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Natural bioluminescence as a genetic marker for ophiuroid species

Samuel Dupont[1,2], Jérôme Mallefet[1] and Yannick Dewael[1]

[1]**Laboratoire de Physiologie Animale, Université catholique de Louvain, Bâtiment Carnoy, 5 Place Croix du Sud, B-1348 Louvain-la-Neuve, Belgium**
[2]**Centre de Recherche sur la Biodiversité, Université catholique de Louvain, Bâtiment Carnoy, 5 Place Croix du Sud, B-1348 Louvain-la-Neuve, Belgium**

ABSTRACT. Bioluminescence is the emission of visible light by living organisms. This amazing property is used in various research fields such as genetics, molecular biology, chemistry, etc. The aim of this work was to gather evidence that bioluminescence could also be used as a genetic marker in various luminescent species. Previous studies with the brittlestar *Amphipholis squamata* have shown that bioluminescence is an excellent marker for intraspecific genetic variability. The method, using maximum luminous capabilities induced by KCl 200mM depolarization, presents numerous advantages over other genetic markers (RAPDs, microsatellites, RFLP, etc.): it is cheaper, faster and easier to use. Since bioluminescence is a frequent phenomenon in brittlestars, the same method was used to compare variability between six different species: *Amphipholis squamata, Amphiura filiformis, A. arcystata, Ophiopsila aranea, O. californica* and a new species of *Amphiodia*. Our results show that *Amphiodia* and both *Ophiopsila* species could be clearly isolated, each in a separated cluster, according to their luminous capabilities. These differences could be explained by physiological properties. On the other hand, an important intraspecific variability was observed for two species (*A. squamata* and *A. filiformis*). We propose that natural bioluminescence is a good marker to study inter- and intraspecific variability, providing useful functional information for physiological and population studies.

KEYWORDS: Ophiuroidea, echinodermata, bioluminescence, phylogeny, biodiversity, population genetics, markers.

INTRODUCTION

Since the 19th century and the theory of evolution, natural scientists have been looking for individual variations within and between populations (RIDLEY, 1997). In order to understand the evolution of these variations, population biologists use different categories of genetic markers: morphological, biochemical or more recently molecular markers (SUNNUCKS, 2000). In the past decade, the contribution of molecular genetics to population biology has been huge. Many new genetic markers appeared with the development of the polymerase chain reaction (PCR) and the advent of routine DNA sequencing. Examination of these markers at the right scales of time, space and change can give information on the distribution and evolution of genetic variants. For example, microevolutionary processes such as migration, natural selection or reproductive success could be investigated using this method. Therefore, measuring genetic variations and the influence of the environment, we can make inferences on the biology of the species (SUNNUCKS, 2000; FÉRAL, 2000).

Bioluminescence is the capability of living organism to produce visible light (HASTINGS & MORIN, 1991). This amazing phenomenon is commonly observed in echinoderms where more than 40% of the luminous species are ophiuroids (HERRING, 1995; MALLEFET, 1999). Few studies have been performed on luminous ophiuroids and they have been restricted to two different fields. Ethological approaches on *Ophiopsila californica* have demonstrated that light emission is used as an anti-predatory signal (BASCH, 1988; GROBER, 1988). Physiological works performed on the small brittlestar *Amphipholis squamata* have shown that emitted light is intracellular and restricted to specialized cells called photocytes (DEHEYN et al., 1996). Moreover, this photogenesis is under complex nervous control (DE BREMAEKER et al., 1996, 2000).

Recent works show that natural bioluminescence is an efficient genetic marker in *A. squamata*. Several parame-

Corresponding author: S. Dupont, e-mail: dupont@bani.ucl.ac.be

ters of the bioluminescence (intensity and kinetics), as revealed by KCl stimulation, present important intraspecific variations (DEHEYN et al., 1997; DUPONT & MALLEFET, 2000), which are heritable (DUPONT et al., 2000a). Moreover, bioluminescence reveals exactly the same variability as that observed with molecular markers such as RAPDs (DUPONT et al., 2000b). Natural bioluminescence, polymorphic and heritable, is then a good genetic marker and presents numerous advantages over molecular markers: it is cheaper, faster and easier to use.

The aim of this work was to test this method on other bioluminescent ophiuroid species. We compared the luminous capabilities of individuals of six species using the KCl stimulation method in order to demonstrate that natural bioluminescence can be used as a genetic marker.

