a Dice Gradient thermal cycler (Takara) under the following conditions: 30 cycles of denaturing at 94 °C for 30 s, annealing

Table 1: Characteristics of Firefly Luciferases

luciferase	AA	M_w[a]	pI[a]	pH optimum	λ_{max} (pH 7.8)	K_m (μM) at pH 7.8 luciferin	ATP
LcLuc1	548	59904	6.74	7.5−8.5	554	14.3	106
LcLuc2	544	59949	8.39	7.5−8.5	543	0.811	21.5
PpeLuc1[b]	552	60971	7.55	8.0−9.5[d]	560[c]	$\sim10^2$	$\sim10^2$
PpeLuc2[b]	545	60649	8.40	9.0−10.0[d]	538[c]	$\sim10^0$	$\sim10^1$
PpLuc[d]	550	60745	6.42	6.5−9.5	558	15 ± 2	160 ± 20
LiLuc[e]	548	60496	6.01	ND[f]	566	90 ± 9	180 ± 14

[a]Compute pI/M_w tool (http://ca.expasy.org/tools/pi_tool.html). [b]Kutuzova et al. (*46*). [c]The pH conditions for these values are not described in Kutuzova et al. (*46*). [d]PpLuc, *P. pyralis* luciferase; data from Branchini et al. (*37*−*39*). [e]LiLuc, *L. italica* luciferase; data from Branchini et al. (*40*). [f]ND, not determined.

for 30 s, and extension at 72 °C for 30 s. The annealing temperatures for *LcLuc1*, *LcLuc2*, *Lc-rp49*, and *actin* were 61, 60, 52, and 54 °C, respectively. To identify the *LcLuc2* transcripts in larva and adult, the product of the first PCR (45 cycles) was diluted 10-fold and used for the nested PCR: 25 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The primer sequences are listed in Table S1 of the Supporting Information. The *Lc-rp49* and *actin* (GenBank accession numbers, AB205198 and AB474250, respectively) were used as endogenous internal controls to standardize the results.

Phylogenetic Analysis. Multiple amino acid sequence alignment was performed using the L-INS-i strategy of Mafft 6.237 (*32*) with a BLOSUM matrix. A phylogenetic tree was reconstructed using MrBayes 3.1.2 (*33*) under mixed models. We conducted two simultaneous chains for 1000000 generations, sampling trees every 100 cycles, discarded the first 2500 trees, and used majority rule consensus of the remaining trees to determine the clade posterior probabilities.

RESULTS AND DISCUSSION

Isolation of the cDNA for LcLuc2. By using RT-PCR and the RACE technique, we isolated the cDNA of a luciferase homologue from a single specimen of the adult male Japanese firefly *L. cruciata* and named it *LcLuc2*. The RT-PCR was performed using the degenerate primers designed for amplifying firefly luciferases and their homologues (*26*). The 0.7 kb PCR fragment was subcloned, and the plasmid DNAs from bacterial colonies were sequenced. When the mRNA from the whole insect body was used for RT-PCR, 12 of 20 clones contained the fragments for *LcLuc1* and 8 clones contained unknown fragments. On the other hand, when the mRNA from the head and thorax (without lantern) was used, 5 of 18 clones contained the fragments for *LcLuc1*, 3 clones for *LcLL1* (*26*), 5 clones for *LcLL2* (*26*), and 1 clone for *LcLuc2*. Four clones contained unknown fragments. LcLuc2, the gene product deduced from the complete cDNA for *LcLuc2*, consisted of 544 amino acid residues with a calculated mass 59949 and pI 8.39 (Table 1). It has been known that firefly luciferases, including PpeLuc1 and PpeLuc2, possess a putative peroxisomal targeting signal (-SKL, -AKM, or -AKL) at the C-terminus (*24*, *34*). The C-terminal sequence of LcLuc2 (-SKI) was not detected as a peroxisomal targeting signal by the PTS1 predictor (http://mendel.imp.ac.at/sat/pts1/cgi-bin/pts1.cgi) (Figure 1A). However, it is unclear whether the -SKI sequence acts as a peroxisomal targeting signal or not in firefly.

Table 2: Amino Acid Sequence Identities among Luciferases of *L. cruciata* and *P. pennsylvanica* and Homologues in *L. cruciata*

	LcLuc1	LcLuc2	LcLL1	LcLL2	PpeLuc1	PpeLuc2
LcLuc1	100	59	41	46	63	56
LcLuc2		100	45	52	59	69
LcLL1			100	43	41	44
LcLL2				100	46	49
PpeLuc1					100	57
PpeLuc2						100

Among firefly luciferases, LcLuc2 showed the highest amino acid sequence identity to the luciferase from *Photuris pennsylvanica*, PpeLuc2 (69%). The amino acid sequence identity between LcLuc1 and LcLuc2 was 59% (Table 2). The motif sequence for the putative AMP-binding domain was conserved (Figure 1A, dashed underline), and seven amino acid residues for the putative luciferin binding in firefly luciferase (dots in Figure 1A) (*22*, *35*) were also conserved in LcLuc2. These results suggested that LcLuc2 is a member of the adenylate-forming enzyme and has a catalytic function of luciferase.

