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ORIGINAL PAPERS

Cloning and molecular properties of a novel luciferase from the Brazilian *Bicellonycha lividipennis* (Lampyridae: Photurinae) firefly: comparison with other firefly luciferases

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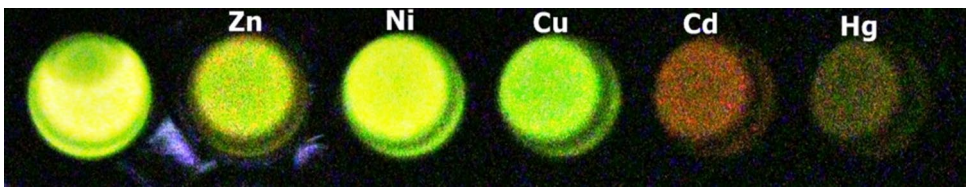
Abstract
Several firefly luciferases eliciting light emission in the yellow-green range of the spectrum and with distinct kinetic properties have been already cloned, sequenced, and characterized. Some of them are currently being applied as analytical reagents and reporter genes for bioimaging and biosensors, and more recently as potential color tuning indicators of intracellular pH and toxic metals. They were cloned from the subfamilies Lampyrinae (Photinini: *Photinus pyralis*, *Macrolampis sp2*; Cratomorphini: *Cratomorphus distinctus*), Photurinae (*Photuris pennsylvanica*), Luciolinae (*Luciola cruciata*, *L. lateralis*, *L. mingrelica*, *L. italica*, *Hotaria parvula*), and Amydetinae (*Amydetes vivianii*) occurring in different parts of the world. The largest number has been cloned from fireflies occurring in Brazilian biomes. Taking advantage of the large biodiversity of fireflies occurring in the Brazilian Atlantic rainforest, here we report the cloning and characterization of a novel luciferase cDNA from the Photurinae subfamily, *Bicellonycha lividipennis*, which is a very common firefly in marshlands in Brazil. As expected, multialignements and phylogenetic analysis show that this luciferase clusters with *Photuris pennsylvanica* adult isozyme, and with other adult lantern firefly luciferases, in reasonable agreement with traditional phylogenetic analysis. The luciferase elicits light emission in the yellow-green region, has kinetics properties similar to other adult lantern firefly luciferases, including pH- and metal sensitivities, but displays a lower sensitivity to nickel, which is suggested to be caused by the natural substitution of H310Y.

Graphical abstract



Keywords Luciferase · Firefly luciferase · Bicellonycha lividipennis · pH-sensitive luciferase · Metal sensitive luciferase

1 Introduction

Luciferases are the enzymes responsible for the catalysis of the bioluminescence reactions which culminates with the production of light with high efficiency [1–3] in organisms such as fireflies. Adult lantern firefly luciferases are pH-sensitive, displaying a bioluminescence color change from yellow–green to red at acidic pH, at higher temperatures and in the presence of heavy metals [4, 5]. Elateridae and

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Phengodidae luciferases emit a wider range of colors, but are not pH-sensitive [4].

Firefly luciferases have been extensively used as analytical reagents to detect ATP, biomass, enzymatic assays and cell viability [6–8]. The luciferases cDNAs have been applied as reporter genes in cell expression studies, bioimaging, biosensors [7, 8] and immunoassays [9]. More recently, firefly luciferases also recruited as ratiometric indicators of pH and heavy metals due to their spectral pH-sensitivity [10, 11]. The quantum yield and spectra of firefly luciferases were also investigated for metals such as zinc and cadmium on bioluminescence [12].

Several firefly luciferases eliciting light emission in the green–yellow range of the spectrum and with distinct kinetic properties have been already cloned, sequenced and characterized. They were cloned from the subfamilies Lampyrinae (Lampyrini; *Lampyris noctiluca* [13], *L. turkastenikus* [14]; Photinini: *Photinus pyralis* [15], *Macrolampis* sp2 [16], Cratomorphini: *Cratomorphus distinctus* [17] *Aspisoma lineatum* [18], Lamprohizinae (*Phausis reticulata* [19]), Photurinae (*Photuris* [20]), Luciolinae (*Luciola cruciata* [21], *L. lateralis* [22, 23], *L. mingrelica* [24], *L. itálica* [25], *Hotaria parvula* [26] and Amydetinae (*Amydetes vivianii* [27]) occurring in different parts of the world.

Most of them were cloned from the adult stage lanterns, emit light in the green-yellow range of the spectrum and are pH sensitive. On the other hand, only few luciferases from the larval stage were cloned and investigated, including those from larvae *P. pennsylvanica* [20], *C. distinctus* [17], *L. cruciata* [21], *L. lateralis* [22, 23], *Pyrocelia atrippenis* [28], *H. parvula* [26] and A. *lineatum* [18]. The firefly luciferases are classified into the following two isotypes: isotype 1 which is the same found in adult and larval lanterns, and isotype 2 which is found in the larval fat body and eggs. Whereas the isotypes from lanterns are pH-sensitive, display flash-like kinetics and are more efficient, the isotypes from fat body are pH-insensitive, display glow-type kinetic and are less efficient, leading to the conclusion that they are more primitive [18].

The three-dimensional structure has been solved for some firefly luciferases [29–32]. Structure–function studies identified residues of the luciferin binding site and residues affecting bioluminescence colors [33–42]. More recently we have identified the putative pH-sensing moiety and metal binding site of firefly luciferases, which involve two electrostatic couples, an external E311/R337 and an internal pair H310/E354, which close the bottom of the luciferin binding site, retaining the excited oxyluciferin-ejected proton near its phenolate group making a favorable environment for green light emission [43]. Comparison of the larval fat body and adult lantern luciferase of *A. lineatum* firefly indicate that the flexibilization of the protein scaffold and substitution at position 310 could be responsible differences in efficiency, kinetics and pH-sensitivity [18].

In Brazil we have already cloned the cDNA and characterized the luciferases from five firefly species and their larvae pertaining to distinct families and tribes, which display distinct BL colors ranging from green to yellow, and pH-sensitivities: (Amydetinae) *A. viviani* [44]; (Lampyrinae: Cratomorphini) *A. lineatum* AL1 and AL2 [18] and *C. distinctus* [17] and (Lampyrinae: Photinini) *Macrolampis* sp2 [11].

