动 物 学 研 究 2008，Oct. 29(5)：477—484 CN 53-1040/Q ISSN 0254-5853
Zoological Research DOI：10.3724/SP.J.1141.2008.05477

Cloning, Expression and Sequence Analysis of A Luciferase Gene from the Chinese Firefly *Pyrocoelia pygidialis*

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Abstract: The cDNA encoding the luciferase from lantern mRNA of one diurnal firefly *Pyrocoelia pygidialis* Pic, 1926 has been cloned, sequenced and functionally expressed. The cDNA sequence of *P. pygidialis* luciferase is 1647 base pairs in length, coding a protein of 548 amino acid residues. Sequence analysis of the deduced amino acid sequence showed that this luciferase had 97.8% resemblance to luciferases from the fireflies *Lampyris noctiluca*, *Lampyris turkestanicus* and *Nyctophila* cf. *caucasica*. Phylogenetic analysis using deduced amino acid sequence showed that *P. pygidialis* located at the base of *Lampyris+Nyctophila* clade with robust support (BP=97%); but did not show a monophyletic relationship with its congeneric species *P. pectoralis*, *P. rufa* and *P. miyako*, all three are strong luminous and nocturnal species. The expression worked in recombinant *Escherichia coli*. Expression product had a 70kDa band and emitted yellow-green luminescence in the presence of luciferin. Five loops in the *P. pygidialis* luciferase, L1 (N198-G208), L2 (T240-G247), L3 (G317-K322), L4 (L343-I350) and L5 (G522-D532), were found from the structure modeling analysis in the cleft, where it was considered the active site for the substrate compound entering and binding. Different amino acid residues between the luciferases of *P. pygidialis* and the three other known strong luminous species can not explain the situation of weak or strong luminescence. Future study of these loops, residues or crystal structure analysis may be helpful in understanding the real differences between the luciferases between diurnal and nocturnal species.

Keywords: *Pyrocoelia*; Diurnal firefly; *Pyrocoelia pygidialis*; Luciferase; Homology modeling

中国萤火虫云南窗萤荧光素酶 cDNA 的克隆表达和序列分析

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摘要：从一种来自中国日行性萤火虫（云南窗萤）发光器官 mRNA 中克隆、测序并表达了有功能的荧光素酶。云南窗萤荧光素酶的 cDNA 序列有 1 647 个碱基，编码 548 个氨基酸残基。从推测得到的氨基酸序列的比对分析得出：云南窗萤的荧光素酶与来自 *Lampyris noctiluca*, *L. turkestanicus* 和 *Nyctophila* cf. *caucasica* 三种萤火虫的荧光素酶有 97.8%的序列一致性。从推测得出的氨基酸序列进行系统发育分析，其结果表明：云南窗萤和 *Lampyris+Nyctophila* 聚在一起, 与同属的发光强夜行性的萤火虫不形成的单系。云南窗萤荧光素酶在大肠杆菌中表达的条带大约 70 kDa，并且在有荧光素存在时发出黄绿色荧光。对荧光素酶的结构模拟和分析表明，云南窗萤荧光素酶基因的氨基端和羧基端结构域之间的裂沟处存在这 5 个多肽环，这正是从其他荧光素酶推测得到的催化荧光反应时的底物结合位点。云南窗萤和窗萤属的其他 3 种萤火虫的荧光素酶相比，有 13 个不同氨基酸位点，位于模拟分子结构的表面。对于这些多肽环、不同氨基酸残基和晶体结构的进一步研究有利于解释日行和夜行性萤火虫荧光素酶的差异。

关键词：窗萤属；日行性萤火虫；云南窗萤；荧光素酶；同源建模
中图分类号：Q969.48+6.2；Q951.3;Q754 **文献标识码**：A **文章编号**：0254-5853-(2008)05-0477-08

Received date：2008-04-17；Accepted date：2008-06-05
Fundation items: Supported partly by the Natural Foundation of Sciences of Yunnan Province (2006C0046Q); Partly by the Chinese Academy of Sciences (O706551141)
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收稿日期：2008-04-17；接受日期：2008-06-05

