# Biofluorescence as a survey tool for cryptic marine species

*by* Rohani Ar

Conservation Biology

Conservation Methods

# Biofluorescence as a survey tool for cryptic marine species

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**Abstract:** *As ecosystems come under increasing anthropogenic pressure, rare species face the highest risk of extinction. Paradoxically, data necessary to evaluate the conservation status of rare species are often lacking because of the challenges of detecting species with low abundance. One group of fishes subject to this undersampling bias are those with cryptic body patterns. Twenty-one percent of cryptic fish species assessed for their extinction risk (International Union for Conservation of Nature [IUCN]) are data deficient. We developed a nondestructive method for surveying cryptically patterned marine fishes based on the presence of biofluorescence (underwater biofluorescence census, UBC). Blue LED torches were used to investigate how widespread biofluorescence was in cryptic reef fishes in the Coral Triangle region. The effectiveness of UBC to generate abundance data was tested on a data-deficient pygmy seahorse species (*Hippocampus bargibanti*) and compared with data obtained from standard underwater visual census (UVC) surveys. We recorded 95 reef fish species displaying biofluorescence, 73 of which had not been previously described as biofluorescent. Of those fish with cryptic patterns, 87% were biofluorescent compared with 9% for noncryptic fishes. The probability of species displaying biofluorescence was 70.9 times greater for cryptic species than for noncryptic species. Almost twice the number of H. bargibanti was counted using the UBC compared with UVC. For 2 triplefin species (*Ucla xenogrammus, Enneapterygius tutuilae*), the abundance detected with UBC was triple that detected with UVC. The UBC method was effective at finding cryptic species that would otherwise be difficult to detect and thus will reduce interobserver variability inherent to UVC surveys. Biofluorescence is ubiquitous in cryptic fishes, making this method applicable across a wide range of species. Data collected using UBC could be used with multiple IUCN criteria to assess the extinction risk of cryptic species. Adopting this technique will enhance researchers' ability to survey cryptic species and facilitate management and conservation of cryptic marine species.*

**Keywords:** biofluorescence, Coral Triangle, cryptic species, cryptobenthic fauna, nondestructive sampling, pygmy seahorse, rarity, underwater visual census

La Bioluminiscencia como Herramienta para Censar Especies Marinas Crípticas

**Resumen:** *Conforme los ecosistemas están sometidos a la creciente presión antropogénica, las especies raras afrontan el riesgo de extinción más alto. Paradójicamente, con frecuencia son pocos los datos necesarios para evaluar el estado de conservación de las especies raras debido a las dificultades existentes en la detección de especies con abundancias bajas. Un grupo de peces sujeto a este sesgo de poco muestreo es aquel conformado por peces con patrones corporales crípticos. El 21% de las especies de peces crípticos evaluados por riesgo de extinción (Unión Internacional para la Conservación de la Naturaleza [UICN]) carece de datos. Desarrollamos un método no-destructivo para el censo de peces marinos con patrones crípticos basado en la presencia de la bioluminiscencia (censo submarino de bioluminiscencia, UBC en inglés). Se utilizaron antorchas de LEDs*

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***Article impact statement**: Underwater biofluorescence census nondestructively collects data needed to assess conservation status of cryptic species.*

*Paper submitted April 9, 2017; revised manuscript accepted September 30, 2017.*



DOI: 10.1111/cobi.13033

*azules para investigar cuán diseminada estaba la bioluminiscencia en los peces crípticos de arrecife en la región del Triángulo de Coral. La efectividad del UBC para generar datos de abundancia se probó con una especie deficiente de datos, el caballito de mar pigmeo (*Hippocampus bargibanti*), y se comparó con los datos obtenidos a partir de censos submarinos visuales (UVC, en inglés) estándar. Registramos 95 especies de peces de arrecife que exhibieron bioluminiscencia, 73 de las cuales no habían sido descritos previamente como bioluminiscentes. De aquellos peces con patrones crípticos, el 87% fue bioluminiscente en comparación con el 9% de los peces no-crípticos. La probabilidad de que una especie exhibiera bioluminiscencia fue 70.9 veces mayor para las especies crípticas que para las no-crípticas. Se contó casi el doble del número de* H. bargibanti *con el uso del UBC en comparación con el UVC. Para dos especies (*Ucla xenogrammus, Enneapterygius tutuilae*) la abundancia detectada con el UBC fue triple que aquella detectada con el UVC. El método UBC fue efectivo para hallar especies crípticas que de otra forma serían difíciles de detectar y por lo tanto reduciría la variabilidad entre observadores inherente a los censos UVC. La bioluminiscencia es ubicua en peces crípticos, lo que hace que este método sea aplicable a lo largo de una amplia gama de especies. Los datos recolectados usando el UBC podrían usarse con múltiples criterios de la UICN para valorar el riesgo de extinción de las especies crípticas. Adoptar esta técnica mejorará la habilidad de los investigadores para censar especies crípticas y facilitar el manejo y la conservación de las especies crípticas marinas.*