Here we report the cloning and characterization of a novel luciferase cDNA from the lanterns of a common marshland firefly species of the Photurinae subfamily occurring in Brazil, *Bicellonycha lividipennis* (Fig. 1) The cDNA and primary structure were compared to those of other firefly luciferases, and the recombinant enzyme was expressed in bacteria, purified, characterized and finally the kinetic, bioluminescence properties, pH- and metal-sensitivities were compared with those of other reported firefly luciferases.

2 Material and methods

2.1 Reagents

D-luciferin, ATP, PMSF, Triton X-100, Sodium chloride, Tris–HCl and sodium citrate were purchased from SIGMA (St.Louis, MI, USA). Dithiothreitol (DTT) and IPTG were purchased from AMBRESCO (Cleveland, OH, USA). Imidazole was purchased from Fisher (Hampton, NH, USA). Ni-agarose was purchased from QIAGEN. Restriction enzymes were purchased from Promega (Madison, WI, USA). Metal salts were purchased from Synth (Diadema,

Fig. 1 *Bicellonycha lividipennis* firefly female: (left) dorsal; (right) ventral

SP, Brazil). Ultrapure water was used for preparation of solutions and all assays.

2.2 Plasmids and cDNAs subcloning

Total RNA from lanterns of eight individuals of *B. lividipennis* was isolated with Trizol reagent and mRNA was purified using oligo-dT resin (Takara). cDNA library was constructed with the SuperScript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning (Life) and cloned into the pSPORT-1 vector. PCR cloning reactions were carried out using KOD-plus polymerase (Toyobo) in the following two steps: first, we used the primers BLampF1 (TCA TAG CAC GTC GAC TTA TAA TTT TGA TTT) and LampF2 (TCA TAG CAC GTC GAC TTA TAA TTT TGA TTT) and the universal T7 and SP6 primers, and the products were sequenced; second, based on the sequence results, we designed the 3′ (CCT GCA TTA CAT ATG GAA GAC AAA AAC) and 5′ (TCA TAG CAC GTC GAC TTA TAA TTT TGA TTT) luciferase primers containing, respectively, the NdeI and SalI sequence sites. The thermal cycling conditions for both reactions were as follows: 95 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min, for 30 cycles. The fragment was subcloned into the pColdII vector (Takara) and two independent clones were sequenced. Both displayed the same sequence and one of them was randomly selected to the downstream analyses.

2.3 Luciferase expression and purification

For luciferase expression, transformed *Escherichia coli* BL21(DE3) strain cells were grown in 100 mL of LB medium at 37 °C up to $OD_{600} = 0.4$ and then induced at 18 °C with 0.4 mM IPTG for 18 h. Cells were harvested by centrifugation at 2500*g* for 15 min at 4 °C and resuspended in 3 mL extraction buffer consisting of 50 mM sodium phosphate buffer pH 7,0, 300 mM NaCl, 10 mM imidazole, 5 mM DTT and protease inhibitor (1 mM PMSF). Cells were lysed by sonication (Mixonix) and centrifuged at 12,000 g for 15 min at 4 °C. The N-terminal histidine-tagged *B. lividipennis* luciferase was further purified by agarose-Nickel affinity chromatography followed by overnight dialysis on a buffer containing 25 mM Tris–HCl Buffer pH 8,0, 10% glycerol, 10 mM NaCl, 1 mM EDTA and 2 mM DTT. The enzyme purification was accompanied by electrophoresis on 10% SDS-PAGE and the estimated purity was about 80%.

2.4 Measurement of luciferase activity

Luciferase bioluminescence intensities were measured using a AB2200 luminometer (ATTO; Tokyo, Japan). The assays were performed by mixing 5 µL of 40 mM ATP/80 mM $MgSO_4$ with a solution consisting of 5 µL of luciferase and 90 µl of 0.5 mM d-luciferin in 0.10 M Tris–HCl pH 8.0 at 22 °C. The effect of heavy metals on the activity of *B. lividipennis* was measured by adding 10 µL of heavy metal salt ($AgNO_3$, $CdSO_4$, $HgCl_2$, $NiSO_4$, $PbCl_2$ and $ZnSO_4$ at 20 mM) in the assay solution. All measurements were done in triplicate of three independent luciferase preparations and averages with standard deviations were reported.

2.5 Kinetics measurements and KM determination

The K_M assays for luciferin were performed by mixing 5 µL of 40 mM ATP/80 mM MgSO4 in a solution containing 5 µL of luciferase, 85 µL of 0.10 M Tris–HCl (pH 8.0) and luciferin at final concentrations between 0.006 and 0.5 mM. The K_M assays for ATP were performed by mixing 5 µL of 80 mM $MgSO_4$ in a solution containing 5 µL of luciferase, 85 µL of 0.5 mM luciferin in 0.10 M Tris–HCl (pH 8.0) and ATP at final concentrations in the range of 0.0006–2 mM. Both assays were performed in triplicate. The K_M values were calculated using Lineweaver–Burk plots taking the peak of intensity (I_0) as a measure of V_0. All measurements are the result of three independent purifications and each luciferase assay was measured in triplicate.

2.6 Determination of k_{cat}

The overall k_{cat} and were determined by calculating the ratio of luminescence activities in *cps* by the number of luciferase molecules based on the specific bioluminescence activities measured with luciferin and ATP (overall k_{cat}). All measurements are the result of three independent purifications and each luciferase assay was measured in triplicate. Because the absolute value of *cps* in photons. s^{-1} could not be determined, the absolute values of k_{cat} in s^{-1} could not be determined, and, therefore, the values were reported in cps (counts for second). Although these values are not absolute, they can be safely used as relative values of catalytic constants.

2.7 pH profile

To determine the optimal pH, the assays were performed with a solution containing 5 µL of a 40 mM ATP and 80 mM MgSO4, 5 µL of 10 mM D-Luciferin, 5 µL of luciferase and 85 µL of buffer. To vary the pH of the enzymatic assay, four different buffers were used: (0.1 M sodium citrate) pH 5.0; 5.5–6.0; (0.1 M sodium phosphate) pH 6.0–8.0; (0.1 M Tris–HCl) pH 8.0–9.0 and (0.1 M CHES pH 9.0–10.0). The activities in different buffers were corrected at intersection points of pH.

