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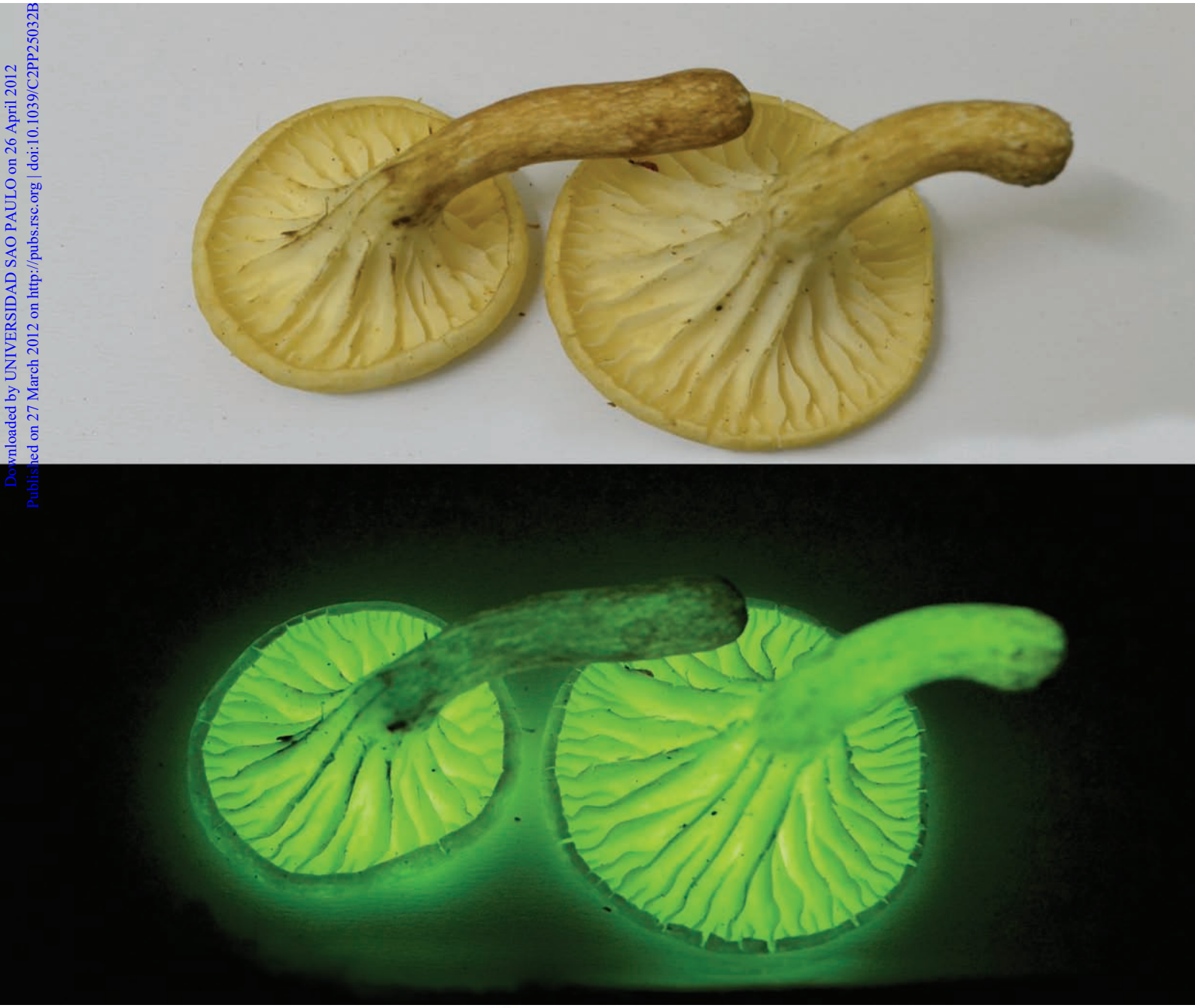

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PAPER

Evidence that a single bioluminescent system is shared by all known bioluminescent fungal lineages†