Luciferases are the enzymes that catalyze the reactions yielding visible light. A great number of bioluminescence reactions depend on various luciferases and widely different substrates (Alipour et al, 2004). Firefly luciferase (EC1.13.12.7) is responsible for the oxidation of firefly D-luciferin with molecular oxygen in the presence of ATP and Mg^{2+} to generate bioluminescence (McElroy, 1969; White et al, 1971; DeLuca, 1976; Wood, 1995; Emamzadeh et al, 2006). As the enzymatic luminescence assay is highly sensitive, rapid, nonradioactive and quantifiable (Gould et al, 1988), the firefly's bioluminescence has been widely applied in fields of life, environment and medical analysis techniques.

Since the cDNA and genomic DNA of the North American firefly *Photinus pyralis* luciferase were reported (De Wet et al, 1985, 1987), the luciferase cDNA and/or genomic DNA of more than 20 firefly species have been cloned, sequenced and/or expressed (Masuda et al, 1989; Tatsumi et al, 1992; Devine et al, 1993; Ohmiya et al, 1995; Sala-Newby et al, 1996; Ye et al, 1997; Viviani et al, 1999; Lee et al, 2001; Choi et al, 2002; Viviani et al, 2004; Alipour et al, 2004; Branchini et al, 2006; Emamzadeh et al, 2006; Li et al, 2006a). The crystal structures of *P. pyralis* and *Luciola cruciata* luciferases were discovered with X-ray diffraction (Conti et al, 1996; Nakatsu et al, 2006). And the substrate binding site and color determination were explained by structure and mutant analysis (Ohmiya et al, 1996; Viviani & Ohmiya, 2000; Viviani et al, 2001; Ugarova & Brovko, 2002; Nakatsu et al, 2006), though the AMP binding site and the structure-function relationship mechanism were still not very clear (Hirokawa et al, 2002; Emamzadeh et al, 2006).

Although fireflies are a well-known luminous beetle, not all species glow in their development from egg to adult. In the case of non-luminous or weak luminous fireflies, it is usually the male adult that has no luminous organs or only has vestiges of luminous organs. Such fireflies are sometimes thought of as diurnal species; analogically, species with both luminous male adults and luminous female adults are called nocturnal fireflies. Though male adults of all species in some firefly genera such as *Pristolycus* (Jeng et al, 2002) and *Cyphonocerus* (Jeng et al, 2006) are non-luminous, other genera ,such as *Pyrocoelia*, include not only diurnal but also nocturnal species (Suzuki, 1997). The function of photic signals, undoubtedly, is believed to be for mating in firefly adults (Branham & Wenzel, 2000, 2003). However, it has been reported that the diurnal fireflies, with weak luminescence or without luminescence, mainly use chemical signals (pheromone) for pair formation (Suzuki, 1997; Branham & Wenzel, 2000).

So far, *Pyrocoelia* luciferases have been cloned from three species: *P. rufa*; *P. miyako* and *P. pectoralis* (Ohmiya et al, 1995; Lee et al, 2001; Rong et al, 2007), all of which, with developed luminous organs, are nocturnal and glow strongly and continuously (Suzuki, 1997; Ohba, 2004; Wang et al, 2007). The luciferase from diurnal fireflies was studied very few, except for one case from a Brazilian twilight active firefly *Macrolampis sp*. (Viviani et al, 2005), which displays an unusual bimodal spectrum. In order to explore the characteristics of the diurnal firefly luciferase and its relationship with those of congeneric nocturnal species, we cloned the luciferase gene of the diurnal firefly *P. pygidialis*, which is distributed in Yunnan (Li et al, 2008), and described its expression and performed sequence analysis.

1 Materials and Methods

1.1 Insect samples

Larvae of *P. pygidialis* were collected from the gardens of the Kunming Institute of Botany (N25.0224° /E102.1225° ; 1968m), the Chinese Academy of Science, in the northern suburb of Kunming city, Yunnan Province, China, on October 19, 2006, by LI Xue-yan. Live fireflies were taken back to the laboratory and used in the experiment.