2.8 Thermostability

The recombinant *B. lividipennis* luciferase was separated in three aliquots at the final concentration of 0.15 mg/mL and incubated at three different temperatures 4 ºC, 22 ºC or 37 ºC. The bioluminescence activity was measured at different times (0 h; 1 h; 2 h, 3 h; 4 h, 5 h, 6 h; 12 h; 24 h e 48 h). The experiments were reproduced three times with luciferase from independent preparations, the average with standard deviations were reported.

2.9 Bioluminescence spectra

Bioluminescence spectra reported here were recorded in ATTO Lumispectra spectroluminometer (Tokyo, Japan) with a cooled CCD camera. In the assay tube, 5 µL of luciferases were mixed with 85 µL of 0.10 M Tris–HCl buffer pH 8.0, 5 µL of specific substrate (10 mM d-luciferin), and 5 µL of 40 mM ATP/80 mM $MgSO_4$. The effect of heavy metals on the bioluminescent spectra of *B. lividipennis* luciferase was assayed by adding 10 µL of metal salt ($AgNO_3$, $CdSO_4$, $HgCl_2$, $NiSO_4$, $PbCl_2$ and $ZnSO_4$ at 20 mM) in the assay solution. Each spectrum was the result of three independent measurements done for three independent luciferase preparations. The estimated peak error was ± 2.5 nm.

2.10 Bioinformatic analysis

Sequence editing was performed in Bioedit 7.0 [45] and protein features analysis (PI and MW) was predicted using ProtParam [46]. We performed a search for Coleoptera luciferase and luciferase-like protein in the NCBI repository (Fig. 4), using the *Drosophila CG6178* amino acid sequence as an outgroup. Multialignment of primary structures of downloaded enzymes plus *B. lividipennis* luciferase was conducted in MAFFT v.7.453 [47] and ClustalOmega. Phylogenetic analysis was carried out using the software MrBayes v3.2.6 [48] and IQiTree2 [49]. IQ-Tree2 was also employed to evaluate the amino acid substitution model (LG + I + G4). In MrBayes, we conducted two independent runs, with four chains and 10,000,000 generations, sampling trees every 1000 generations. The first 25% of trees were discarded and the remaining trees were concatenated to create the consensus tree. In IQtree, we used 1000 ultrafast bootstraps.

3 Results and discussion

3.1 cDNA structure and protein sequence

The ORF sequence of *B. lividipennis* firefly luciferase has 1638 bp that encodes 545 amino acids (Fig. 2). The estimated molecular weight is 60.4 kDa and theorical pI 6.3. As expected, this firefly luciferase protein also has the peroxisomal targeting tripeptide signal, Serine–Lysine–Leucine (SKL), at the C-terminus, which drives the protein to the peroxisomes [50–52] (Gould et al. [50–52]). The close relative *Photuris pennsylvanica* luciferase isotype (BAA05006.1) has the peroxisomal tripeptide AKL (Fig. 3). Noteworthy, when compared to other firefly luciferases, the primary structure is shorter than those of *Photuris pennsylvanica* isozymes (552 residues—BAA05006.1 and BAA05005.1) and other adult lanterns luciferases of Lampyrinae (547–550 residues) and Luciolinae (548).

3.2 Phylogenetic analysis

As expected, the phylogenetic reconstructions based on adult firefly luciferases using the Bayesian and Maximum-Likelihood (ML) approaches, reconstructed quite well the traditional phylogeny of Lampyridae. The luciferase of *B. lividipennis* was clustered with the close relative *Photuris pennsylvanica* adult isoenzymes (BAA05005.1, and BAA05006.1) from the same subfamily, displaying 72.7 and 73.4% identity, with high branch support to the monophyly of the Photurinae subfamily. Next, it clustered near *Amydetes viviani* luciferase from the Amydetinae subfamily (Fig. 4), consistently with the molecular phylogeny based on mitochondrial and nuclear genes [53]. However, the relationship among sister clades is still unsolved, displaying distinct relationships using different methods (sister clade of Amydetinae subfamily in ML approach; sister clade of Photurinae subfamily in Bayesian approach (data not shown). New luciferase sequences from closely Lampyridae taxa (e.g., genus *Pyrogaster* and *Psilocladus*) may improve the resolution power to these closely related tribes/subfamilies.

On the other hand, the lantern firefly luciferases (isotypes 1), including *B. lividipennis* luciferase, clustered in a separate group from the respective larval fat body isozymes (isotypes 2). Indeed, the luciferase of adult lantern *B. lividipennis* has a considerably higher identity (74.0–78.0%) with luciferases from the adults of the Lampyrinae and Amydetinae subfamilies, including *Lampyris, Pyrocoelia, Cratomorphus, Lucidina, Macrolampis, Photinus*, and *Amydetes,* than with the larval/eggs luciferases isozymes from the close relative *P. pennsylvanica* (PPU31240,) of the same Photurinae subfamily, which displayed 56.8% identity. This is expected because the luciferase isozymes from the larval fat body, which are pH-insensitive, and those from the lanterns, which are pH-sensitive, are paralogous.