Luminescence Properties of Recombinant LcLuc1 and LcLuc2. The coding regions of *LcLuc1* and *LcLuc2* were cloned into the expression vector, and the recombinant proteins expressed in *E. coli* were purified by nickel chelate chromatography. SDS−PAGE analysis showed that the purified proteins of LcLuc1 and LcLuc2 were homogeneous (Figure 2A). From the bacterial cells of the 200 mL culture, the yields of LcLuc1 and LcLuc2 were 400 and 70 μg, respectively. To compare the luminescence activity between LcLuc1 and LcLuc2, the I_{max} was determined at pH 7.8 using the purified recombinant proteins (0.1 pmol) in our assay conditions. The I_{max} values (n = 3) of LcLuc1 and LcLuc2 were $8.60 \times 10^5 \pm 3.86 \times 10^4$ and $4.96 \times 10^5 \pm 3.89 \times 10^4$ rlu (relative light units), respectively. In the absence of luciferase, the I_{max} value showed 6.33 ± 0.882 rlu. Thus, the luminescence activity of LcLuc2 was comparable to that of LcLuc1, indicating that LcLuc2 is an active luciferase. The pH optima for LcLuc1 and LcLuc 2 were determined to be 7.5−8.5 (Figure S1 of the Supporting Information, Table 1). When CoA was added to the reaction mixture, the luminescence intensity of LcLuc2 was stimulated (data not shown), as observed in LcLuc1 and other firefly luciferases (*6*, *14*). The emission maxima of the luminescence reaction (λ_{max}) by LcLuc1 and LcLuc2 were 554 nm (pH 7.8, half-width = 65 nm) and 543 nm (pH 7.7, half-width = 55 nm), respectively (Figure 2B,C). When the luminescence reaction was carried out under acidic conditions, λ_{max} of LcLuc1 was red shifted to around 600 nm, which is similar to the case in other firefly luciferases (*36*), but λ_{max} of LcLuc2 was unchanged (Figure 2B,C). The K_m values of firefly luciferin and ATP for LcLuc1 and LcLuc2 were determined (Table 1, Figure S2 of the Supporting Information). The K_m of firefly luciferin and ATP for several firefly luciferases have been reported, and these values were in good agreement with that of LcLuc1. On the other hand, the K_m values of firefly luciferin and ATP for LcLuc2 were smaller than those of other firefly luciferases (Table 1) (*37*−*40*).

Determination of Fatty Acyl-CoA Synthesis in LcLuc2. Firefly luciferases from *L. cruciata* (LcLuc1), *P. pyralis* (PpLuc), and *Lucidina biplagiata* (LbLuc) had the catalytic function of fatty acyl-CoA synthesis (*6*, *23*, *24*, *41*). We examined fatty acyl-CoA synthetic activity of LcLuc2 using ^{14}C-labeled lauric acid as a substrate and found the formation of the lauroyl-CoA formation (Figure 3A). This result indicated that LcLuc2 is also a

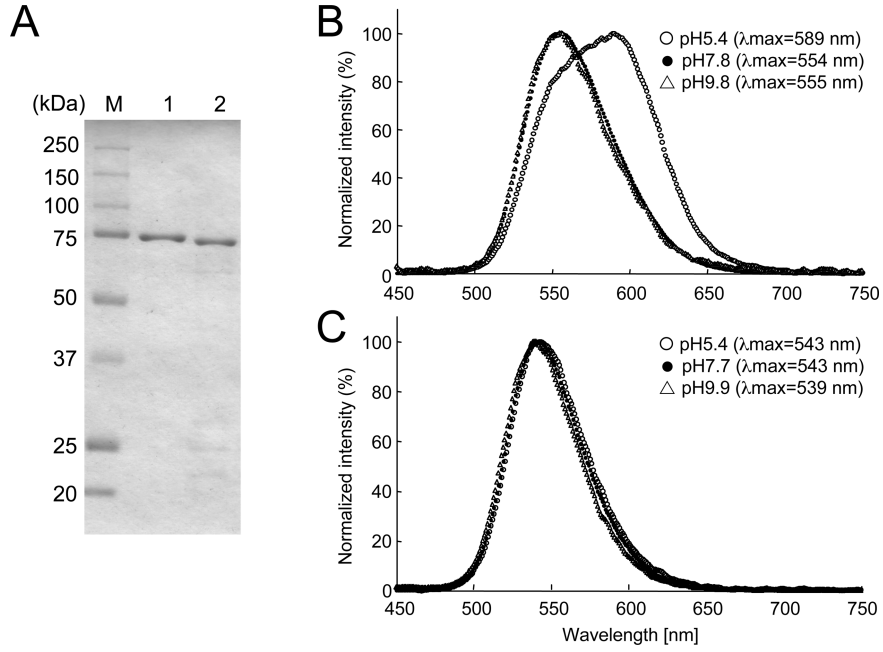

FIGURE 2: (A) SDS−PAGE analysis of purified proteins. Approximately 0.5 μg of recombinant proteins was loaded. Lane M, molecular marker; lane 1, LcLuc1; lane 2, LcLuc2. Luminescence spectra of recombinant LcLuc1 (B) and LcLuc2 (C). The reaction mixture contains 30 pmol (LcLuc1 at pH 5.4, 7.8, and 9.8), 18.5 pmol (LcLuc2 at pH 5.4 and 7.7), and 55.3 pmol (LcLuc2 at pH 9.9) of the proteins.