1.2 RNA extraction and RT-PCR

The lanterns of two larvae were dissected and pulverized under liquid nitrogen with a mortar and pestle. Total RNA was extracted using the RNAiso reagent (TaKaRa, #D312). The first strand of cDNA was synthesized at 42℃ for 60min in the presence of 200 U/μL M-MuLV Reverse transcriptase (TaKaRa, #DRR019A), 20U RNase inhibitor, dNTP mixture (final concentration each at 1 mmol/L), and dT adaptor: 5'-GTTTTCCCAGTCACGACTTTTTTTTTTTTTTT T-3' as the reverse primer. The specific primer sequences used for selective amplification of *P. pygidialis* luciferase gene were: 5'-ACGCGCTAATATCATTGCA-3' (sense primer), based on the luciferase gene of *Lampyris noctiluca* (GenBankTM/EBI accession number X89479), and the antisense primer-M13: 5'-GTTTTCCCAGTCA-CGAC-3'. The RT-PCR amplification of cDNA was

carried out by use of the cDNA first strand and under the following condition: initial denaturation at 95℃ for 5min, a 36-cycles amplification (94℃ for 40s, 48℃ for 30s and 72℃ for 2 min) and the final extension was performed at 72℃ for 8 min. PCR products were analyzed on 1.0% agarose gel electrophoresis and the expected fragment was purified with the Agarose gel DNA Purification kit (TaKaRa, DV805A). The cDNA product was sequenced by an automatic sequencer ABI sequencer PRISM 3730 (Shanghai Sangon Biological Engineering Technology & Services Co, Shanghai, China).

1.3 T-A cloning and expression

The following primer set was used to introduce *Nde*I and *Bam*HI restriction endonuclease sites at the 5' and 3' ends, respectively, in the PCR-amplified cDNA: 5'-TTGCACATATGGAAGATGATCATAA-3' and 5'-ATAAACGGATCCAATTACAGTTTTGATTTTTTTC-3' (underlined sequences represent the endonuclease site). The PCR product was ligated with pMD-18-T Simple Vector (TaKaRa, DV805A) and was transformed into the Top 10 competent cells. The PMD-18T-Pyluc vector was digested with *Nde*I and *Bam*HI sequentially and ligated into the corresponding clone sites on the pET-28a plasmid. PET28a-Luc recombinants were transformed into BL21 (DE3) competent cells by heat-shock treatment. Positive colonies were incubated in the fresh Luria-Bertain medium containing Kanamycin (50μg/mL) at 37℃ until the optical density of liquid medium at 600 nm (OD600) reached 0.5-0.7 (mid-log phase). The culture was induced with IPTG (isopropyl β-D-thiogalactoside, 0.1mmol/L) and the incubation was continued for an additional 8-16 hours at 25℃. The bacterial cells were precipitated (5000× g, 20min) and the pellet was suspended in lyse buffer PBS (0.14 mol/L NaCl, 26 mmol/L KCl, 10mmol/L Na_2HPO4, 2mmol/L KH_2PO_4, pH7.4) and frozen and thawed for 3-4 cycles to disrupt the bacterial cells. The homogenate was centrifuged at 12 000 g for 5 min and the supernatant was stored at -20℃. The cell lysate was centrifuged and the supernatant was analyzed by 12% SDS-PAGE.

1.4 Luciferase activity

To verify the luciferase expressed in *E. coli*, the cell extracts were assayed for luciferase activity by checking light generation in the dark. A volume of 20 μL of substrate mixture consisting of 1 mmol/L luciferin, 50 mmol/L Tris buffer, pH7.8, 2mmol/L ATP and 10mmol/L $MgSO_4$ was added to 50μL luciferase extraction solution in a quartz cell. A photo of the luminescent wells was taken with a Sony DSC-707 digital camera with 30 s exposure.