3.3 Expression and purification

The recombinant firefly luciferase was expressed in *E. coli* and purified by nickel-agarose chromatography. Part of enzyme was retained in the insoluble fraction after cell

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   1  M  E  D  K  N  I  K  T  G  P  P  P  F  Y  P  L  E  Q  G  T  A  G  E  Q  L
   1 ATGGAAGACAAAAACATCAAGACGGGACCTCCTCCATTCTATCCTTTAGAACAAGGAACTGCAGGAGAACAATTG
  26  H  K  A  M  K  R  Y  A  E  Q  P  E  A  L  V  F  T  D  A  H  T  E  F  E  F
  76 CACAAAGCTATGAAAAGATATGCCGAACAACCGGAAGCTCTTGTATTTACAGATGCTCACACTGAATTTGAATTT
  51  T  Y  K  E  Y  F  S  L  S  C  R  L  A  E  S  L  K  R  H  G  L  G  L  Q  H
 151 ACATATAAGGAATACTTTTCACTGTCTTGTCGTTTAGCAGAAAGTTTGAAAAGGCATGGATTAGGTTTGCAACAT
  76  R  I  A  V  C  S  E  N  C  A  Q  F  F  I  P  V  C  G  A  L  F  I  G  V  A
 226 AGAATTGCTGTCTGCAGTGAAAATTGTGCTCAGTTTTTTATTCCTGTTTGCGGTGCATTATTTATAGGAGTTGCT
 101  V  A  P  T  N  D  I  Y  N  E  R  E  L  F  N  S  L  N  I  S  Q  P  N  I  V
 301 GTTGCACCCACCAATGATATTTACAACGAACGTGAATTATTCAACAGTTTAAATATTTCACAACCAAATATAGTA
 126  F  T  S  R  R  S  L  Q  K  I  V  G  V  Q  S  R  V  P  A  I  K  K  I  C  I
 376 TTTACTTCAAGAAGATCATTACAAAAAATTGTAGGAGTGCAATCAAGAGTACCTGCAATTAAAAAAATTTGTATA
 151  L  D  S  K  K  D  Y  M  G  Y  Q  S  M  Y  S  F  M  K  T  H  V  P  A  D  F
 451 TTAGATTCCAAAAAAGACTACATGGGATATCAATCTATGTACTCATTTATGAAAACCCACGTTCCCGCCGACTTC
 176  D  A  Q  K  F  V  P  E  T  F  D  V  M  H  T  A  L  Y  M  N  S  S  G  S  T
 526 GACGCTCAAAAATTTGTACCAGAAACATTTGACGTTATGCACACAGCACTTTACATGAATTCCTCAGGTTCTACG
 201  G  L  P  K  G  V  K  L  S  H  Q  N  L  I  V  R  F  S  H  C  R  D  P  V  F
 601 GGATTACCAAAAGGTGTAAAGCTTTCCCACCAAAATCTCATAGTCAGATTTTCTCATTGCAGAGATCCTGTGTTT
 226  G  N  Q  I  I  P  D  T  S  I  L  S  L  V  P  F  H  H  A  F  G  M  N  T  T
 676 GGCAATCAGATTATTCCAGATACTTCTATTTTATCACTTGTACCGTTTCATCATGCTTTTGGCATGAATACAACA
 251  L  G  Y  L  I  C  G  F  H  I  V  L  M  Y  R  F  E  E  E  L  F  L  R  S  L
 751 TTGGGATATTTAATATGTGGATTCCACATAGTGCTTATGTATAGATTTGAAGAAGAATTATTTTTACGATCACTT
 276  Q  D  Y  K  I  Q  S  T  I  L  V  P  T  V  L  S  F  L  S  K  S  Q  E  V  E
 826 CAAGATTACAAAATTCAAAGTACAATATTAGTTCCTACGGTATTATCATTTTTATCTAAAAGCCAAGAAGTTGAA
 301  K  Y  D  L  S  N  L  Y  E  I  A  S  G  G  A  P  L  A  R  E  I  G  E  A  A
 901 AAATATGATTTATCAAATTTGTATGAAATTGCTTCTGGTGGTGCACCACTTGCAAGGGAAATTGGAGAAGCAGCA
 326  A  K  R  F  N  L  P  G  I  R  Q  G  F  G  L  T  E  T  T  S  A  F  I  I  T
 976 GCAAAACGTTTTAACCTACCTGGAATACGTCAAGGTTTCGGGCTTACAGAAACTACTTCAGCTTTTATAATTACT
 351  A  E  G  D  D  K  P  G  A  V  G  K  V  V  P  F  M  S  V  K  V  V  D  L  D
1051 GCTGAAGGAGATGATAAACCGGGTGCAGTAGGAAAAGTTGTTCCATTTATGTCTGTAAAAGTTGTTGATCTGGAC
 376  T  G  K  T  L  G  V  N  E  K  G  E  I  C  A  K  G  P  M  L  M  K  G  Y  V
1126 ACGGGTAAAACTTTGGGTGTAAATGAAAAAGGTGAAATATGTGCTAAAGGTCCCATGCTAATGAAAGGATACGTC
 401  D  N  P  E  A  T  A  A  L  I  D  K  D  G  W  L  H  S  G  D  I  G  Y  W  D
1201 GATAACCCAGAAGCTACAGCTGCACTGATTGACAAAGATGGGTGGTTACACTCTGGTGATATAGGATATTGGGAC
 426  E  D  G  H  F  F  I  V  D  R  L  K  S  L  I  K  Y  K  G  Y  Q  V  P  P  A
1276 GAAGATGGTCATTTCTTCATTGTTGATCGCTTAAAATCATTGATTAAATATAAGGGTTATCAAGTACCACCTGCA
 451  E  L  E  S  I  L  L  Q  H  P  L  I  F  D  A  G  V  A  G  V  P  D  V  E  A
1351 GAATTGGAGTCAATTTTGTTACAACATCCGTTAATTTTTGATGCAGGAGTTGCGGGTGTTCCTGATGTAGAAGCG
 476  G  E  L  P  A  A  V  V  V  L  E  E  G  K  T  M  T  E  Q  E  V  V  D  Y  V
1426 GGTGAACTTCCTGCGGCTGTTGTTGTTTTAGAAGAAGGAAAAACTATGACAGAACAAGAAGTTGTAGATTACGTA
 501  A  S  Q  V  T  S  S  K  K  L  R  G  G  V  K  F  V  D  E  V  P  R  G  L  T
1501 GCTTCGCAAGTGACTTCTTCAAAAAAGTTACGTGGCGGTGTCAAATTTGTGGACGAAGTACCAAGAGGTCTAACT
 526  G  K  I  D  T  R  K  I  K  E  I  L  T  K  A  R  K  S  K  L  *
1576 GGAAAAATTGATACAAGAAAAATTAAAGAAATTCTTACTAAAGCAAGGAAATCAAAATTATAA
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Fig. 2 Nucleotide and amino acid sequence of *B. lividipennis* firefly luciferase. * represents a stop codon

lysis. As expected, the N-terminal histidine-tagged luciferase has great affinity to nickel column and almost all enzyme is retained in the nickel with little loss in the washout fraction. The expression and purifications were followed by SDS-PAGE (Fig. 5). The average concentration of purified luciferases was around 0.2 mg/L of bacterial culture, which was quite lower than other firefly luciferases such as *Amydetes viviani* which were expressed and purified by the same procedures [44]. The specific bioluminescence activity of *B. lividipennis* luciferase, however, is comparable to other firefly luciferases expressed in our laboratory (Table 1).