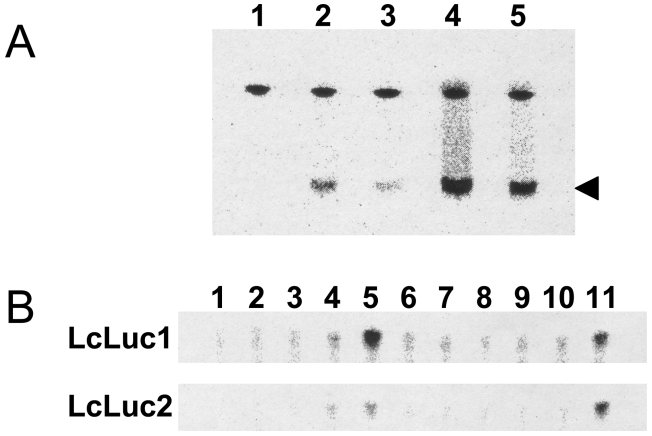

FIGURE 3: Determination of fatty acyl-CoA synthetic activity in LcLuc1 and LcLuc2. (A) Detection of [^{14}C]lauroyl-CoA (arrowhead) produced by the reaction with [1-^{14}C]lauric acid, ATP, and CoA. Lane 1, no enzyme; lane 2, LcLuc1 (1 pmol); lane 3, LcLuc2 (1 pmol); lane 4, LcLuc1 (10 pmol); lane 5, LcLuc2 (10 pmol). (B) Detection of [^{32}P]AMP produced by the reaction with [α-^{32}P]ATP, CoA, and various substrates. Lane 1, no substrate; lane 2, acetic acid (C2:0); lane 3, octanoic acid (C8:0); lane 4, decanoic acid (C10:0); lane 5, lauric acid (C12:0); lane 6, myristic acid (C14:0); lane 7, oleic acid (C18:1); lane 8, linoleic acid (C18:2); lane 9, α-linolenic acid (C18:3); lane 10, arachidonic acid (C20:4); lane 11, firefly luciferin.

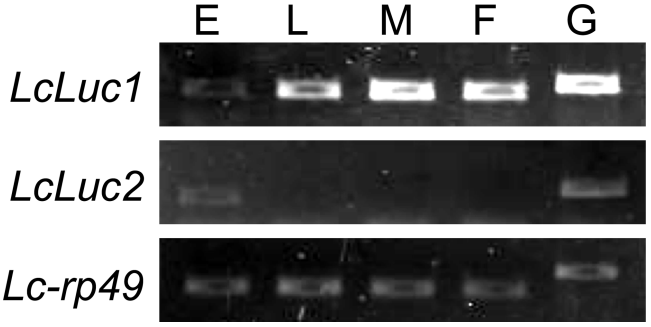

FIGURE 4: Detection of transcripts of *LcLuc1* and *LcLuc2* in *L. cruciata*: E, eggs; L, larval lantern; M, adult male lantern; F, adult female lantern; G, genome; *Lc-rp49*, internal control.

bifunctional enzyme, having luminescence activity and fatty acyl-CoA synthetic activity. The adenylation activity for various fatty acids was determined by detecting the formation of [^{32}P]AMP from [α-^{32}P]ATP. The results showed that medium-chain fatty acids, decanoic acid (C10:0) and lauric acid (C12:0), were preferable substrates for LcLuc2 (Figure 3B), similar to the case for other firefly luciferases (*23*, *24*, *41*).

Gene Expressions of LcLuc1 and LcLuc2 in L. cruciata. To investigate the gene expressions of *LcLuc1* and *LcLuc2* in eggs, larva, adult male, and adult female of *L. cruciata*, RT-PCR was performed using gene-specific primer sets (Table S1 of the Supporting Information). The mRNAs prepared from eggs and single lantern of larva and adult were reverse-transcribed and used as a template for RT-PCR. The primer sets for RT-PCR of *LcLuc1*, *LcLuc2*, and *Lc-rp49* were designed to contain introns in the amplified regions. The results showed that *LcLuc1* was expressed strongly in the lanterns of larva, adult male, and adult female. The *LcLuc1* was also expressed in eggs. On the other hand, under our PCR condition, the expression of *LcLuc2* was detected only in eggs (Figure 4). When the PCR product for *LcLuc2* was applied to the nested PCR, the gene expression of *LcLuc2* was detected slightly in lanterns of larva, adult male, and adult female (data not shown). These results suggested that the gene expressions of *LcLuc1* and *LcLuc2* are independently regulated; furthermore, LcLuc1 functions in firefly lanterns of larva, adult male, and adult female, whereas both LcLuc1 and LcLuc2 function in eggs.

