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Supplementary Materials for

Resurrecting the ancient glow of the fireflies

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(available at advances.sciencemag.org/cgi/content/full/6/49/eabc5705/DC1)

Tables S1, S3 and S5
PDB Summary Validation Report 6K4C
PDB Summary Validation Report 6K4D

Supplementary Materials

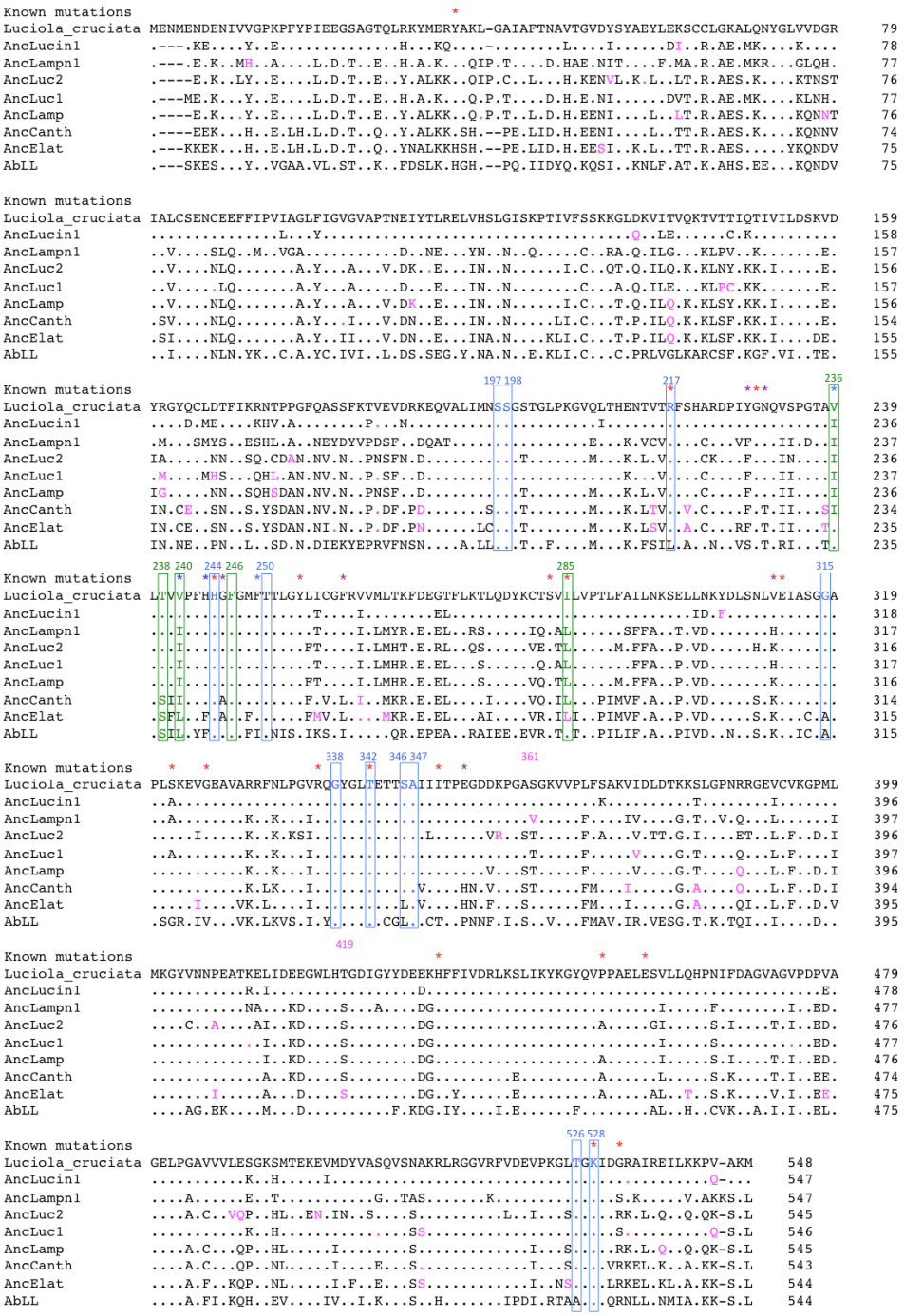

Fig. S1. Multiple-sequence alignment of ancestral luciferases. The amino acid residues of the ancestral luciferases, which are identical to *L. cruciata* LcLuc1, are