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Since the early 20th century, many researchers have attempted to determine how fungi are able to emit light. The first successful experiment was obtained using the classical luciferin–luciferase test that consists of mixing under controlled conditions hot (substrate/luciferin) and cold (enzyme/luciferase) water extracts prepared from bioluminescent fungi. Failures by other researchers to reproduce those experiments using different species of fungi lead to the hypothesis of a non-enzymatic luminescent pathway. Only recently, the involvement of a luciferase in this system was proven, thus confirming its enzymatic nature. Of the 100 000 described species in Kingdom Fungi, only 71 species are known to be luminescent and they are distributed unevenly amongst four distantly related lineages. The question we address is whether the mechanism of bioluminescence is the same in all four evolutionary lineages suggesting a single origin of luminescence in the Fungi, or whether each lineage has a unique mechanism for light emission implying independent origins. We prepared hot and cold extracts of numerous species representing the four bioluminescent fungal lineages and performed cross-reactions (luciferin × luciferase) in all possible combinations using closely related non-luminescent species as controls. All cross-reactions with extracts from luminescent species yielded positive results, independent of lineage, whereas no light was emitted in cross-reactions with extracts from non-luminescent species. These results support the hypothesis that all four lineages of luminescent fungi share the same type of luciferin and luciferase, that there is a single luminescent mechanism in the Fungi, and that fungal luciferin is not a ubiquitous molecule in fungal metabolism.

Introduction

Bioluminescence (BL) has evolved independently at least 40 times in different lineages of organisms.[1,2] All luminous systems involve the catalytic oxidation of a substrate (a luciferin) by a respective enzyme (a luciferase) or photoprotein.[3] The luciferases, however, are not necessarily homologous to each other, and each luciferin has a specific structure depending upon the luminescence system. Thus, a given luciferin and its particular luciferase are commonly found only within a single *lineage*, with the exception of a few marine bioluminescent organisms from six phyla [*e.g.*, Sarcomastigophora (protozoa), Cnidaria, Ctenophora, Mollusca, Arthropoda, and Chordata (pisces)] that share a common luciferin known as coelenterazine, although their luciferases differ or are currently unknown.[1,4]

Despite the excellent progress achieved in the past century in understanding the biological and evolutionary aspects of various bioluminescent systems, there are still some luminous organisms that remain poorly investigated. Bioluminescent fungi are one of those.

Fungal BL is a common phenomenon seen on land, first described by Aristotle (384–322 BC).[3,5] All known bioluminescent fungi are saprotrophic (or rarely plant pathogenic), mushroom-forming species that belong to the Agaricales lineage of the Basidiomycota.[6] Over 9000 species representing *ca.* 350 genera in 26 families comprise order Agaricales.[7] It should be noted that this order is a tiny fraction of Kingdom Fungi that conservatively contains 1.5 million species, although only 100 000 have been formally described to date.[8] Of this diversity, only 71 species have been verified as bioluminescent and they belong to four distantly related lineages.[7,9] Five luminescent species belong to the Armillaria lineage and are commonly known as Honey Mushrooms whose mycelium causes "foxfire". The luminescent *Armillaria* species are members of the family Physalacriaceae, where they represent a lineage sister to *ca.* 100 non-luminescent species. Twelve luminescent species belong to the Omphalotus lineage (*Neonothopanus, Omphalotus*) and are

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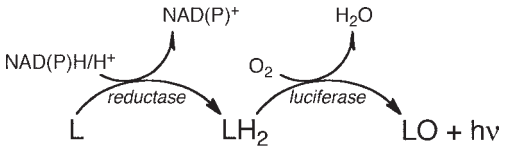

L: luciferin, LH_2: reduced luciferin, LO: oxyluciferin

Scheme 1

commonly called the Jack-o-Lantern Mushrooms. This lineage represents a small part of family Omphalotaceae that contains *ca.* 250 non-luminescent species. The highest diversity, 52 luminescent species, belongs to the Mycenoid lineage (*Mycena*, *Panellus*, *Prunulus*, *Roridomyces*) where they are phylogenetically scattered amongst over 500 non-luminescent species of family Mycenaceae. Two species represent the newly discovered Lucentipes lineage ("*Mycena*" *lucentipes*, "*Gerronema*" *viridilucens*) and belong to a formally unnamed family of the hydropoid clade distantly related to the Physalacriaceae, Omphalotaceae and Mycenaceae.[10]