Introns in LcLuc1 and LcLuc2. We identified two introns in the same region of *LcLuc1* and *LcLuc2* by PCR using genomic DNA and exon-specific primer sets. Each intron was located at the same position, but the length and nucleotide sequence were different between *LcLuc1* and *LcLuc2* (Figure 1B). This result indicated that *LcLuc1* and *LcLuc2* are encoded at different loci on the *L. cruciata* genome. The complete intron−exon structure

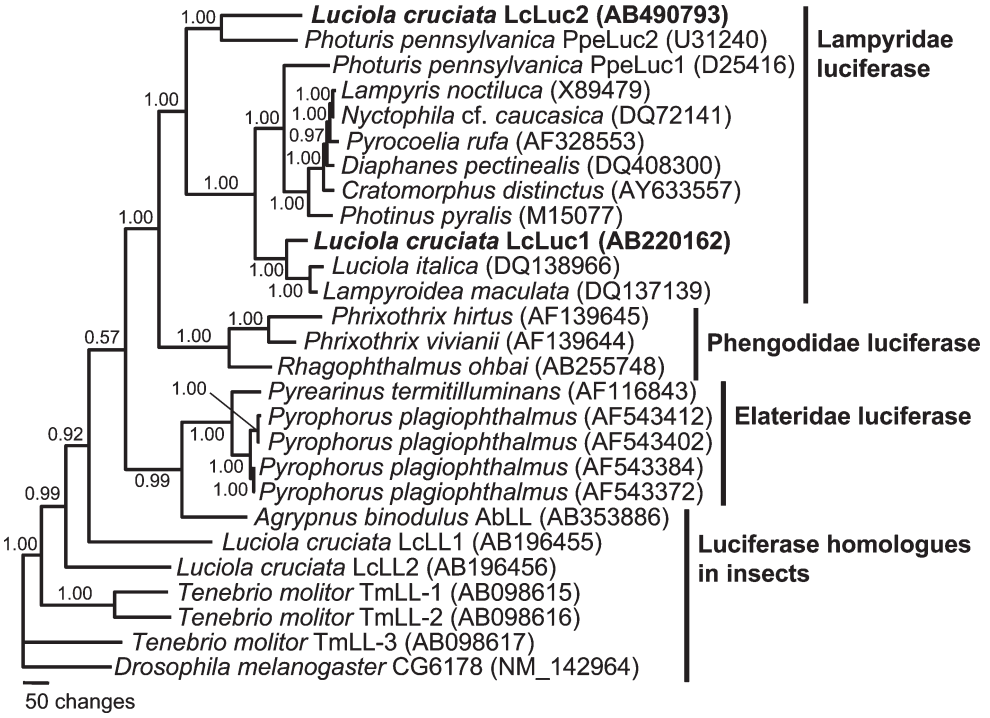

FIGURE 5: Bayesian phylogenetic tree of beetle luciferases and their homologues. The posterior probabilities above 0.9 are indicated on the nodes. GenBank accession numbers are shown in parentheses.

of the firefly luciferase gene was determined in PpLuc (*19*) and *Luciola lateralis* luciferase (*42*), and each genomic luciferase gene contained six introns at the same positions. The introns A and B found in *LcLuc1* and *LcLuc2* corresponded to the intron 2 and intron 3 in *PpLuc* and *L. lateralis* luciferase, suggesting that the intron−exon structure of firefly luciferase is conservative.

Comparison with PpeLuc1 and PpeLuc2 in P. pennsylvanica. The cDNAs of firefly luciferase have been isolated from over 20 species of the family Lampyridae (*6*), but a single type of gene was isolated from each of those species, and these genes are closely related to each other (> 60% amino acid sequence identity). The exception is the case of the North American firefly, *P. pennsylvanica*. Two types of luciferase cDNA, *PpeLuc1* and *PpeLuc2*, were isolated from the lanterns of the adult specimens (*43*−*45*): *PpeLuc1* (GenBank accession numbers D25415 and D25416) and *PpeLuc2* (GenBank accession numbers U31240 and AX074300). The amino acid identity between PpeLuc1 and PpeLuc2 was 57%. This suggested that two types of luciferases might be present in *P. pennsylvanica*. However, the cDNA clones of *PpeLuc1* and *PpeLuc2* described in Ye et al. (*44*) were obtained from more than one specimen of firefly; hence it is unclear that a single specimen possesses both PpeLuc1 and PpeLuc2. The light emission peaks of recombinant PpeLuc1 and PpeLuc2 were at 560 and 538 nm, respectively (*44*), and the binding constants for firefly luciferin and ATP and the pH effects were also reported (*46*). These data imply that PpeLuc1 and PpeLuc2 in *P. pennsylvanica* seem to correspond to LcLuc1 and LcLu2 in *L. cruciata*, respectively (Table 2). To confirm these relationships, we then performed phylogenetic analysis.