1.5 Sequence and phylogenetic analysis

With 28 GenBank-registered amino acid luciferase sequences, initial alignment was conducted using Clustal X (Thompson et al, 1997), then confirmation of the alignment was done manually using BioEdit (Version 4.7.8). The phylogenetic tree was constructed using the neighbor-joining method (Saitoh & Nei, 1987) and the software PAUP* 4.0 beta version (Swofford, 2002); the consistency of the branches was analyzed with a bootstrap value of 1000. The other sequences used in the phylogenetic analysis were obtained from GenBank and the accession numbers are as follows: *P. rufa* (AF328553, AY447202 and AY447203); *P. pectoralis* (EF155570); *P. miyako* (L39928); *Diaphanes pectinealis* (DQ408300); *L. noctiluca* (X89479, AY447204); *L. turkestanicus* (AY742225); *P. pyralis* (M15077); *Cratomorphus distinctus* (AY633557); *Nyctophila cf. caucasica* (DQ072141); *L. cruciata* (M26194); *Luciola lateralis* (X66919); *Luciola mingrelica* (S61961); *Luciola italica* (DQ138966); *Hotaria parvula* (L39929); *H. unmunsana* (AF420006, AF486800); *H. papariensis* (AF486802, AF486803); *H. tsushimana* (AF486801, AF486804); *Lampyroidea maculate* (DQ137139); *Photuris pennsylvanica* (U31240); *Phrixothrix hirtus* (AF139645); *Phrixothrix vivianii* (AF139644); *Rhagophthalmus ohbai* (AB255748).

1.6 Structure and homology modeling analysis of *P. pygidialis* luciferase

The structure of the *P. pygidialis* luciferase was modeled with the protein homology modeling SWISS-MODEL server using the crystal structure of *L. cruciata* luciferase (Protein Data Bank code: 2d1sA) as a template (swissmodel.expasy.org) (Guex & Peitsch, 1997; Schwede et al, 2003; Arnold et al, 2006). Analysis and comparison of the structures were carried out using Swiss-PdbViewer ver3.7. The domain structure map for the predicted amino acid sequence of *P. pygidialis* luciferase was performed using ProSite (Gattiker et al, 2002).

2 Results

2.1 RNA extraction, RT-PCR and sequence analysis

From the light organs of two larvae of the firefly *P. pygidialis*, total RNA was isolated, and its quality and quantity were verified by 1.0% agarose gel

electrophoresis. With the gene-specific primers, based on the luciferase gene of *L. noctiluca*, RT-PCR was performed to amplify *P. pygidialis* luciferase genes from the first strand of cDNA. The molecular sizes of the RT-PCR products (cDNA) were 1.7kb and identical to that expected (Fig. 1).

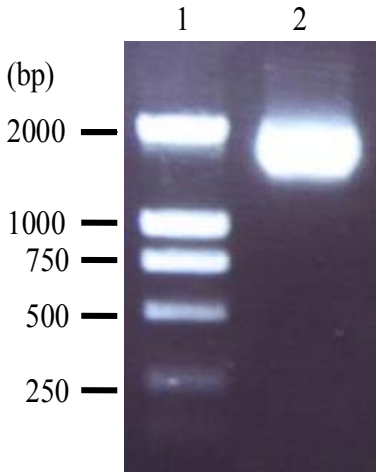

Fig. 1 RT-PCR products of luciferase gene on 1.0% agarose gel

Lane 1, nucleotide acid molecule size marker，DL2000; Lane 2, luciferase gene of *Pyrocoelia pygidialis*.

The nucleotide sequence of PCR products was sequenced and its amino acid sequence was deduced. The result of the complete nucleotide and amino acid sequences is shown in Fig. 2, the 1647bp luciferase gene has an open reading frame of 548 amino acid residues. The nucleotide sequence of *P. pygidialis* has been deposited in GenBank as entry EU826678.