MATERIAL AND METHODS

Six species of luminous ophiuroids were collected in different locations (Table 1). Animals were anaesthetised by immersion in 3.5% w/w $MgCl_2$ in artificial sea water. Arms were removed from the disc, measured and stimulated with KCl 200mM to trigger the maximum light emission. Measurements of light capabilities were carried out in a dark room using a luminometer (Berthold FB 12); three parameters were measured to characterize the light response as described for *A. squamata* (MALLEFET et al., 1992). The maximum intensity of light was expressed in megaquanta per seconds and per millimeter of arm (Lmax in $Mq.s^{-1}.mm^{-1}$) and two kinetic parameters were expressed in seconds: the time elapsing between the application of the KCl stimulus and the beginning of the light production (Latency time, Lt), and the time between the beginning of the light production and the maximum of light production (Tlmax). Data were considered as coordinates using the three luminous parameters. Euclidian distances were computed and observations were hierarchically clustered using Ward's maximum-variance method (WARD, 1963). Each mean value is expressed with its standard error of mean (mean ± SEM); analysis of variance (ANOVA) and t-tests were used to determine the significance of the observed differences between the groups. All statistical methods used are designed under the assumption that the data are normally distributed. Tests have been used to check that the data are a random sample from a normal distribution. Since the sample size was less than or equal to 2000, the SHAPIRO-WILK (1965) statistic, W, was used. When data were not normally distributed or when heteroscedasticity occurred, a logarithmic transformation of data was performed as indicated by SOKAL and ROHLF (1995). Analyses were performed using Statistical Analysis System (SAS institute).

TABLE 1

Luminous species of ophiuroids used in this study

	Sampling site
AMPHIURIDAE	
Amphipholis squamata Delle Chiaje, 1828	Tindari, Italy (38°08'N 15°03'E)
Amphiura filiformis Müller, 1776	English Channel, Belgium (54°N 8°E) Fiskebäckskil, Sweden (58°16'N 11°26'E)
Amphiura arcystata Clarck, 1911	Santa Barbara, USA (34°25'N 119°57'W)
Amphiodia n. sp.	Santa Barbara, USA (34°24'N 119°49'W)
OPHIOCOMIDAE	
Ophiopsila aranea Forbes, 1843	Banyuls-sur-Mer, France (42°29'N 3°08'E)
Ophiopsila californica Clarck, 1921	Santa Barbara, USA (34°25'N 119°57'W)

RESULTS

Qualitative description of light emission

In the studied species, bioluminescence is represented by a diversity of colours, localizations and patterns. Two different colours are observed: *Amphiura filiformis* is the only species producing blue luminescence while other species emit in the green. Bioluminescence is always restricted to the arms except in the undescribed *Amphiodia* species where a weak light is also observed in the disc. Representative patterns of light emission of each species are presented in Fig. 1. Luminous reaction presents rapid kinetics in nearly all the species (Table 2): the latency time is short (Lt<2 s) and the maximum intensity of light is quickly reached (Tlmax< 25 s). A contrasting kinetic is observed for *O. aranea* where the luminous reaction is significantly slower (Tlmax>40 s). On average, maximum intensity of light is significantly different between the species ($p<0.01$ except between *A. arcystata – A. squamata* and *O. aranea - O. californica*). *Amphiodia* always produces intense light (30713±2564 $Mq.s^{-1}$.mm-1), followed in level of intensity by both *Ophiopsila* species and *A. filiformis*. *A. squamata* and *A. arcystata* produce a much weaker light, at least ten times less intense than the other species. In addition, there is important intraspecific variability between the three species: *A. filiformis, A. squamata* and *O. aranea*.

Quantitative description of light emission

Inter- and intraspecific differences were formalised by cluster analysis (Ward's methods). As a consequence of the huge variability within the species, *A. filifomis* was analysed separately. Fig. 2 presents the tree inferred from euclidian distances calculated on the basis of the three luminous parameters for *O. aranea, O. californica, A. arcystata, Amphiodia* n. sp. and *A. squamata*, where three different colour morphs were observed on the basis of the

TABLE 2

Luminous parameters for each species (*Amfiura filiformis*, *A. arcystata*, *Amphipholis squamata, Amphiodia n. sp., Ophiopsila aranea* and *O. californica*), colour morphs of *A. squamata* and main clusters for *A. filiformis* (Fig.4) and *O. aranea* (Fig.2). Mean ± standard error of mean; n=number of ophiuroids.