shown as a dot. The substrate-binding site residues (Ser^{197}, Ser^{198}, Arg^{217}, His^{244}, Thr^{250}, Gly^{315}, Gly^{338}, Thr^{342}, Ser^{346}, Ala^{347}, Thr^{526}, and Lys^{528} reference the positions in AncLamp) (*7*) are outlined in blue. The sites relevant to the AncLamp luminescence spectra predicted by the crystal structure (Ile^{236}, Thr^{238}, Ile^{240}, Phe^{246}, and Leu^{285}) are outlined in green (shown on structure in Fig. 3). The more ambiguously identified sites (posterior probability less than 0.1 higher than the second most probable amino acid) are shown in magenta (depicted on structures in fig. S3). The 'know mutations' lines indicate the reported site-directed mutation sites (listed in table S5) that cause luminescence wavelength shifts. The residue sites related to hypsochromic-, bathochromic-, and both-shift are indicted with asterisks in blue, red, and purple, respectively. AbLL, *A. binodulus* fatty acyl-CoA synthetase.

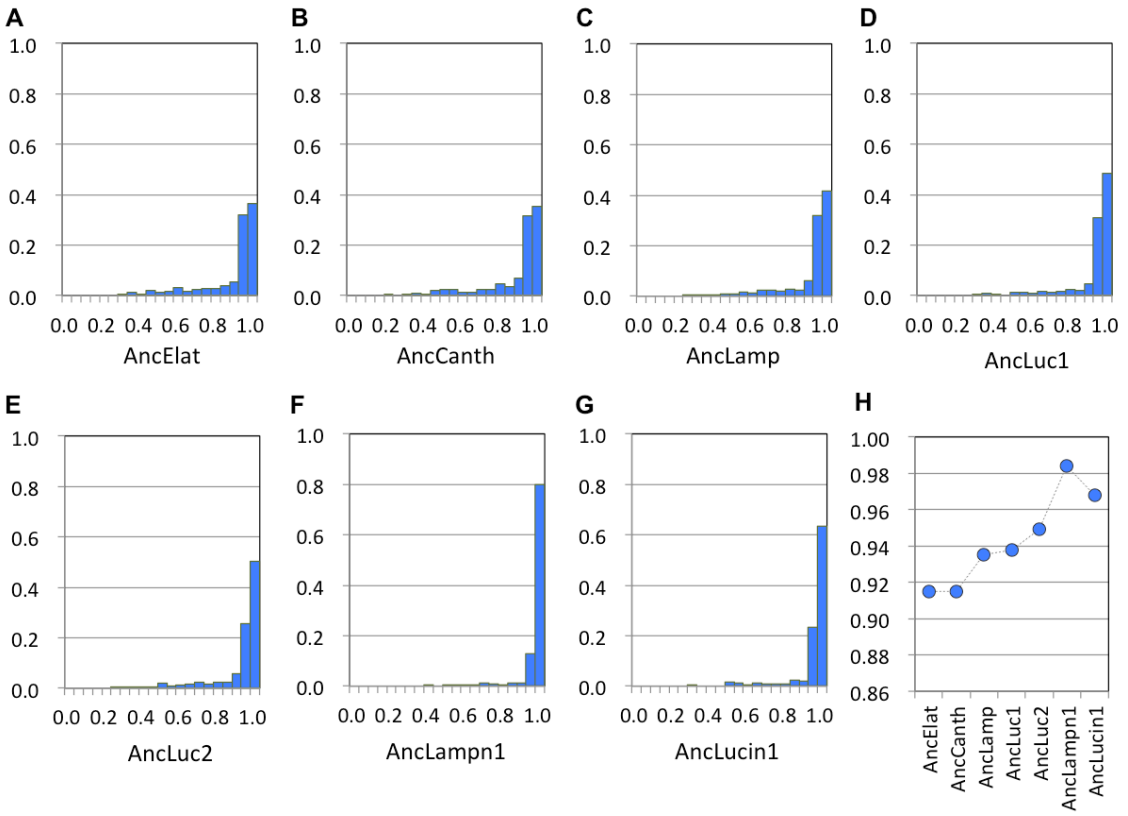