2.2 Cloning and expression in *E. coli* BL21 (DE3)

*Nde*I and *Bam*HI restriction sites were appended to the *P. pygidialis* luciferase cDNA prior to being inserted into the pET-28a expression plasmid. Under the control of T7 promoter, luciferase was expressed in BL21(DE3) prokaryotic cells. The positive clones were verified by checking their sequences. The modification of a base pair, without amino acid change, was found. The protein synthesis in BL21 (DE3) cells were analyzed by 12% SDS-PAGE (Fig. 3). The luciferase expressed by the *P. pygidialis* luciferase gene was present as a band of about 70kDa in the cells transformed with recombinant vectors, but not in the cells infected with empty pET-28a plasmid (negative control). The Vector's initial codon and stop codon could have been under the T7 promoter or other reasons may have caused the expressed luciferase molecular mass difference compared to the calculated value.

By checking luciferase activity of light generation in the dark, the bioluminescence emission of the luciferase from *P. pygidialis* was shown in the photo (Fig. 4), as a yellow-green emission.

2.3 Sequence analysis and phylogenetic implication

The deduced amino acid sequences of *P. pygidialis* luciferase gene were compared with those of known luciferase genes. The result of pairwise comparisons was not shown. The deduced amino acid sequence of the *P. pygidialis* luciferase was found to have a high identity value (97.8%) to those of *L. noctiluca*, *L. turkestanicus* and *Nyctophila* cf. *caucasica*. And 60%−70% sequence identity with those of Luciolinae. It had low similarity (48%−53%) with the Phengodidae and Rhagophthalmidae groups.

Phylogenetic analysis using deduced amino acid sequence showed that *P. pygidialis* did not form monophyly with its congeneric species *P. pectoralis*, *P. rufa* and *P. miyako*, but located at the base of *Lampyris+Nyctophila* clade with a high bootstrap (97%) in NJ tree (Fig. 5).

2.4 Structure analysis and homology modeling

Using the ProSite (expasy.org/cgi-bin/prosite) online motif analysis, the structure of *P. pygidialis* luciferase was hypothesized. Tab. 1 defines part motifs, their sites and functions in *P. pygidialis* luciferase. *P. pygidialis*luciferase had two special motifs: Amidation site and Serpins signature compared to *L. maculata* luciferase (Emamzadeh et al, 2006). The c-terminal and microbody (peroxisome) targeting tripeptide is SKL, similar with other *Pyrocoelia* species. The putative AMP-binding sequence is 196-IMNSSGSTGLPK-206, corresponding to the *L. cruciata* luciferase P-loop (Nakatsu et al, 2006). The sequence is highly conserved among the various Lampyrinae firefly species (Alipour et al, 2004), small differences occurring in the Rhagophthalmidae and the Phengodidae luciferases. Three dimensional structure of *P. pygidialis* luciferase (K6-L540) and putative loops [L1 (N198-G208), L2 (T2

Tab. 1 Part motifs and sites in *Pyrocoelia pygidialis* luciferase

Amino acid position	Motif information
196 IMNSSGSTGLPK 207	Putative AMP-binding domain signature
458 ILLQHP 468	SERPIN Serpins signature
542 MGKK 545	Amidation site
546 SKL 548	Microbodies C-terminal targeting signal