3.4 Kinetic constants

The KM values for ATP and for luciferin were 277 µM and 46 µM, respectively (Table 1). These values are higher than the KM values reported for other adult firefly luciferases, including *Photuris pennsylvanica* Ppe2 isozyme (luciferin $K_M = 46$ µM), with the exception of the K_M values for ATP for *P. pyralis* firefly luciferase (250 µM) and *P. hirtus* railroadworm (230 µM). Similar high K_M values for luciferin were reported for the *P. termitiluminans* larval

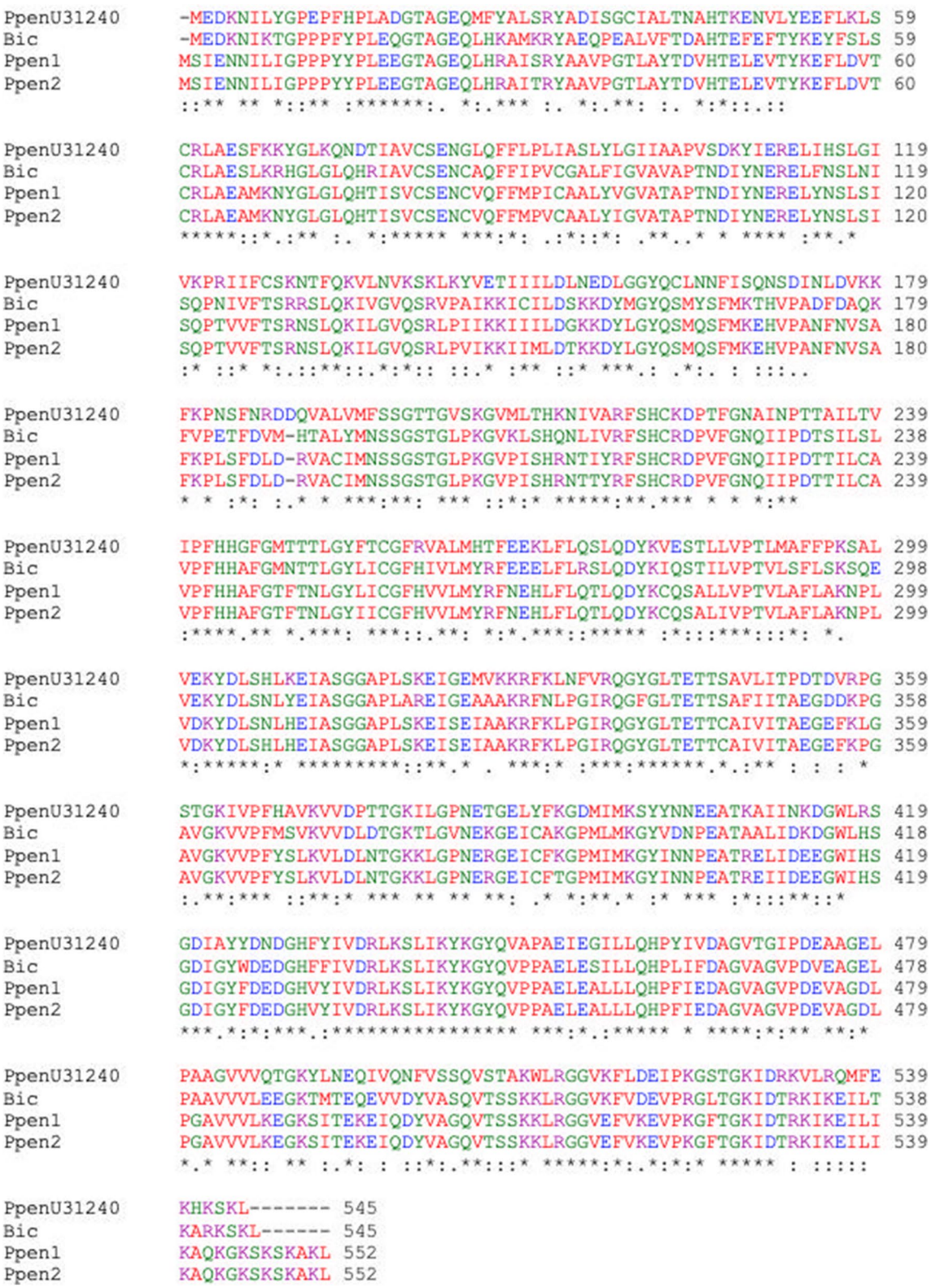

Fig. 3 Multialignement of firefly luciferases from *Photuris pennsylvanica* larval/eggs stages (PpenU31240—PPU31240), adult isoenzymes (Ppen1—BAA05006.1, Ppen2—BAA05005.1) and *B. lividipennis* adult lanterns (Bic). (Red) apolar; (Green) polar neutral; (Blue) negatively charge; (Pink) positively charged