Fig. S2. Posterior probability distributions of the residue sites. (**A**) AncElat, (**B**) AncCanth, (**C**) AncLamp, (**D**) AncLuc1, (**E**) AncLuc2, (**F**) AncLampn1, and (**G**) AncLucin1. Horizontal and vertical axes show posterior probability bins and fractional frequency of inferred sites, respectively. (**H**) Average posterior probabilities are plotted over the ancestral luciferases.

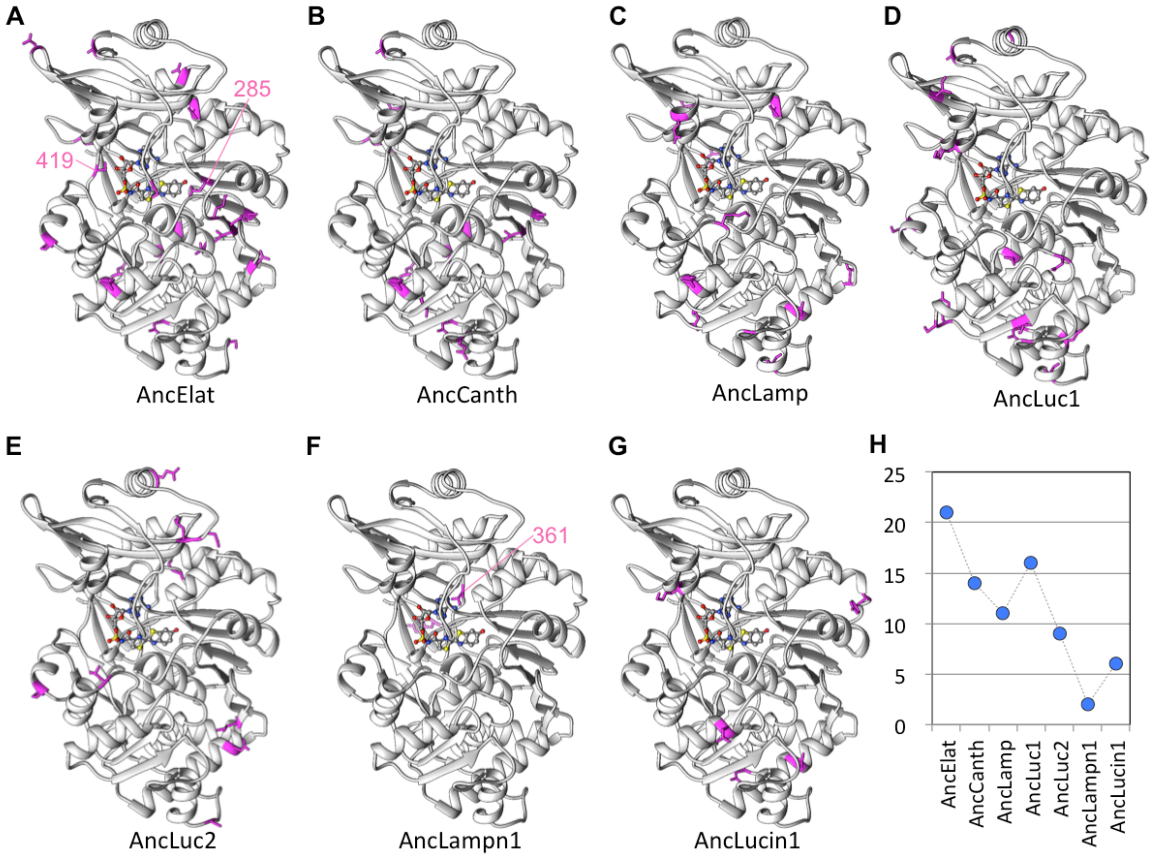

Fig. S3. Ambiguous residues. The residues, for which the posterior probability is less than 0.1 higher than the second most probable amino acid (listed in table S1), are mapped in magenta for (**A**) AncElat, (**B**) AncCanth, (**C**) AncLamp, (**D**) AncLuc1, (**E**) AncLuc2, (**F**) AncLampn1, and (**G**) AncLucin1 on the crystal structure of AncLamp. The sites that might have direct interaction with the substrate-binding site residues, namely Val^{361} in AncLampn1, and Leu^{285} & Ser^{419} in AncElat (also indicated in fig. S1) are denoted with residue number. (**H**) Total numbers of ambiguous residues are plotted over the ancestral luciferases.