The uncertainty about the participation of a luciferase in fungal BL hampered for decades the understanding of the biochemical pathways involved in light emission. Only recently, the involvement of a luciferase in the fungal BL system was proved, thus confirming the enzymatic nature of fungal BL first proposed in the 1960s by using the classical luciferin/luciferase test, which consists of mixing hot (substrate) and cold (enzyme) water extracts.[11–14] Ever since, the use of the classical luciferin/luciferase test allowed the verification of the presence of the fungal luciferin or the luciferase in any extract obtained from a particular species regardless its taxonomic affinity. As judged by the results obtained to date, the fungal system requires a luciferin, a NAD(P)H-dependent reductase, NADPH or NADH, and a luciferase (Scheme 1).[12,14,15]

Evidence of a common substrate and enzymes in the four evolutionary lineages of bioluminescent fungi would support the hypothesis of the involvement of the same enzymatic mechanism in all known species, further suggesting a single early origin of bioluminescence in the mushroom-forming order Agaricales. Moreover, it would also help to shed some light on the unsuccessful results previously reported with the mycenoid lineage – more specifically with cell-free extracts of the fungus *Panellus stipticus*.[15,16]

Experimental

Fungal species

Seven different species of bioluminescent fungi were used: *Gerronema viridilucens* (Instituto de Botânica, SP307883), Brazil, São Paulo State, Iporanga, Parque Estadual Turístico do Alto Ribeira, Sep. 2003;[17,18] *Armillaria mellea* (San Francisco State University (SFSU), NW 444), USA, California, Mendocino County, Jackson State Forest, 17 Nov. 2007; *Mycena fera* (SFSU, PR-6462), Puerto Rico, El Verde Research Station, in front of apartments, 23 Jan. 2007; *Mycena citricolor* (Centraalbureau voor Schimmelcultures (CBS), 193.57), Costa Rica, Turrialba; and *Mycena luxperpetua* (SFSU, PR-6463), Puerto Rico, El Verde Research Area, almost to ridge above footbridge over the Q. Sonadora, 15 Jan. 2007.[6] *Mycena luxaeterna* fruiting bodies were collected and preserved in liquid nitrogen in March 2008 in Brazilian Atlantic Rainforest, Iporanga municipality, São Paulo State.[6] *Neonothopanus gardneri* fruiting bodies were collected and sun dried in Fazenda Boa Vista, Gilbués municipality, Piauí State, Brazil, Feb. 2008.[19] Non-bioluminescent species used included: *Filoboletus gracilis* (SFSU, PR-6530), Puerto Rico, Bisley Watersheds, Tana Woods Plot trail near road; *Mycena singeri* (SFSU, PR-6456), Puerto Rico, El Verde Research Station, in front of apartments, 15 Jan. 2007 (this represents a non-luminescent strain of a species reported from São Paulo State as luminescent by Desjardin *et al.*[20]) and *Mycena nivicola* (SFSU, BAP 671), USA, California, Sierra County, Yuba Pass, 4 June 2008.

Culture conditions

Armillaria mellea, *M. fera*, *M. citricolor*, *M. luxperpetua*, *F. gracilis*, *M. singeri* and *M. nivicola* mycelia were cultivated at 25 °C on Petri dishes (100 mm diameter) using a non-buffered 2.0% (w/v) agar medium containing 1.25% (w/v) malt extract (Difco). Media were prepared using an autoclave (Steris, Amsco Century SG-116 Gravity Sterilizer) set at 120 °C for 30 min and a laminar flow hood (Labconco). Mycelia were harvested after 10 d. They were cut in cubes to *ca.* 2 cm^2 (*ca.* 1.08 g) and immediately used in the chemiluminescence assays. *Gerronema viridilucens* mycelium was cultivated as above mentioned, but using a sugar cane molasses (82.2° Bx, Pol 56%) medium and 0.10% (w/v) yeast extract (Oxoid). *Mycena luxaeterna* and *Neonothopanus gardneri* fruiting bodies were additionally dried in a vacuum desiccator with $CaCl_2$ (Merck) and stored at room temperature until use.