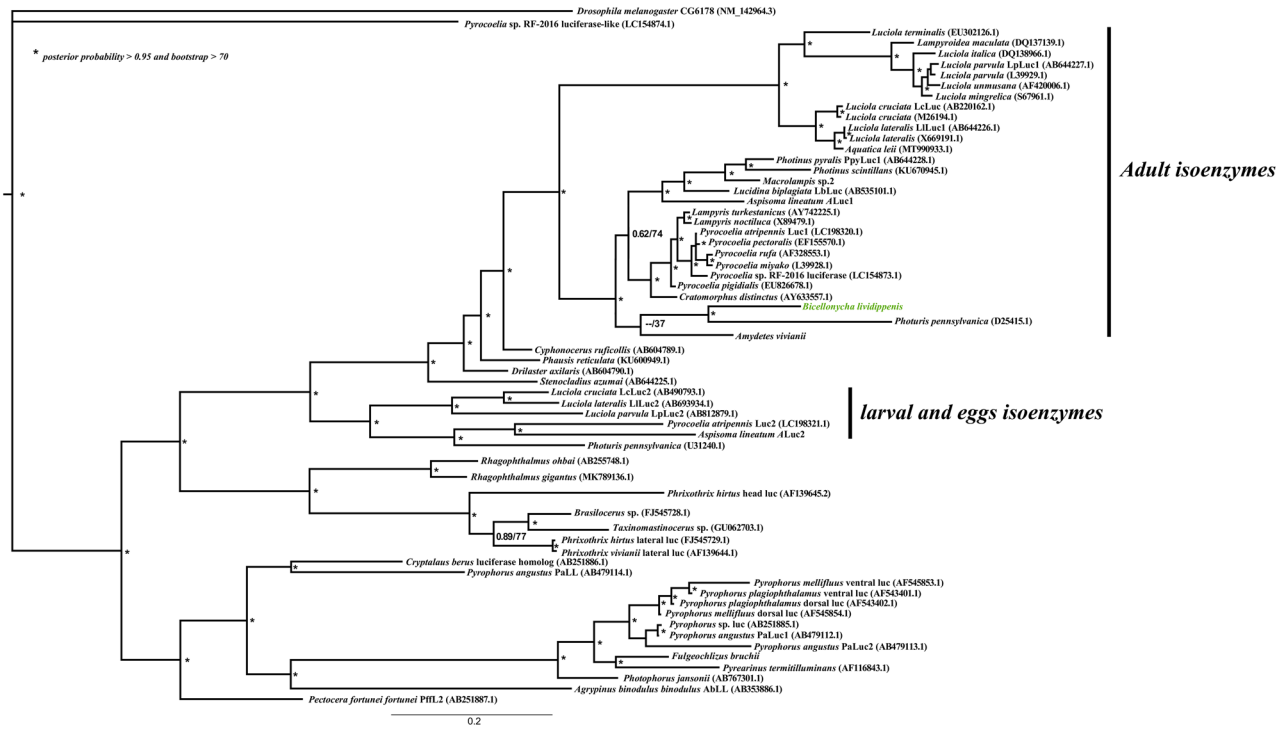

Fig. 4 Phylogenetic tree of firefly luciferases showing the relationship of *B. lividipennins* luciferase (in green). The asterisk in the node represents high supported branches in both approaches

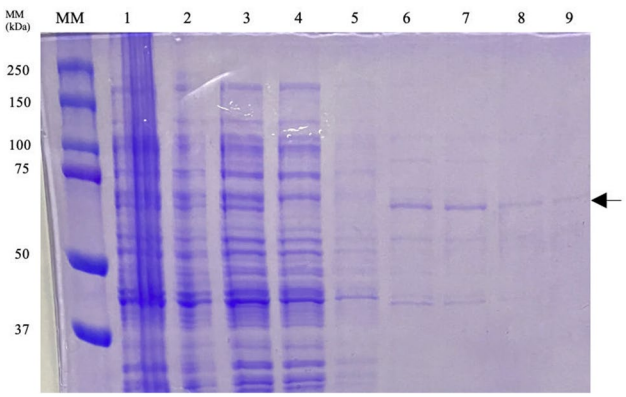

Fig. 5 SDS-PAGE analysis of recombinant *B. lividipennis* firefly luciferase expression and purification. MM: Molecular marker—precision plus protein dual-color standards (Bio-rad). 1: Crude extract; 2: Cell lysate-insoluble fraction; 3: Cell lysate-soluble fraction; 4: Outflow – proteins that did not bind to nickel column; 5: Washout; 6–9: Elution fraction with 250 mM imidazole. Arrow indicates the *B. lividipennis* firefly luciferase

click beetle luciferase (80 µM) and for *Phrixotrix viviani* railroadworm green emitting luciferase (64 µM).

We determined and compared the catalytic constants and efficiencies of *B. lividipennis* firefly luciferase with other known cloned beetle luciferases (Table 1). The luciferase of *B. lividipennis* displayed similar *k*cat to other firefly luciferases. The catalytic efficiencies for *B. lividipennis* luciferase, however, were smaller than those reported for other firefly luciferases, due to high values of KM for luciferin and ATP. The catalytic efficiency for ATP is similar to that of *A. lineatum* larval fat body luciferase [44] and to *P. termitilluminans* larval click beetle luciferase.

3.5 pH profile

Firefly luciferases have optimum pH close to 8.0. The luciferase of *B. lividipennis* firefly has an optimum pH close to 7.5, with a wider curve with high activity (more than 60%) between pH 7.0 and 9.0 (Fig. 6a). Similar profile was detected for other firefly luciferases, such as *A. viviani* luciferase [44] and *A. lineatum* pH-sensitive luciferase [18].

3.6 Thermostability

The thermostability of *B. lividipennis* luciferase was analyzed at 4 °C, 22 °C and 37 °C. The enzyme is quite unstable, retaining only about 60% activity after 48 h at 4 °C and 22 °C, and losing about 90% of its activity during 1 h at 37 °C (Fig. 6b).