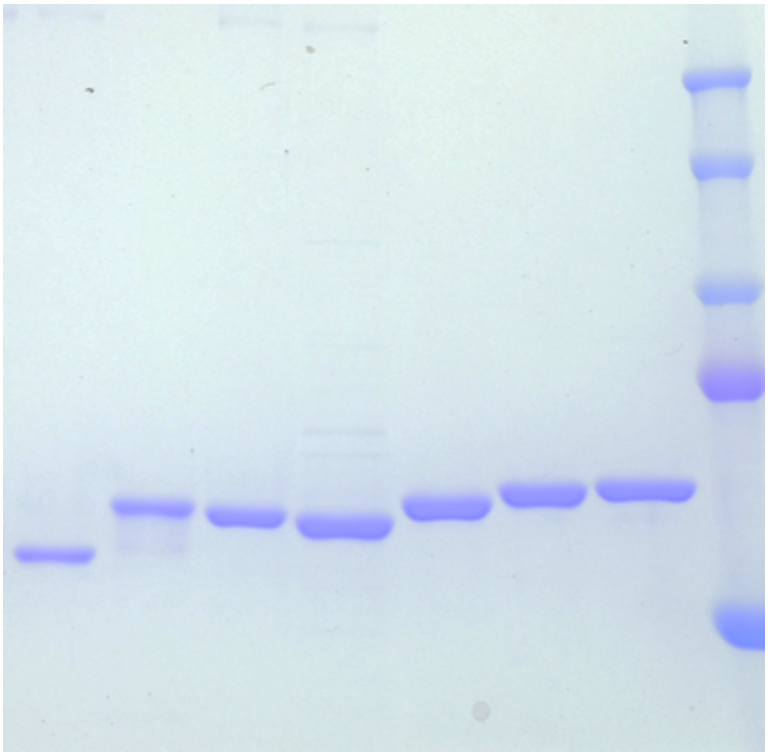

Fig. S4. SDS-PAGE gel stained by CBB. 430 ng of each protein was loaded. From left: AncElat, AncCanth, AncLamp, AncLuc2, AncLuc1, AncLucin1, AncLampn1, and molecular marker (from top, 250, 150, 100, 75, 50 kDa; Precision Plus Protein Dual Standards, Bio-Rad).