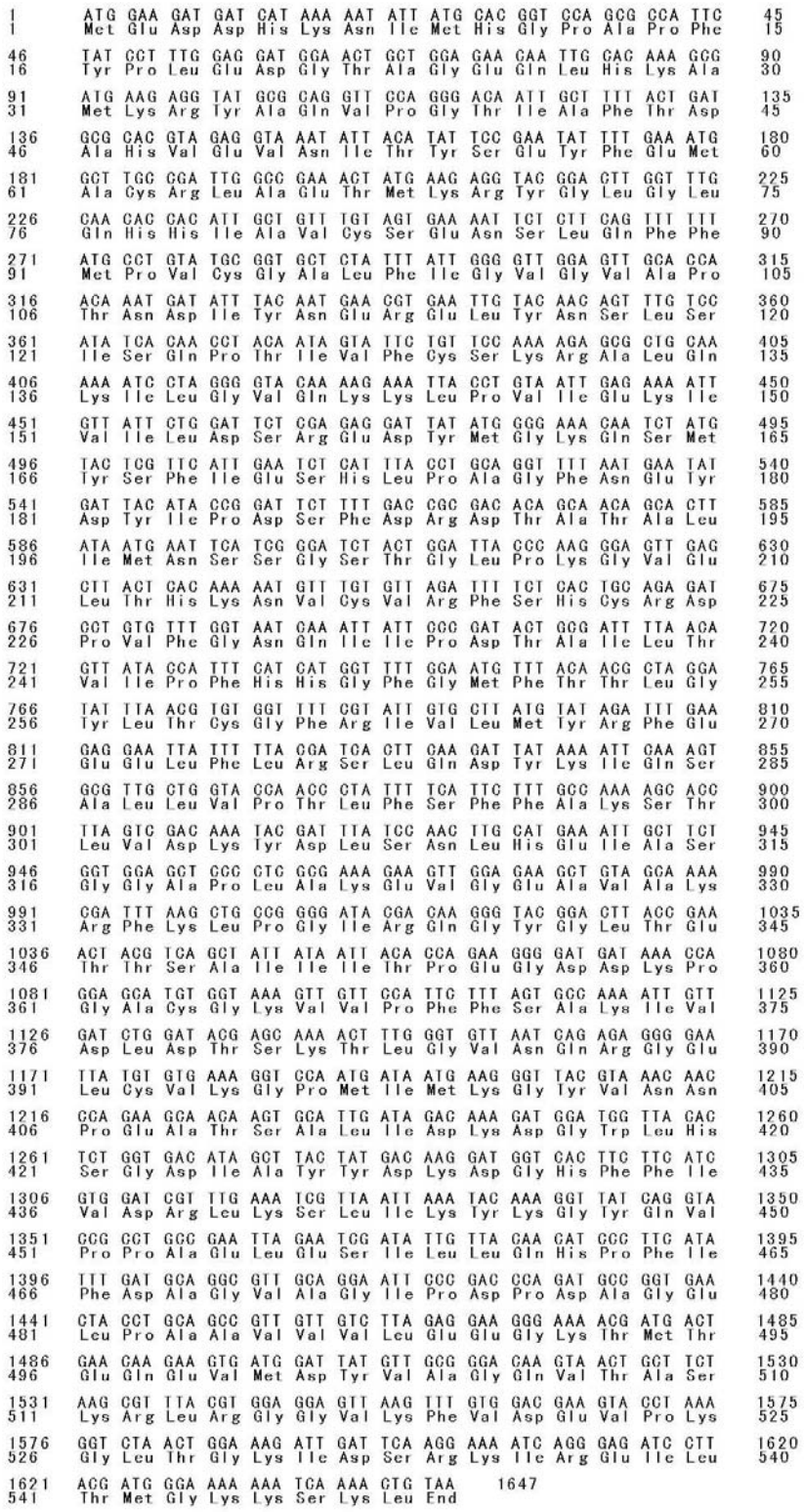

Fig. 2 The nucleotide and deduced amino acid sequences of *Pyrocoelia pygidialis* luciferase gene

40-G247), L3 (G317-K322), L4 (L343-I350) and L5 (G522-D532)] of the luciferase cleft area are shown in Fig. 6. It also shows one of the different amino acid residues between *P. pygidialis* luciferase and its congeneric species.