Phylogenetic Tree. The phylogenetic tree for full-ORFs of beetle luciferases and their homologues was reconstructed by the Bayesian method, suggesting that LcLuc2 and PpeLuc2 together form a sister group to the clade of the other firefly luciferases with high posterior probabilities (100%) (Figure 5). Previous phylogenetic studies on Lampyridae based on 18S and 16S rDNAs have shown that the subfamily Photurinae (including the genus *Photuris*) is more closely related to the subfamily Lampyrinae (including *Photinus* and *Lampyris*) than to Luciolinae (including *Luciola* and *Lampyroidea*) (*47*, *48*). This suggested that the clade of *PpeLuc2* and *LcLuc2* had arisen by gene duplication before Lampyrinae and Luciolinae diverged. Recently, Day et al. (*49*) isolated genomic fragments similar to both *PpeLuc2* and *LcLuc2* by PCR from five lampyrid species: *Photuris* sp., *Photuris congener*, *Luciola italica*, *Lampyroidea maculata*, and *Lamprohiza splendidula*. These findings strongly support our hypothesis that firefly luciferase underwent the gene duplication event before Lampyridae was diverged.

Luminescence Spectra of the Extracts from Eggs and Lanterns. The extracts were prepared from eggs and lanterns of larva, adult male, and adult female and were used for measuring the luminescence spectra at various pH conditions (Figure 6). As the luminescence intensities of the extracts from eggs were very low, the spectral data were integrated 20 times at pH 7.3 and 50 times each at pH 5.5 and 9.8. The results showed that the luminescence spectra of the lantern extracts from larva, adult male, and adult female were in agreement with that of LcLuc1 but not with that of Lcluc2 (Figure 7A,C). This suggested that LcLuc1 is the major luciferase responsible for light emission of the lanterns in larva, adult male, and adult female of *L. cruciata*. This is also consistent with the finding that the transcript level of *LcLuc1* was high whereas that of *LcLuc2* was very low in lanterns (Figure 4). On the other hand, the spectral maximum of the egg extract was between those of LcLuc1 and LuLuc2 at pH 7.8 (Figure 7A) and in good agreement with that of LcLuc2 under acidic condition (Figure 7B). These results may be explained by the hypothesis that similar amounts of LcLuc1 and LcLuc2 are present in eggs. To test this hypothesis, recombinant LcLuc1 and LcLuc2 having the same luminescence activity were mixed, and the luminescence spectra were measured at pH 7.8 and 5.7. At pH 7.8, the spectral maximum from the mixture of LcLuc1 and LcLuc2 was between those of LcLuc1 and LcLuc2 (Figure 7C), whereas the spectral maximum at pH 5.7 was in agreement with

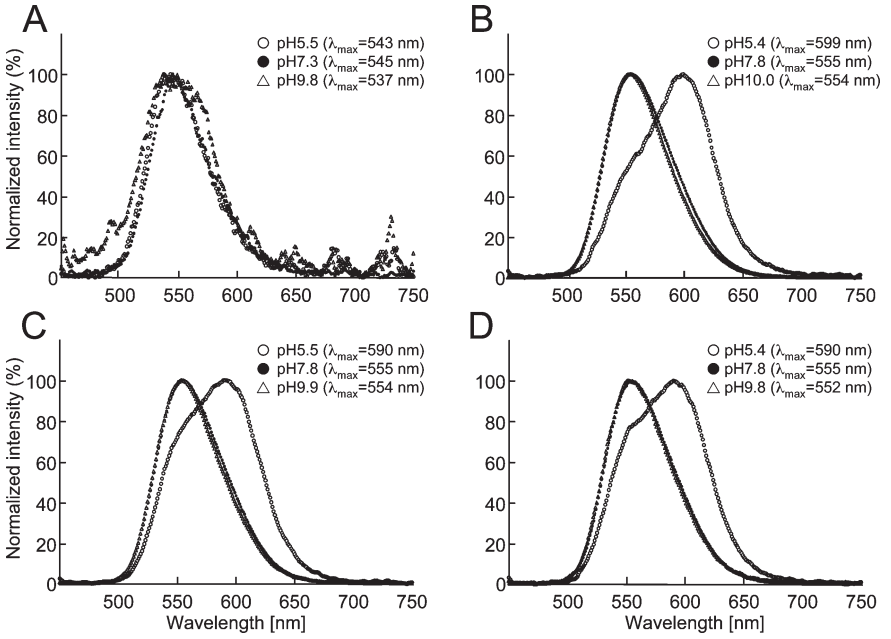

FIGURE 6: Luminescence spectra of the extract from eggs and lanterns of *L. cruciata* at various pH conditions: (A) eggs; (B) larval lantern; (C) adult male lantern; (D) adult female lantern.