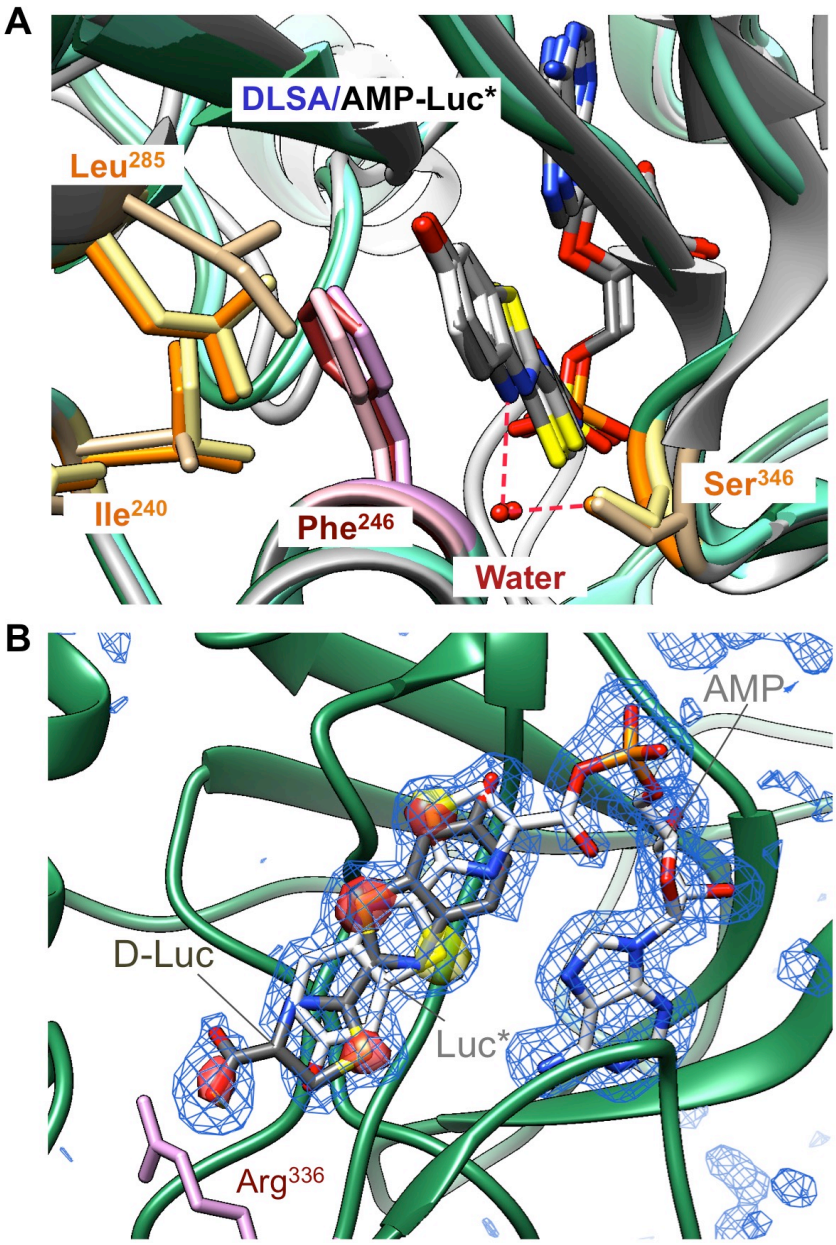

Fig. S5. The substrate-binding site structure of AncLamp. (**A**) Interaction between Ser^{346} (Ser^{349} in LcLuc1) and DLSA/AMP-Luc in AncLamp - DLSA (green), LcLuc1 - DLSA (white), and AncLamp - substrates (D-luciferin/ATP) (light green) complex structures. In all structures, a water molecule (Water) mediate hydrogen bond networks from Oγ of Ser to the nitrogen atom of thiazole plane of DLSA/AMP-Luc. The hydrogen bonds in the AncLamp – DLSA only are shown by red dotted lines. (**B**) The substrate-binding site structure of AncLamp - substrates (D-luciferin/ATP) complex. The all ligand omitting *Fo* – *Fc* map is shown in blue mesh (contoured at 10 σ). Also the *Fo* – *Fc* D-Luc omitting map (red, 30 σ), and the Luc* omitting map (yellow, 30 σ)

are superposed. The probable non-reactive D-luciferin (D-Luc, carbon atoms are shown in gray) and AMP and luciferyl moiety in the probable reaction intermediate (Luc*, carbon atoms are shown in white) are indicted with stick models. The carboxylate moiety of the non-reactive D-luciferin makes an electro-static interaction with Arg^{336} in this configuration.