Hot and cold extracts

Hot extracts were prepared using either lyophilized mycelia/fruiting bodies (20 mg) of *G. viridilucens*, *M. luxaeterna* and *N. gardneri* or fresh mycelia (*ca.* 1.0 g) from *A. mellea*, *M. fera*, *M. citricolor*, *M. luxperpetua*, *F. gracilis*, *M. singeri* and *M. nivicola*. All necessary permits were obtained for the acquisition of cultures and specimens included in this research. For material from São Paulo State, permits were issued by Instituto Florestal to CVS; for Piauí State, the fruiting bodies were collected and sent to CVS by the land owners Mr Marino G. de Oliveira (Fazenda Boa Vista, Gilbués, PI), and Dr Ismael Dantas (Fazenda Cana Brava, Teresina, PI); for Puerto Rico, permits were issued by El Verde Research Station to D. Jean Lodge; for California, no permits were required for collecting in Jackson State Forest or at Yuba Pass, Sierra Co.

The dried powder of mycelia/fruiting bodies was weighted (*ca.* 20 mg per vial) in amber vials and then sealed with septa. A stream of argon was introduced into each vial using two syringes with needles in order to exchange the air in the vials with argon. Then, the powder inside each vial was extracted using 2.0 mL of hot (80 °C) extraction buffer [100 mM phosphate buffer pH 7.5, containing 1 mM 2-mercaptoethanol (Sigma) and 5 mM Na_4EDTA (Sigma)]. Another syringe was used to inject the

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extraction buffer. Vials were maintained in a water bath at 80 °C for 1 min with a stream of argon to facilitate the homogenization, and then rapidly cooled in an ice bath. The high temperature allows a suitable extraction of the luciferin and the final centrifugation step (3000 × *g* for 5 min at 4 °C) isolates the insoluble particulate material and yields a suitable homogenate. In order to obtain higher light emissions, and avoid non-enzymatic oxidation of luciferin, it is strongly recommended to maintain the luciferin extract under argon atmosphere and ice bath until use.

Cold extracts were prepared likewise, but using either 80 mg of lyophilized mycelia/fruiting bodies or *ca.* 1.0 g of fresh mycelia (*ca.* 2 mg of total proteins) mixed in 5 mL of cold extraction buffer [*ibid.*] with a potter homogenizer. The extract was centrifuged and the supernatant was also kept in ice until use. The protein concentration was measured using the Bradford assay.[21]

Chemiluminescence assay

Chemiluminescence assays were conducted in 12 × 50 mm test tubes at 25 ± 1 °C, using a Turner TD-20/20 luminometer with integration time set to 0.2 s. Light emission intensities were measure in relative light units (RLU) and sensitivity was adjusted in 100% unless otherwise indicated. The standard chemiluminescence assay was accomplished as previously described.[11] In summary, it was conducted by the addition of 200 µL of cold extract, 50 µL of 1 g L^{-1} bovine serum albumin solution (BSA, Sigma), 50 µL of hot extract and 50 µL of NADPH (Sigma) in extraction buffer (100 mM). NADPH triggers the reaction. NADH can also be used, however the light intensity is lower when compared to NADPH.

The species *A. mellea*, *G. viridilucens*, *N. gardneri*, *M. luxaeterna* and *F. gracilis* were used in all possible combinations to prepare hot and cold extracts. A second set of experiments was also conducted using the standard chemiluminescence assay, but only with mycenoid species *M. luxaeterna*, *M. fera*, *M citricolor*, *M. luxperpetua*, *M. singeri* and *M. nivicola*. All the experiments were conducted in triplicate with an observed average error of 10%. The integrals for each light emission profile (Fig. S1†) were calculated from 0 to 180 s using the Microcal Origin® 7.0 software.

Results and discussion

Cross-reactivity of hot/cold extracts from Armillaria, Lucentipes, Mycenoid and Omphalotus lineages

Bioluminescent bacteria, dinoflagellates and coleopteran beetles are examples of phylogenetically delimited bioluminescent systems, *i.e.*, luciferase and luciferin extracted from different species within the same phylogenetic lineage can be cross-reacted, resulting in light emission.[4] As far as we know, a complete set of cross-reactions of all possible combinations of luciferin/enzymes (hot/cold extracts) has never been accomplished using the four known lineages of bioluminescent fungi.