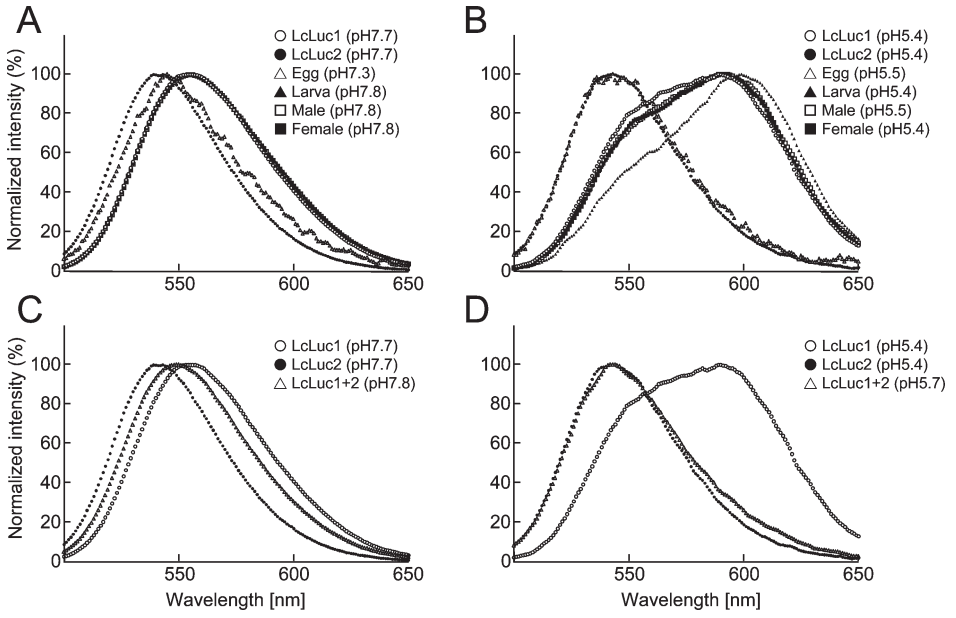

FIGURE 7: Comparisons of luminescence spectra between recombinant luciferases (LcLuc1 and LcLuc2) and extracts from eggs and lanterns (of larva, adult male, and adult female) at pH 7.3−7.8 (A) and pH 5.4−5.5 (B). Luminescence spectra of the mixture of LcLuc1 (0.6 pmol) and LcLuc2 (1.1 pmol) at pH 7.7−7.8 (C) and pH 5.4−5.7 (D). Note: the luminescence intensity of 0.6 pmol of LcLuc1 is almost the same as that of 1.1 pmol of LcLuc2 at pH 7.8.

that of LcLuc2 (Figure 7D). Therefore, we concluded that both LcLuc1 and LcLuc2 are responsible for the dim glow in *L. cruciata* eggs.

Conclusion. We identified a luciferase isotype gene from a single specimen of the Japanese firefly, *L. cruciata*, and assigned it to *LcLuc2*. The gene product, LcLuc2, was a functional luciferase having fatty acyl-CoA synthetic activity. The light emission peak of LcLuc2 was 543 nm, and no spectral change in luminescence was observed under acidic condition (pH insensitive). The transcript of *LcLuc2* was detected in eggs. The luminescence spectra of lantern extracts from larva, adult male, and adult female were in agreement with that of LcLuc1, suggesting that LcLuc1, not LcLuc2, seemed to be the major enzyme responsible for the luminescence *in vivo* in larva and adult fireflies. On the other hand, the luminescence spectrum of egg extract was between those of LcLuc1 and LcLuc2, suggesting that both of these luciferases are responsible for the dim glow in firefly eggs.

ACKNOWLEDGMENT

The authors thank Professor Makoto Ojika (Nagoya University) for helpful discussions.

SUPPORTING INFORMATION AVAILABLE

Relative luminescence intensities (I_{max}) of the purified LcLuc1 (0.17 pmol, panel A) and LcLuc2 (0.10 pmol, panel B) under