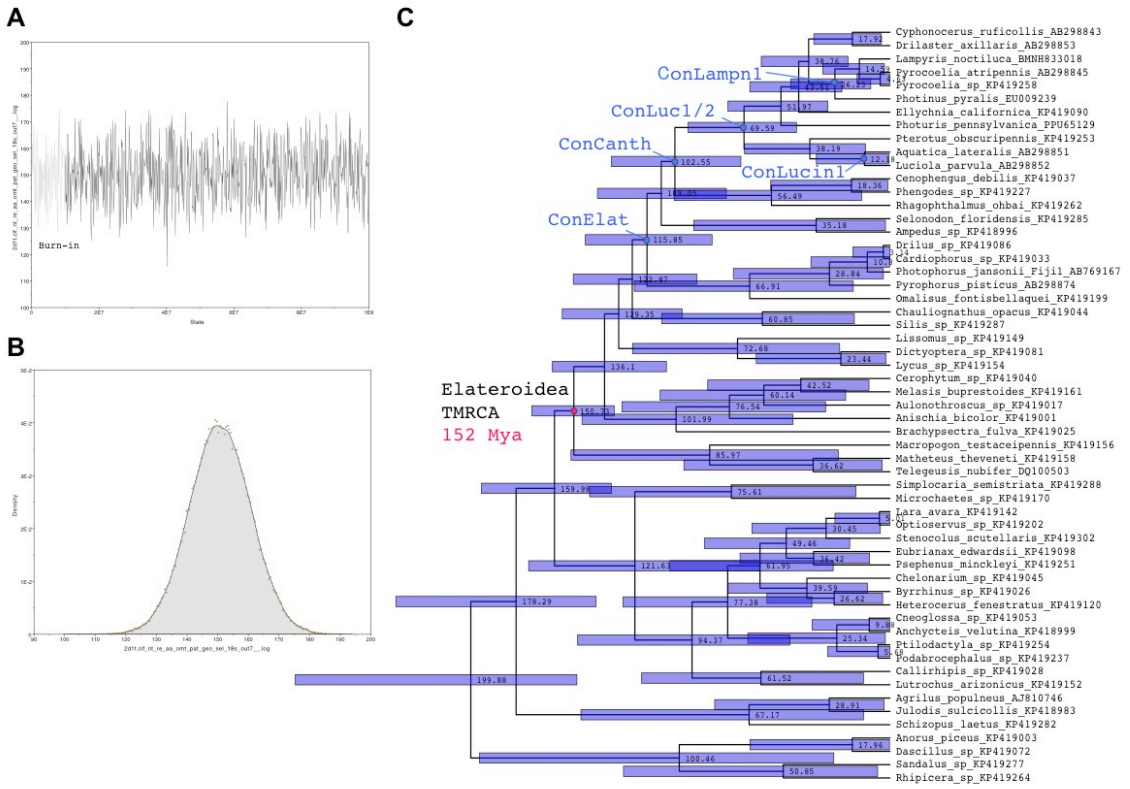

Fig. S6. Geological dating. (**A**) The trajectory and (**B**) Distribution of Elateroidea common ancestor geological age during MCMC simulation for 10^8 iterations. The initial 10^7 iterations are burn-in process. (**C**) The phylogeny of Elateriformia species. The leaf nodes are indicated with species names and GenBank accession number of corresponding 18S rRNA gene sequence. The median of estimated geological age and 95% HPD (purple bar) are indicted on each node. The reference Elateroidea TMRCA (*16*) is highlighted with a magenta circle, and the nodes corresponding to the hypothesized ancestral species, which genomes encoded AncElat, AncCanth, AncLampn1, and AncLucin1 are indicated with blue circles and labeled as ConElat, ConCanth, ConLampn1, and ConLucin1, respectively. ConLuc1/2 is the first species, which had the duplicated genes of AncLuc1 and AncLuc2.

Table S2. X-ray crystallography data collection and refinement statistics.

Protein[b]	AncLamp - DLSA complex (6K4C)[a]	AncLamp - ATP - D-luciferin complex (6K4D)[a]
Space group	*C222₁*	*C222₁*
Cell constants (a, b, c Å)	48.3, 123.4, 177.4	48.8, 123.7, 177.0
Wavelength (Å)	1.0000	1.0000
Resolution range - data collection (Å)	20.00 - 2.10 (2.21 - 2.10)	20.00 - 1.70 (1.79 - 1.70)
R_{sym}	0.032 (0.073)	0.014 (0.030)
Completeness (%)	99.7 (100.0)	96.3 (95.4)
$\langle I/\sigma I \rangle$	14.0 (6.9)	75.6 (22.5)
Resolution range - refinement (Å)	20.00 - 2.10 (2.17 - 2.10)	20.00 - 1.70 (1.73 - 1.70)
No. reflections	31197(3317)	55829 (2520)
R_{cryst}	0.208 (0.266)	0.163 (0.226)
R_{free}	0.244 (0.328)	0.189 (0.280)
RMSD length (Å)	0.007	0.017
RMSD angle (°)	0.98	1.22

[a] Values for the highest resolution bin are in parentheses.