Previous studies performed by our group confirmed that enzymes mediate fungal bioluminescence.[11] When the cold extract was heated or precipitated with ammonium sulfate or filtered using a 3 kDa molecular weight cut off filter, the filtrate obtained did not lead to light emission upon its reaction with the hot extract and NADPH.[11]

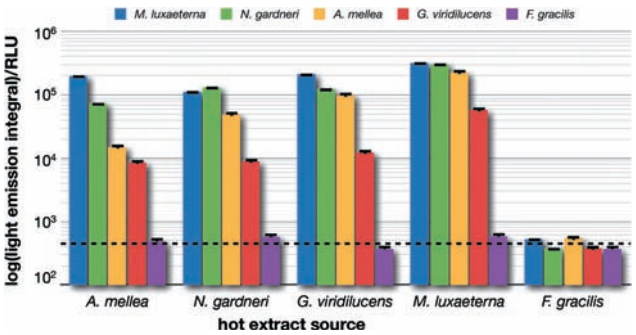

Fig. 1 Integral of light emission obtained with the chemiluminescence assay with the four known lineages of bioluminescent fungi. Total amount of light were obtained by integrating the light emission curves (see ESI, Table S1, Fig. S1†), from 0 to 180 s, to each possible combination using: *A. mellea*, *N. gardneri*, *G. viridilucens*, *M. luxaeterna* and *F. gracilis* (control, non-luminescent). Colors refer to species used as cold extract source and the dotted line is the average baseline of the equipment. Reactions were initiated by the addition of NADPH. [NADPH] = 100 mM, [BSA] = 140 mg L^{-1}, hot extract: 50 µL, cold extract: 200 µL, final volume: 350 µL. All the experiments were made in triplicate with an average error of 10%.

Cell-free light emission was obtained using hot/cold extracts from all combinations of the bioluminescent fungi *Armillaria mellea* (Armillaria lineage), *Gerronema viridilucens* (Lucentipes lineage), *Neonothopanus gardneri* (Omphalotus lineage) and *Mycena luxaeterna* (Mycenoid lineage) (Fig. 1; Table S1, Fig. S1†). Cross-reactions involving the non-luminescent fungus *Filoboletus gracilis* (a mycenoid species distantly related to *M. luxaeterna* in family Mycenaceae) as a source of luciferin or enzymes resulted in no detectable light emission.

Chemiluminescence assays using the non-luminescent fungus *F. gracilis* showed that this species contains neither luciferin nor the enzymes. Hot or cold extracts prepared from *F. gracilis* do not lead to light emission when cross-reacted with hot or cold extracts prepared from any of the bioluminescent fungi belonging to the four lineages. A direct consequence of this result is that the fungal luciferin cannot be expected to be a ubiquitous molecule of fungal metabolism or a widespread biomolecule such as FMN, the bacterial luciferin. In fact, addition of either FMN plus n-dodecanal or firefly luciferin [(4S)-2-(6-hydroxy-1,3-benzothiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid] in the presence of ATP/Mg^{2+} did not lead to any light emission observed with the tube luminometer, which is in accordance with Airth and McElroy.[22]

All reactions involving the fungi *N. gardneri* or *M. luxaeterna*, no matter as source of luciferin or luciferase, were responsible for the highest values of light emission observed. This fact may indicate a naturally great amount of luciferin/luciferase available in those fungi, making them good species for luciferin and luciferase extraction.