		Lmax ($Mq.s^{-1}.mm^{-1}$)	Lt (s)	Tlmax (s)	n
Ophiopsila aranea	**Mean**	**14679.85±5848.51**	**2.94±1.24**	**51.70±4.82**	**18**
"	Cluster 1 (Fig.2)	3826.52±1195.6	4.16±1.77	43.49±5.08	12
"	Cluster 2 (Fig.2)	36386.52±14186.21	0.48±0.11	68.12±6.50	6
Ophiopsila californica	**Mean**	**19803.89±8230.06**	**0.50±0.02**	**1.88±0.65**	**10**
Amphiura arcystata	**Mean**	**323.10±160.33**	**0.78±0.10**	**9.89±1.19**	**9**
Amphiura filiformis	**Mean**	**8055.39±1270.11**	**0.51±0.04**	**12.69±1.36**	**59**
"	Cluster 1 (Fig.4)	9616.45±2678.60	0.44±0.03	4.95±0.38	12
"	Cluster 2 (Fig.4)	5923.98±4925.69	1.06±0.18	2.38±0.40	4
"	Cluster 3 (Fig.4)	26929.12±5742.82	0.35±0.06	1.16±0.20	6
"	Cluster 4 (Fig.4)	4834.97±724.56	0.35±0.03	24.36±2.20	17
"	Cluster 5 (Fig.4)	7954.76±934.70	0.20±0.00	8.81±1.48	7
"	Cluster 6 (Fig.4)	2824.78±1593.83	0.83±0.08	15.15±1.74	13
Amphipholis squamata	**Mean**	**637.43±153.47**	**1.18±0.09**	**5.84±0.43**	**35**
"	Orange	1.17±0.18	1.62±0.15	4.04±0.29	11
"	Dark-brown	177.25±49.55	1.20±0.11	5.05±0.63	13
"	Spotted	1817.51±215.76	0.71±0.06	8.58±0.46	11
Amphiodia n. sp.	**Mean**	**30713.70±2563.66**	**0.67±0.05**	**3.52±0.41**	**26**