various pH conditions [●, MES buffer; ○, MOPS buffer; ▲, HEPES buffer; △, Tris buffer; ♦, glycine−NaOH buffer (37.5 mM); luciferin, 100 μM; CoA, 250 μM; $MgCl_2$, 5 mM] (Figure S1); Lineweaver−Burk plots of ATP and luciferin for LcLuc1 (A and B) and LcLuc2 (C and D) [bars indicate mean ± SEM (n = 3)] (Figure S2); primer list used for PCR (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Lawrence, J. F. (1982) Coleoptera, in Synopsis and Classification of Living Organisms (Parker, S. P., Ed.) pp 482−553, McGraw-Hill, New York.
2. Branham, M. A., and Wenzel, J. W. (2003) The origin of photic behavior and the evolution of sexual communication in fireflies (Coleoptera: Lampyridae). *Cladistics 19*, 1–22.
3. Harvey, E. N. (1952) Bioluminescence, Academic Press, New York.
4. Ohba, N. (2004) Mystery of fireflies, Yokosuka City Mus., Japan (in Japanese).
5. De Cock, R., and Matthysen, E. (1999) Aposematism and bioluminescence: experimental evidence from glow-worm larvae (Coleoptera: Lampyridae). *Evol. Ecol. 13*, 619–639.
6. Inouye, S. (2010) Firefly luciferase: an adenylate-forming enzyme for multicatalytic functions. *Cell. Mol. Life Sci. 67*, 387–404.
7. White, E. H., McCapra, F., Field, G. F., and McElroy, W. D. (1961) The structure and synthesis of firefly luciferin. *J. Am. Chem. Soc. 83*, 2402–2403.
8. Kishi, Y., Matsuura, S., Inoue, S., Shimomura, O., and Goto, T. (1968) Luciferin and luciopterin isolated from the Japanese firefly, *Luciola cruciata*. *Tetrahedron Lett. 9*, 2847–2850.
9. Green, A. A., and McElroy, W. D. (1956) Crystalline firefly luciferase. *Biochim. Biophys. Acta 20*, 170–176.
10. Kajiyama, N., Masuda, T., Tatsumi, H., and Nakano, E. (1992) Purification and characterization of luciferases from fireflies, *Luciola cruciata* and *Luciola lateralis*. *Biochim. Biophys. Acta 1120*, 228–232.
11. McElroy, W. D., Seliger, H. H., and White, E. H. (1969) Mechanism of bioluminescence, chemiluminescence and enzyme function in the oxidation of firefly luciferin. *Photochem. Photobiol. 10*, 153–170.
12. White, E. H., Rapaport, E., Seliger, H. H., and Hopkins, T. A. (1971) The chemi- and bioluminescence of firefly luciferin: an efficient chemical production of electronically excited states. *Bioorg. Chem. 1*, 92–122.
13. Wood, K. V. (1995) The chemical mechanism and evolutionary development of beetle bioluminescence. *Photochem. Photobiol. 62*, 662–673.
14. Airth, R. L., Rhodes, W. C., and McElroy, W. D. (1958) The function of coenzyme A in luminescence. *Biochim. Biophys. Acta 27*, 519–532.
15. Fraga, H., Fernandes, D., Fontes, R., and Esteves da Silva, J. C. G. (2005) Coenzyme A affects firefly luciferase luminescence because it acts as a substrate and not as an allosteric effector. *FEBS J. 272*, 5206–5216.
16. Ribeiro, C., and Esteves da Silva, J. C. G. (2008) Kinetics of inhibition of firefly luciferase by oxyluciferin and dehydroluciferyl-adenylate. *Photochem. Photobiol. Sci. 7*, 1085–1090.
17. Leitão, J. M. M., and Esteves da Silva, J. C. G. (2010) Firefly luciferase inhibition. *J. Photochem. Photobiol. B101*, 1–8.
18. de Wet, J. R., Wood, K. V., Helinski, D. R., and DeLuca, M. (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A. 82*, 7870–7873.
19. de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., and Subramani, S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol. 7*, 725–737.
20. Tatsumi, H., Masuda, T., Kajiyama, N., and Nakano, E. (1989) Luciferase cDNA from Japanese firefly, *Luciola cruciata*: cloning, structure and expression in *Escherichia coli*. *J. Biolumin. Chemilumin. 3*, 75–78.
21. Conti, E., Franks, N. P., and Brick, P. (1996) Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes. *Structure 4*, 287–298.
22. Nakatsu, T., Ichiyama, S., Hiratake, J., Saldanha, A., Kobashi, N., Sakata, K., and Kato, H. (2006) Structural basis for the spectral difference in luciferase bioluminescence. *Nature 440*, 372–376.
23. Oba, Y., Ojika, M., and Inouye, S. (2003) Firefly luciferase is a bifunctional enzyme: ATP-dependent monooxygenase and a long chain fatty acyl-CoA synthetase. *FEBS Lett. 540*, 251–254.
24. Oba, Y., Sato, M., Ojika, M., and Inouye, S. (2005) Enzymatic and genetic characterization of firefly luciferase and *Drosophila* CG6178 as a fatty acyl-CoA synthetase. *Biosci., Biotechnol., Biochem. 69*, 819–828.
25. Oba, Y., Ojika, M., and Inouye, S. (2004) Characterization of *CG6178* gene product with high sequence similarity to firefly luciferase in *Drosophila melanogaster*. *Gene 329*, 137–145.