3 Discussion

The cDNA encoding luciferase in *P. pygidialis* was

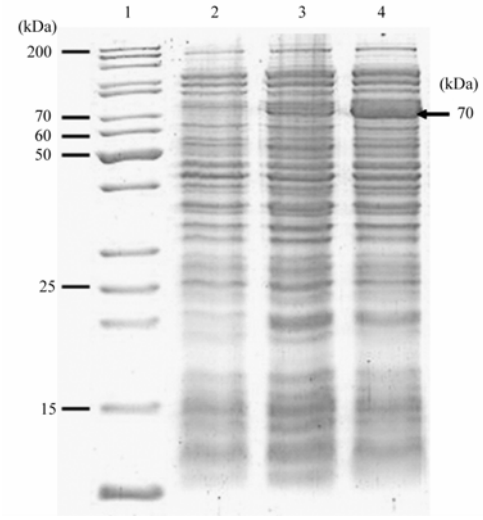

Fig. 3 SDS-PAGE analysis of *Pyrocoelia pygidialis* luciferase expressed in *E. coli* cells

Molecular weight standards were used as size marker (lane 1). The BL21 (DE3) *E. coli* cells were transformed by empty pET-28A (lane 2) and pET28a-Luc luciferase recombinant (lane 3, 4). The solid arrow on the right indicates the luciferase band of 70 kDa.

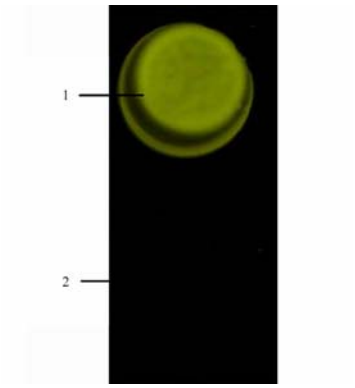

Fig. 4 Photo of a glowing clone, taken using a digital camera (Sony DSC-707) by long exposure and high light-sensitivity

1: *Pyrocoelia pygidialis* luciferase luminescence activity;
2: the negative control (DE3 with pET-28a only).

cloned and expressed functionally in *E. coli* (Fig. 4). The ORF of this cDNA consisted of 1647 bp encoding a polypeptide of 548 amino acid residues. Phylogenetic analysis using deduced amino acid seq-