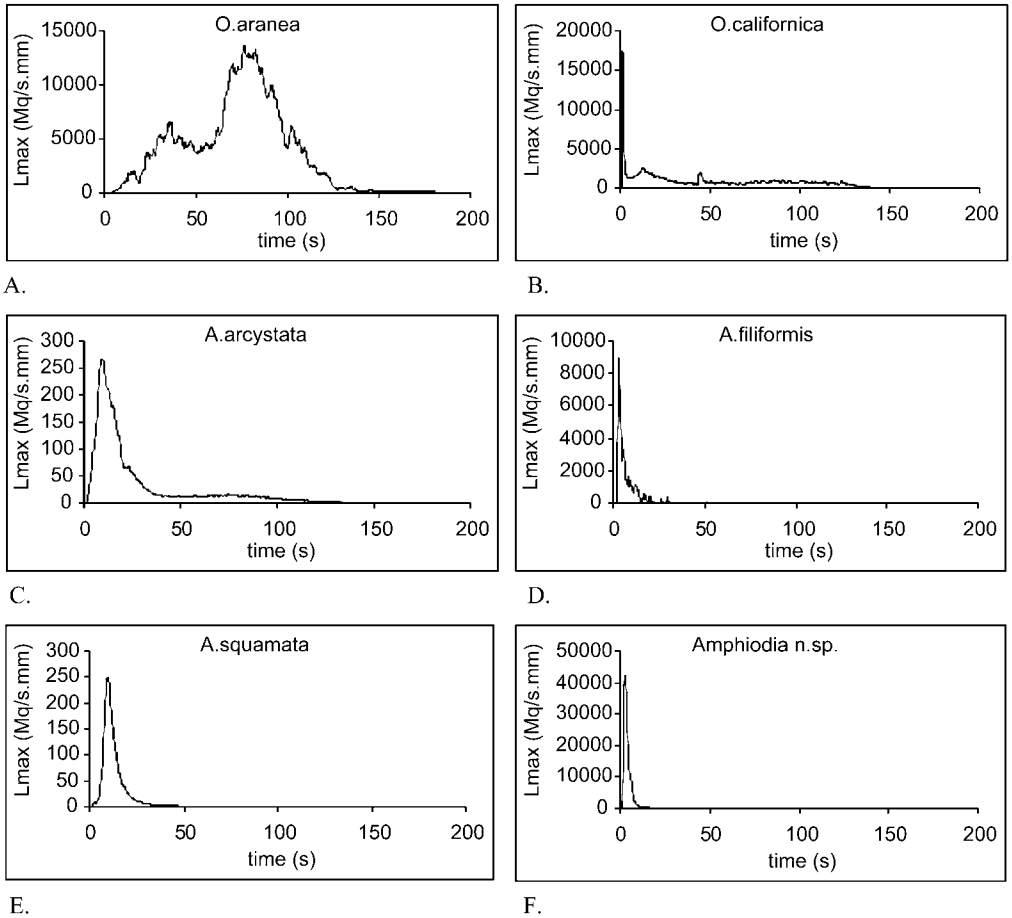


Fig. 1. – Representative recordings of light emitted by an arm stimulated by KCl 200mM (A, *Ophiopsila aranea;* B, *O. californica*; C, *Amphiura arcystata;* D, *A. filiformis;* E, *Amphipholis squamata;* F, *Amphiodia* n.sp.).

pigmentation of arms and discs: Orange, Dark-brown and Spotted (see DUPONT & MALLEFET, 2000). Ward's clustering method revealed six clusters separated by minimal distance of 0.01. Clusters 1 and 2 correspond to *O. aranea*. Cluster 3 contains individuals of *A. arcystata* and *A. squamata* of Dark-brown and Spotted morphs where Orange one constitutes the cluster 4. These four clusters are greatly separated from clusters 5 and 6 containing respectively *Amphiodia* n. sp. and *O. californica*.

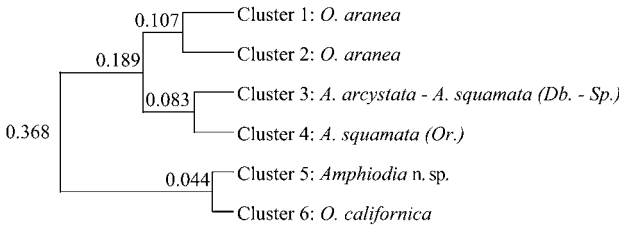
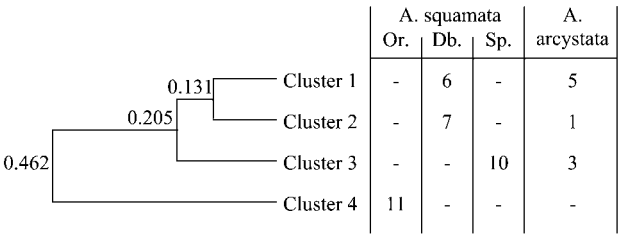


Fig. 2. – Tree inferred from euclidian distances between bioluminescence parameters of *Ophiopsila aranea, O. californica, Amphiodia* n. sp.*, Amphiura arcystata, A. filiformis* and the three colour morphs of *Amphipholis squamata* (Or, orange, Db, dark-brown and Sp., spotted). Distances within a cluster are inferior to 0.01.

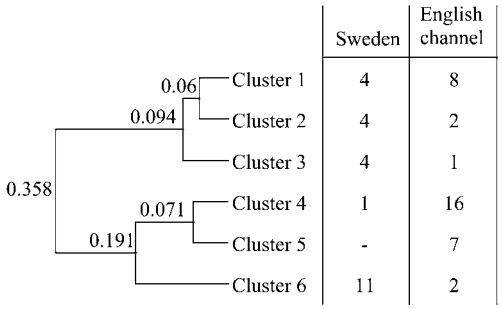
Since it was impossible to isolate *A. arcystata* from *A. squamata,* the same analysis was performed on these species only (Fig. 3). Ward's method then reveals four clusters and allowed the separation of the three colour morphs of *A. squamata* (clusters 1 and 2 are the individuals of the Dark-brown morph; cluster 3, individuals of the Spotted morph and cluster 4, individuals of the Orange ones). Nevertheless, we are unable to isolate *A. arcystata* from Dark-brown and Spotted morphs of *A. squamata* (clusters 1 to 3).