[b] In parentheses PDB accession codes.

Table S4. Hydrogen bonds on DLSA molecule.

DLSA moiety	AncLamp (6K4C) [a]		LcLuc1 (2D1S) [a]	
Luciferyl-moiety	SLU^{601} O10 - HOH^{704} O	2.34	SLU^{2001} O10 - HOH^{2135} O	2.73
	SLU^{601} N7 - HOH^{810} O	2.99	SLU^{2001} N7 - HOH^{2002} O	2.82
	SLU^{601} O39 - Lys^{527} Nζ	2.96	SLU^{2001} O39 - HOH^{2014} O (- Lys^{531} Nζ)	2.63 (2.77)
AMP-moiety	-	-	SLU^{2001} O18 - Ser^{200} Oγ	3.08
	-	-	SLU^{2001} O19 - Ser^{201} N	3.30
	-	-	SLU^{2001} O19 - Ser^{201} Oγ	2.98
	-	-	SLU^{2001} O18 - Thr^{345} N	3.11
	SLU^{601} O18 - Thr^{341} Oγ1	3.31	SLU^{2001} O18 - Thr^{345} Oγ1	3.00
	-	-	SLU^{2001} O18 - HOH^{2223} O	2.80
	SLU^{601} O19 - His^{243} Nε2	2.87	SLU^{2001} O19 - His^{247} Nε2	2.85
	SLU^{601} O19 - HOH^{812} O	2.71	-	-
	SLU^{601} O20 - HOH^{812} O	3.14	SLU^{2001} O20 - HOH^{2014} O	3.33
	SLU^{601} O27 - Asp^{420} Oδ1	3.22	SLU^{2001} O27 - Asp^{424} Oδ1	3.23
	SLU^{601} O27 - Asp^{420} Oδ2	2.29	SLU^{2001} O27 - Asp^{424} Oδ2	2.56
	-	-	SLU^{2001} O27 - HOH^{2385} O	2.86
	SLU^{601} O28 - Asp^{420} Oδ1	2.59	SLU^{2001} O28 - Asp^{424} Oδ1	2.70
	SLU^{601} O28 - Asp^{420} Oδ2	2.98	-	-
	SLU^{601} O28 - HOH^{830} O	2.75	SLU^{2001} O28 - HOH^{2136} O	2.80
	SLU^{601} N31 - HOH^{830} O	3.20	SLU^{2001} N31 - HOH^{2136} O	2.91
	SLU^{601} N29 - HOH^{924} O	2.93	SLU^{2001} N29 - HOH^{2410} O	2.99
	SLU^{601} N37 - Gly^{314} N	3.28	SLU^{2001} N37 - Gly^{318} N	3.10
	SLU^{601} N38 - Gly^{337} O	2.54	SLU^{2001} N38 - Gly^{341} O	2.84
	-	-	SLU^{2001} N38 - HOH^{2564} O	2.71

[a]The number shows the distance between donor and acceptor atoms in Å.

Table S6. Stability of the recombinant proteins.

Protein	RLU/ng protein (0 h on ice) [a]	RLU/ng protein (12 h on ice) [a]	Remaining activity (%)
AncElat	1.77	1.41	79.8
AncCanth	6014.62	5195.45	86.4
AncLamp	49863.41	47656.04	95.6
AncLuc1	40471.84	37617.22	92.9
AncLuc2	57978.09	52424.62	90.4
AncLampn1	32913.34	40851.94	124.1
AncLucin1	13723.18	16697.27	121.7

[a] Luminescence reaction was initiated by injecting the 430 ng of purified recombinant luciferase into the mixture of D-luciferin (final conc. 10 μM D-luciferin, 5 mM $MgCl_2$, 100 μM ATP, 50 mM Tris-HCl, pH 7.8). Relative light intensity (RLU) was measured using a luminometer Centro LB960 (Berthold), and integrated from 2 to 32 s after mixing (total 30 s).

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