Identical conclusions can be postulated from the results obtained from tests among species within the Mycenoid lineage. Chemiluminescence cross-reaction assays were conducted using extracts prepared from the following species of *Mycena*

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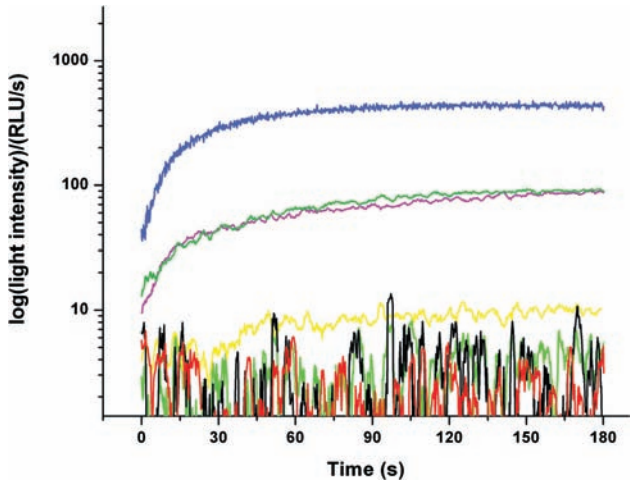

Fig. 2 Light emission profile obtained with the chemiluminescence assay with the mycenoid lineage of bioluminescent fungi. Light intensity time courses obtained with *M. luxaeterna*, *M. fera*, *M citricolor*, *M. luxperpetua*, *M. singeri* (non-luminescent) and *M. nivicola* (non-luminescent). Reactions were initiated by the addition of NADPH. [NADPH] = 100 mM, [BSA] = 140 mg L^{-1}, hot extract: 50 µL, cold extract: 200 µL, final volume: 350 µL. Colors in the plot refer to the following combination of cold/hot extracts: (blue) *M. luxaeterna*/*M. fera*, (purple) *M. luxaeterna*/*M. citricolor*, (green) *M. luxaeterna*/*M. luxperpetua*, (yellow) *M. luxaeterna*/*M. singeri*, (black) *M. singeri*/*M. luxaeterna*, (light green) *M. luxaeterna*/*M. nivicola*, (red) *M. nivicola*/*M. luxaeterna*.

representing three distantly related bioluminescent mycenoid lineages (Perry & Desjardin, personal communication): the luminescent *M. luxaeterna*, *M. fera*, *M citricolor* and *M. luxperpetua*, and the non-luminescent *M. nivicola* nom. prov. and a non-luminescent strain of *M. singeri* (Fig. 2). Extracts prepared with *M. luxaeterna* were chosen to validate the presence/absence of luciferin or enzymes in other fungal species due to the higher intensity observed in its self-chemiluminescence assay and due to the large amount of available fruiting bodies. As in the first set of experiments, light emission was only observed using the hot and cold extracts from luminescent species, and in all possible combinations. Despite the non-luminescent species *M. singeri* and *M. nivicola* belonging to the same phylogenetic lineage as luminescent *Mycena* species, no light was registered by the luminometer in self- and cross-reactions in all combinations with luminescent species. Thus, these non-luminescent fungi contain neither luciferin nor the enzymes required for BL. It must be pointed out that the chemiluminescence assay is very sensitive and specific and is a very precise method to determine the presence of luciferin and enzymes.

Phylogenetic significance

Data obtained from chemiluminescence assays (see Experimental) support the hypothesis of a shared unique enzymatic mechanism operating in all known bioluminescent lineages of fungi, since both hot and cold extracts containing luciferin and enzymes respectively are *sine quibus non* to achieve light emission. In addition, one may also conclude that the luciferin, the enzymes and the emitter required are similar in each lineage. These results support a single evolutionary origin of the bioluminescent pathway in the Agaricales, and suggest that this pathway has become inactivated, lost or unrecognized in a large number of taxa. Such hypotheses are currently being tested within a phylogenetic framework by Perry and Desjardin, and will be published separately.

Conclusions

Seventy-one species of Fungi are known to be bioluminescent, out of 100 000 described species. All known BL fungi belong to the mushroom-forming group the Agaricales, where they are scattered among four phylogenetic lineages. Cross-reactions in all possible combinations of hot (substrate/luciferin) and cold (enzyme/luciferase) water extracts from species representing each of the four bioluminescent lineages resulted in light emission. In comparison, cross-reactions of these extracts with extracts from closely related non-luminescent species yielded no light emission. The most parsimonious explanation of these results is that each of the four bioluminescent lineages shares the same or similar luciferin and luciferase, whereas these compounds are absent in non-luminescent species. This suggests a single luminescent pathway in the Fungi that arose early in the evolution of the mushroom-forming Agaricales.

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