	A. squamata			A. arcystata
	Or.	Db.	Sp.	
Cluster 1	-	6	-	5
Cluster 2	-	7	-	1
Cluster 3	-	-	10	3
Cluster 4	11	-	-	-

Fig. 3. – Tree inferred from euclidian distances between bioluminescence parameters of *Amphiura filiformis* and the three colour morphs of *Amphipholis squamata* (Or, orange, Db, dark-brown and Sp, spotted) and the quantity of ophiuroids of both species in each cluster. Distances within a cluster are inferior to 0.01.

A. filiformis from two different locations (Sweden and the English channel) were analysed with the same methods (Fig. 4). Two group of three clusters (1 to 3 and 4 to 6) are separated by an important distance. This great intraspecific variability cannot be explained by the geographical distribution since all the clusters (except cluster 5) are consist of individuals from the both locations.



	Sweden	English channel
Cluster 1	4	8
Cluster 2	4	2
Cluster 3	4	1
Cluster 4	1	16
Cluster 5	-	7
Cluster 6	11	2

Fig. 4. – Tree inferred from euclidian distances between bioluminescence parameters of *Amphiura filiformis* from two different location (Sweden and English Channel) and the quantity of ophiuroids from each location in each cluster. Distances within a cluster are inferior to 0.01.

DISCUSSION

Looking at bioluminescence, we can see that each species possesses its own particularities: *A. filiformis* is the only one to emit blue light, only *Amphiodia* is able to produce light with its disc, *O. aranea* has a very slow kinetic of light emission, etc. Luminescence is induced by KCl depolarization and characterized by three parameters (Lmax, Lt and Tlmax). Ward's clustering method on euclidian matrix of distance computed on these parameters constitutes an excellent tool giving functional information at inter- and intraspecific levels.

Interspecific variability

Most of the species were isolated using their bioluminescence capabilities. Surprisingly, great differences were observed compared to what was expected with classical phylogeny (SMITH et al., 1995). For example, both species of the genus Ophiopsila, *O. aranea* and *O. californica,* are not closely related. *O. aranea* is clustered with *A. arcystata* and *A. squamata* while *O. californica* is linked to *Amphiodia* n. sp..

These paradoxical results could be due to differences in the nervous control of the photogenesis. Previous works have shown that calcium ions are required to trigger light emission in ophiuroids (MALLEFET et al., 1994, 1998; DEWAEL & MALLEFET, 2000). Nevertheless, the type of calcium channel involved in the luminous control differs from one species to another: L-type channels are involved in the luminous control of *O. californica* whereas another uncharacterized channel type would be implicated in *O. aranea* (DEWAEL & MALLEFET, 2000).

Therefore, we can postulate that our method is useful to reveal physiological differences between species. A comparative study of the nervous control of these species is in progress in order to confirm this hypothesis.

Intraspecific variability

The same method, combining bioluminescence and cluster analysis, was used to reveal variability at the species level. Two species present important intraspecific variability: *A. filiformis* and *A. squamata*.

In *A. squamata*, this variability reflects the polychromatism. Each colour morph is isolated according to its luminous capabilities. This result confirms observations in several populations around the world (Deheyn et al., 1997, 2000; Dupont & Mallefet 2000; Dupont et al., 2000b). Moreover, genetic variations revealed by RAPDs demonstrate that genetic structure is homogenous within each colour morph of a same population (Dupont et al., 2000b). Since there is a link between polychromatism, bioluminescence and genetics, it was suggested that polychromatism and/or bioluminescence might be good indicators of genotype variability.

We propose that this idea could be extended to other ophiuroid species. In the case of *A. filiformis*, the two studied populations (English Channel and Sweden) were not differentiated with the method employed. Moreover, this variability could not be explained by any morphological character such as polychromatism. Assuming that bioluminescence is an indicator of genetic variability in *A. filiformis*, we postulate that most of the genetic variation occurs within population. Similar conclusions were reached by McCormack et al. (2000). They used RAPDs analysis applied to individuals of *A. filiformis* from two geographical locations. Analysis of molecular variance showed that a minimum of 93% of phenotypic variance occurred among individuals within populations.

Conclusion

We propose a new method, using natural bioluminescence properties and cluster analysis, to measure variability within or between species. At the interspecific level, it might give information about differences in the nervous control of luminescence. At the intraspecific level, it could be used as an easy-to-use genetic marker providing information for population genetics of luminous ophiuroids.

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