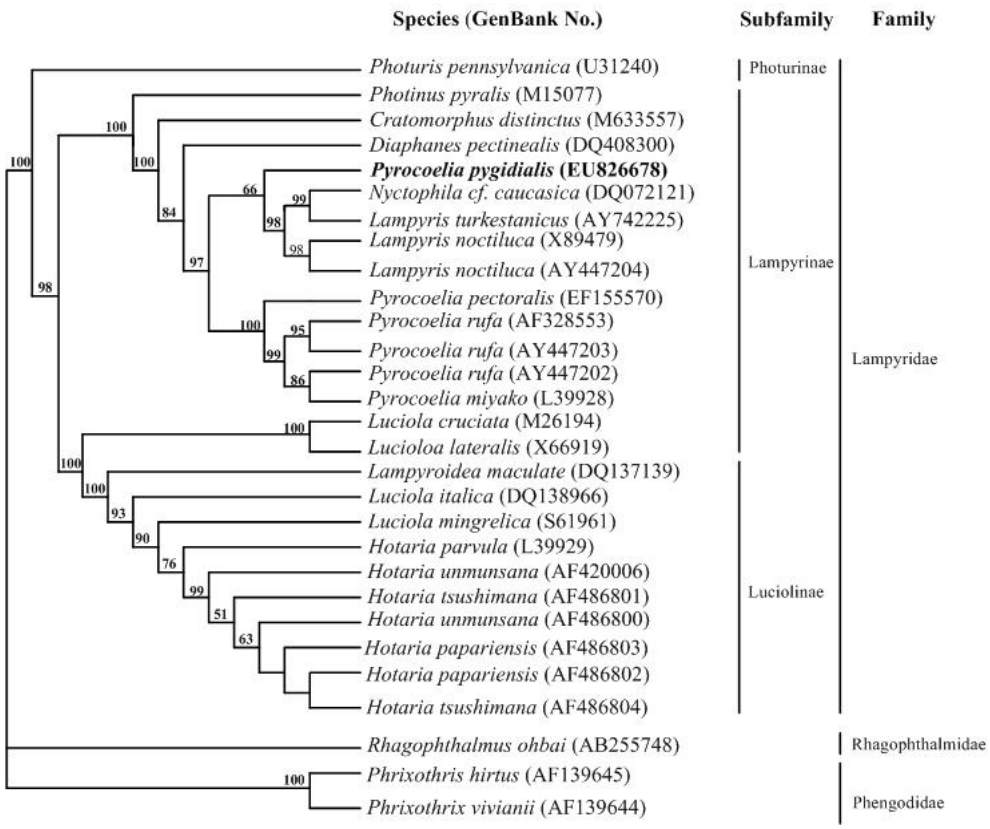

Fig. 5 A phylogenetic tree for aligned amino acid sequences of *Pyrocoelia pygidialis* luciferase and the known luciferase

The bootstrap values exceeding 50% were shown. *Rhagophthalmus ohbai* (Rhagophthalmidae), *Phrixothris vivianii* and *Phrixothris hirtus* (Phengodidae) were chosen as outgroups.

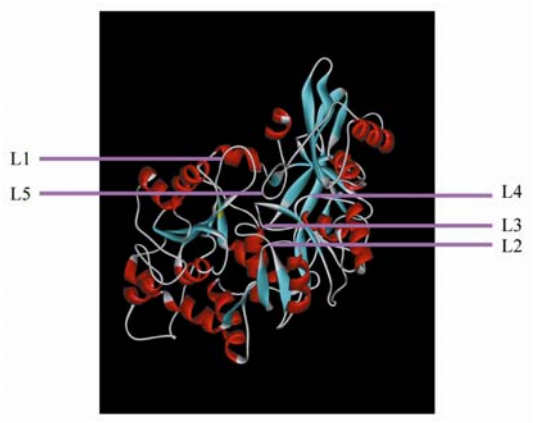

Fig. 6 Structural modeling of *Pyrocoelia pygidialis* Luciferase

The ribbon presentation of structural models were built using SWISS-MODEL server, based on crystal structure of *L. cruciata* (PDB code: 2d1sA). The α-Helixes are in red, the β-sheets are in blue, and the random coils are in grey. Five conserved loops (with purple line) located at the putative active area. The yellow position was the amino acid residue E210, while the correspondent position in other *Pyrocoelia* luciferases is aspartic acid. Note: E is glutamic acid.

uence showed that *P. pygidialis* did not form a monophyletic group with its congeneric species *P. rufa*, *P. pectoralis* and *P. miyako*, all three of which are strong luminous species. The analysis of 16S mitochondrial DNA sequences also supported *P. pygidialis*, together other weakly luminous congeneric species from Japan or China, constituted a separate group from those strong luminous taxa (Suzuki, 1997; Li et al, 2006b; Wang et al, 2007). *P. pygidialis*, with weakly luminous male adults, is considered a diurnal firefly male, deduced from the morphology of its photic organs, i.e., degenerate photic organ of two-spot on the 7th ventrite (Li et al, 2008).

Five peptide loops (Fig. 6) located in the *P. pygidialis* luciferase cleft area between the C- and N-terminal domains, the active site for the substrate compound is entering and binding (Conti et al, 1996; Ohmiya et al, 1996; Nakatsu et al, 2006). Thirteen different residues of the luciferases of *P. pygidialis* with other known luciferases of *Pyrocoelia* species were located on the surface of the luciferase molecule. Future studies on these loops, residues or crystal structure analysis may be helpful in understanding the real difference between the luciferases from diurnal and nocturnal species.

Due to the lack of luciferase sequences from other weak luminous *Pyrocoelia* species (Suzuki, 1997), it is still too early to say that which is primitive for weak and strong luminous species in the Asian genus *Pyrocoelia* (Li et al, 2008). Nevertheless, it seems that weak luminous species, with degenerate photic organs in morphology, possibly evolved from a separated lineage to those possessing strong luminous species.

Acknowledgements: We are grateful to Dr WANG Wen for his special support to this project. Thanks to all other colleagues of Dr WANG's Max-Plank group for assistances in the laboratory work, too.

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