Table 1 Comparative bioluminescence and kinetic properties of *B.lividipennis* firefly luciferase with some other Brazilian firefly and beetle luciferases

Luciferase	λmax (nm) [half-band][a]	Specific act (10^9 cps/mg)	K_M (μM)		k_{cat}[b] (10^{-6} c s^{-1}) [SD]	k_{cat}/K_M [SD]		References
			ATP	LH_2		ATP	LH_2	
Lampyridae								
Amydetinae								
Amydetes viviani	547 [81]	890	9	9	109	12.1	12.1	[27, 44]
Lampyrinae photinini								
Macrolampis sp2	575 [86]	1198	83	20	125	1.5	6.25	[16]
Photinus pyralis	567 [81]	1037	250	5	116	0.46	23	[15]
Cratomorphini								
Aspisoma lineatum (adult lantern)	573 [88]	693	4	7	69 [±7]	11 [±3.2]	9.8 [±3.6]	[18]
Aspisoma lineatum (fat body)	561 [88]	40	7	3	6 [±1.5]	0.85 [±0.5]	2 [±0.9]	[18]
Cratomorphus distinctus	550	–	–	15	–	–	–	[17]
Photurinae								
Bicellonycha lividipennis	567	10.5	277	46	212	0.76	4.59	
Photuris pennsylvanica (Ppe1)	538	–	–	20	–	–	–	[54]
Click beetles								
Pyrearinus termitilluminans	546 [87]	290	370	80	29	0.78	0.3625	[55]
Phengodidae								
Phrixothrix hirtus	626 [82]	70	230	7	8.3	0.04	1.2	[56]
Phrixotrix vivianii	558 [89]	37.9	330	64	3.79	0.011	0.059	[56]

[a]The peak estimated error was ±2.5 nm

[b]The overall catalytic constant was calculated from the total light intensity in counts per second of the bioluminescence reaction starting with ATP and D-luciferin

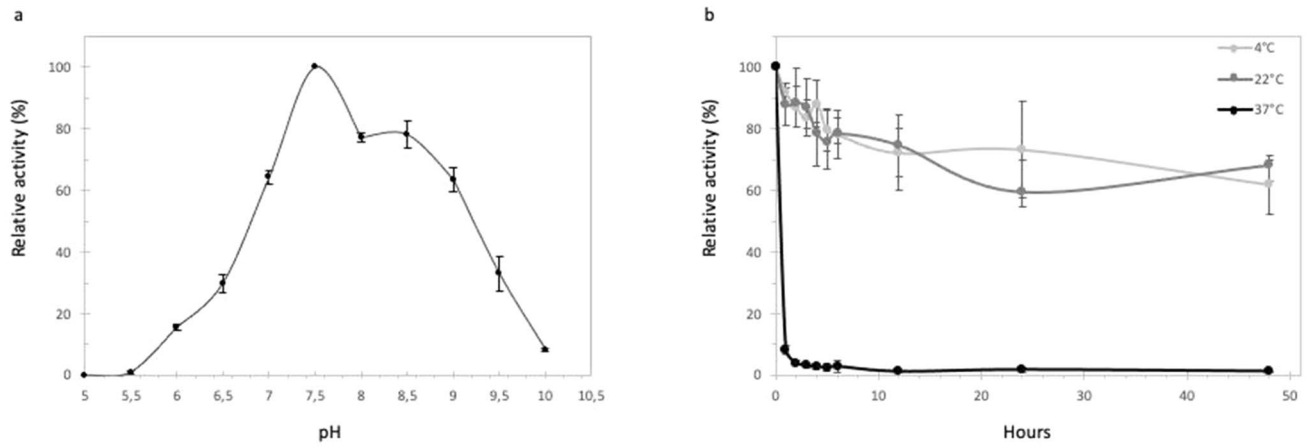

Fig. 6 pH and thermostability profiles of *B. lividipennis* firefly luciferase activity: **a** pH profile; **b** effect of temperature on the enzyme stability

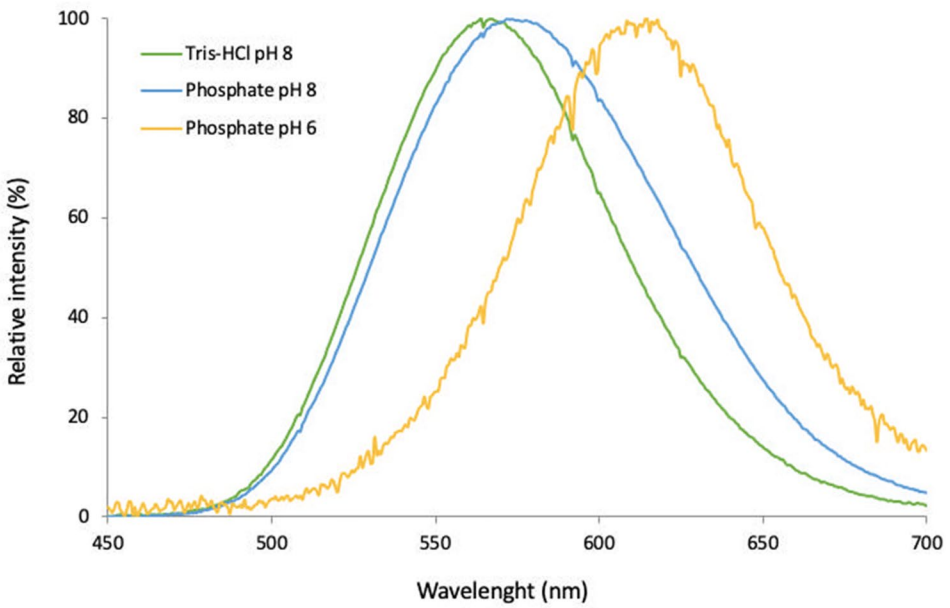

Fig. 7 Bioluminescence spectra for *B. lividipennis* firfly luciferase at different pH

3.7 Bioluminescence spectra and pH-sensitivity

The *B. lividipennis* luciferase emits yellow–green light with an emission peak at ~ 567 nm at pH 8.0 and, as expected, was pH-sensitive, shifting the spectrum to the red region with a peak at 614 nm at pH 6.0 (Fig. 7).

3.8 Effect of heavy metals

Such as in the case of other firefly luciferases, heavy metals such as cadmium, mercury, lead and zinc promoted the green–red change of the bioluminescence spectrum of *B. lividipennis* luciferase. Among the metal ions tested here, however, nickel was the only metal that did not shift the spectrum (Figs. 8, 9).