26. Oba, Y., Sato, M., Ohta, Y., and Inouye, S. (2006) Identification of paralogous genes of firefly luciferase in the Japanese firefly, *Luciola cruciata*. *Gene 368*, 53–60.
27. Oba, Y., Sato, M., and Inouye, S. (2006) Cloning and characterization of the homologous genes of firefly luciferase in the mealworm beetle, *Tenebrio molitor*. *Insect Mol. Biol. 15*, 293–299.
28. Oba, Y., Iida, K., Ojika, M., and Inouye, S. (2008) Orthologous gene of beetle luciferase in non-luminous click beetle, *Agrypnus binodulus* (Elateridae), encodes a fatty acyl-CoA synthetase. *Gene 407*, 169–175.
29. Oba, Y., Kumazaki, M., and Inouye, S. (2010) Characterization of luciferases and its paralogue in the Panamanian luminous click beetle *Pyrophorus angustus*: A click beetle luciferase lacks the fatty acyl-CoA synthetic activity. *Gene 452*, 1–6.
30. Oba, Y., Iida, K., and Inouye, S. (2009) Functional conversion of fatty acyl-CoA synthetase to firefly luciferase by site-directed mutagenesis: a key substitution responsible for luminescence activity. *FEBS Lett. 583*, 2004–2008.
31. Inouye, S., and Sahara, Y. (2008) Soluble protein expression in *E. coli* cells using IgG-binding domain of protein A as a solubilizing partner in the cold induced system. *Biochem. Biophys. Res. Commun. 376*, 448–453.
32. Katoh, K., Misawa, K., Kuma, K., and Miyata, T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res. 30*, 3059–3066.
33. Huelsenbeck, J. P., and Ronquist, F. (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics 17*, 754–755.
34. Gould, S. J., Keller, G.-A., and Subramani, S. (1987) Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. *J. Cell Biol. 105*, 2923–2931.
35. Branchini, B. R., Magyar, R. A., Murtiashaw, M. H., and Portier, N. C. (2001) The role of active site residue arginine 218 in firefly luciferase bioluminescence. *Biochemistry 40*, 2410–2418.
36. Viviani, V. R. (2002) The origin, diversity, and structure function relationships of insect luciferases. *Cell. Mol. Life Sci. 59*, 1833–1850.
37. Branchini, B. R., Hayward, M. M., Bamford, S., Brennan, P. M., and Lajiness, E. J. (1989) Naphthyl- and quinolylluciferin: green and red light emitting firefly luciferin analogues. *Photochem. Photobiol. 49*, 689–695.
38. Branchini, B. R., Magyar, R. A., Murtiashaw, M. H., Anderson, S. M., and Zimmer, M. (1998) Site-directed mutagenesis of histidine 245 in firefly luciferase: a proposed model of the active site. *Biochemistry 37*, 15311–15319.
39. Branchini, B. R., Magyar, R. A., Murtiashaw, M. H., Anderson, S. M., Helgerson, L. C., and Zimmer, M. (1999) Site-directed mutagenesis of firefly luciferase active site amino acids: a proposed model for bioluminescence color. *Biochemistry 38*, 13223–13230.
40. Branchini, B. R., Southworth, T. L., DeAngelis, J. P., Roda, A., and Michelini, E. (2006) Luciferase from the Italian firefly *Luciola italica*: molecular cloning and expression. *Comp. Biochem. Physiol. B145*, 159–167.
41. Oba, Y., Furuhashi, M., and Inouye, S. (2010) Identification of a functional luciferase gene in the non-luminous diurnal firefly, *Lucidina biplagiata*, *Insect Mol. Biol. 19*, 737−743.
42. Cho, K. H., Lee, J. S., Choi, Y. D., and Boo, K. S. (1999) Structural polymorphism of the luciferase gene in the firefly, *Luciola lateralis*. *Insect Mol. Biol. 8*, 193–200.
43. Zenno, S., Shiraishi, S., Inouye, S., and Saigo, K. (1994) Patent Appl. No. JP6303982.
44. Ye, L., Buck, L. M., Schaeffer, H. J., and Leach, F. R. (1997) Cloning and sequencing of a cDNA for firefly luciferase from *Photuris pennsylvanica*. *Biochim. Biophys. Acta 1339*, 39–52.
45. Weber, E. R., Wood, K. V., and Hall, M. P. (2000) Patent Appl. No. WO 01-04310A.
46. Kutuzova, G. D., Hannah, R. R., and Wood, K. V. (1997) Bioluminescence color variation and kinetic behavior relationships among beetle luciferases, in Bioluminescence and Chemiluminescence (Hastings, J. W, Kricka, L. J., and Stanley, P. E., Eds.) pp 248−252, John Wiley and Sons, Chichester, U.K.
47. Sagegami-Oba, R., Takahashi, N., and Oba, Y. (2007) The evolutionary process of bioluminescence and aposematism in cantharoid beetles (Coleoptera: Elateridae) inferred by the analysis of 18S ribosomal DNA. *Gene 400*, 104–113.
48. Stanger-Hall, K. F., Lloyd, J. E., and Hillis, D. M. (2007) Phylogeny of North American fireflies (Coleoptera: Lampyridae): implications for the evolution of light signals. *Mol. Phylogenet. Evol. 45*, 33–49.
49. Day, J. C., Goodall, T. I., and Bailey, M. J. (2009) The evolution of the adenylate-forming protein family in beetles: multiple luciferase gene paralogues in fireflies and glow-worms. *Mol. Phylogenet. Evol. 50*, 93–101.