3.9 Structural and functional relationships

The luciferase of *B.lividipennis* firefly falls within the lantern yellow–green emitting luciferases group. It displays flash-like kinetics, similar optimum pH to other firefly luciferases, and pH- and metal-sensitivities. It is noteworthy that this adult lantern luciferase has the same length of the larval *P. pennsylvanica* firefly luciferase but is 7 residues shorter than adult *P. pennsylvanica* alozyme. Even then, *B.lividipennis* firefly luciferase has some structural

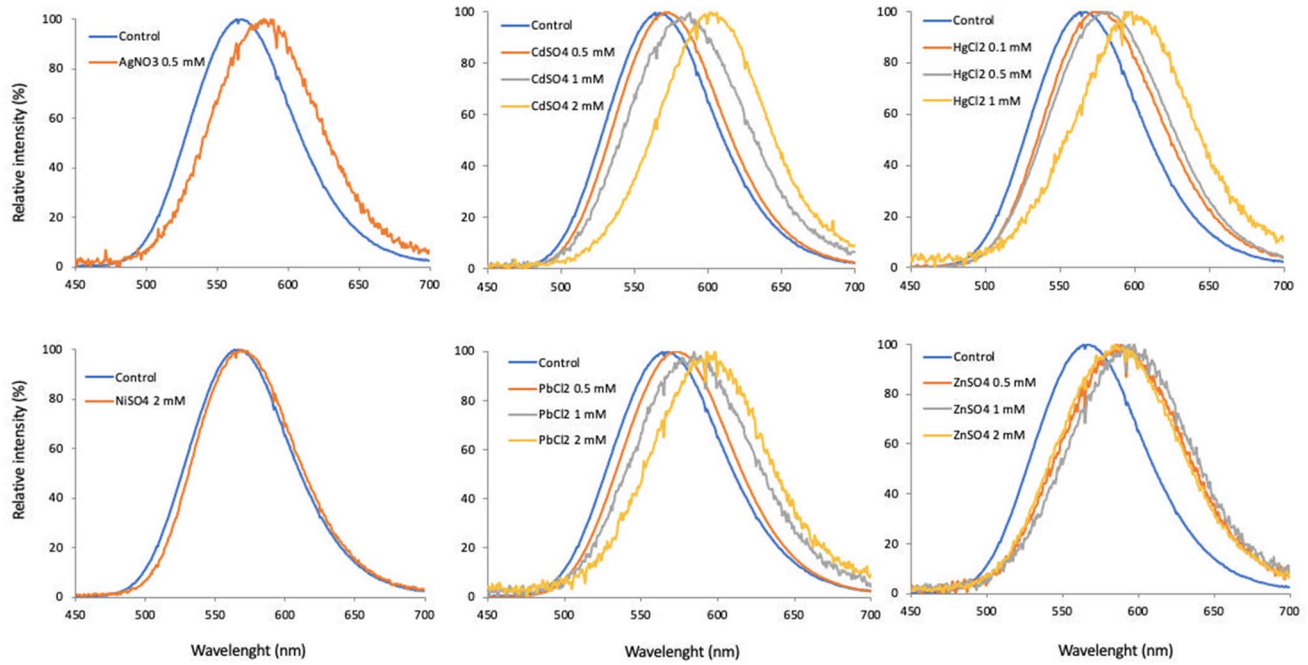

Fig. 8 Effect of distinct metals in different concentrations on the bioluminescent spectra of *B. lividipennis* firefly luciferase

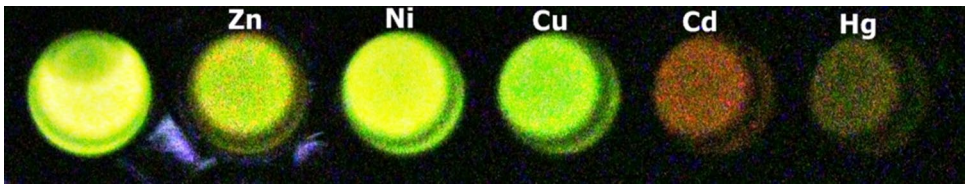

Fig. 9 Effect of different metals on *Bicellonycha lividipennis* firefly luciferase bioluminescence color, showing the distinct sensitivities to different metals

Fig. 10 Alignment of firefly luciferases showing the luciferin binding site and color modulating residues (yellow shadow) and pH- and metal-sensitive sites (gray and blue shadows): (Bic) *Bicellonycha lividipennis* lantern luciferase; (PpenA) *Photuris pennsylvanica* adult lantern luciferase and (PpenL) larval pH-insensitive luciferase; (Me^{2+}) binding metal (Zn, Cd, Hg)

features important for pH-sensitivity more similar to those of the adult alozyme of *P. pennsylvanica* and other firefly luciferases which are pH-sensitive. Among these features there are more similar substitutions in the loop 223–235 and more conserved luciferin binding site motif 241–246 (Fig. 10). The pH-insensitive larval isozyme of *P. pennsylvanica*, on the other hand, differs from the *B. lividipennis* luciferase by substitutions in the loop 223–235. The loop 223–235 was previously shown to be important for BL color in different beetle luciferases [40]. This luciferase

also displays I241 instead of V241. The presence of the larger I241 was shown to be important to stabilize the luciferin binding pocket in more blue-shifted and less sensitive luciferases [35]. Furthermore, in *P. pennsylvanica* pH-insensitive luciferase the position 310 has lysine (K310) instead of histidine, and D354 instead of E354. The substitutions at positions 310 and 354 were shown to be important for pH- and metal sensitivities [11, 16, 40].

Indeed, one of the interesting differences which may explain the natural lower spectral sensitivity of this luciferase to Nickel in relation to other metals, when compared to other metal-sensitive luciferases, is the substitution of the usual histidine at position 310 by Tyrosine (Y310). Whereas previous studies showed that histidine at this position is important for Nickel binding [11], the presence of Tyrosine at this position in a firefly luciferase is unprecedent. It is possible that the hydroxyl group of tyrosine may not coordinate Nickel as well as other divalent heavier metals.

4 Concluding remarks

The luciferase from *B. lividipennis* firefly was cloned. It is more similar to other adult lantern firefly luciferases, especially to the adult isozyme of *Photuris pennsylvanica* also from the Photurinae subfamily, agreeing with the traditional phylogeny of Lampyridae subfamilies. It shows similar kinetic properties to other firefly luciferases, emitting in the yellow–green region instead of green, and being pH-sensitive. However, whereas the bioluminescence spectrum is sensitive to zinc, cadmium and mercury, it is not sensitive to Nickel. The lower metal sensitivity to nickel is thought to be caused by the substitution H310Y in the metal binding site.

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Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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