**Palabras Clave:** bioluminiscencia, caballito de mar pigmeo, censo visual submarino, especie críptica, fauna criptobéntica, muestreo no-destructivo, Triángulo de Coral

**摘要:** *生物荧光在调查隐秘海洋物种中的应用*
*随着人类对生态系统的影响与日俱增, 珍稀物种正面临极高的灭绝风险。矛盾的是, 由于探测低丰度物种的难度较大, 评估珍稀物种的濒危等级所必需的数据时常匮乏。其中有一大类身体含有隐秘图案的鱼类的评估, 就受到低样本量的抽样偏差影响。来自世界自然保护联盟 (IUCN) 的数据显示, 21%的这类鱼类因为数据缺乏而无法准确评估其灭绝风险。这里, 我们发展了一个基于生物荧光 (水下生物荧光调查, UBC) 的非损伤取样方法来调查有隐秘图案的海洋鱼类。我们用蓝光LED手电探究了珊瑚大三角地区这类岩礁鱼类中生物荧光的普遍性, 并用一个数据资料缺乏的倭海马 (Hippocampus bargibanti) 检验了UBC法获取数据的效力, 同时与标准的水下视觉调查法 (UVC) 所得到的数据进行了比较。我们一共记录到95种有生物荧光的岩礁鱼类, 其中73种之前没有关于生物荧光的记载。在有隐秘图案的鱼中, 87%有生物荧光, 这在没有隐秘图案的鱼中仅有9%。隐秘种展现出生物荧光的概率是非隐秘种的70.9倍。例如, 用UBC法记录的巴氏豆丁海马 (H. bargibanti) 的数量是用UVC法的近两倍。而在两种三鳍鱼 (Ucla xenogrammus和Enneapterygius tutuilae) 研究中, 用UBC法检测到的丰度是UVC法的三倍。我们的研究表明, UBC法可有效用于寻找这些难以用其它方法探测到的隐秘物种, 因此可以减少UVC调查方法中出现的观察者之间的误差。由于在隐秘的鱼类物种普遍存在生物荧光, 使得这个方法可以大范围应用于许多物种。用UBC法收集的数据可以用于IUCN的多重标准, 来评估隐秘种的灭绝风险。同时, 采取这种技术可以提高研究者对隐秘种的调查能力, 并有利于隐秘的海洋物种的管理和保护。* ***【翻译:胡怡思;审校:聂永刚】***

**关键词:**珊瑚大三角, 隐秘种, 倭海马, 稀有性, 隐秘底栖动物, 水下视觉调查, 非损伤取样, 生物荧光

## Introduction

Numerically, rare species comprise the majority of species in any ecosystem (Gaston 1994), yet comparatively little research is conducted on these species (Kunin & Gaston 1993; Jones et al. 2002; McClenachan et al. 2012). Rare organisms have a higher risk of extinction, and therefore a greater need for data to determine their true conservation status, risk of extinction, and changes in abundance and distribution (Diamond 1984; Soulé 1987; IUCN 2014). A taxon-specific problem when assessing fish species' risk of extinction is the difficulty in adequately assessing population size when applying International Union for Conservation of Nature (IUCN) criteria (Dulvy et al. 2003). As a result, extinction-risk assessments in fishes are frequently based on criteria such as population declines as inferred from reduced fisheries catches or from life-history characteristics such as geographic range and fecundity (Dulvy et al. 2004). These methods are effective for species exploited by fisheries but are not as efficient for those species not targeted or otherwise difficult to survey.

Although surveying rare species is a challenge, it is more problematic when species are difficult to detect. Many of the species thought to be most threatened are cryptic, which often leads to a classification of data deficient when describing their conservation status (Pearson et al. 2007; Chades et al. 2008). In contrast to other reef fishes, cryptobenthic fishes often have small geographic ranges (Goatley & Brandl 2017), which can increase a species' vulnerability to extinction (IUCN 2014). Moreover, survey methods unable to record accurate estimates of a species' abundance can produce false absence data. Consequently, management agencies have difficultly determining whether and why a cryptic species is at risk of extinction, and development of an appropriate conservation strategy is a challenge (Mackenzie & Royle 2005; Chades et al. 2008).

A range of solutions have been developed for detecting cryptic species in the terrestrial environment. Auditory surveys are frequently used to survey birds and amphibians (Celis-Murillo et al. 2009; Dorcas et al. 2009), whereas camera traps are increasingly used to monitor cryptic mammals (Tobler et al. 2008). Nonlethal methods include infrared digital cameras and hair sampling to detect rare and cryptic species such as the snow leopard (*Panthera uncia*) and small-bodyweight mammals (Jackson et al. 2006; Paull et al. 2012). These established methods are difficult or impossible to implement in the marine environment, where there are thousands of cryptic species of unknown conservation status that play an important role in ecosystem trophodynamics (Depczynski & Bellwood 2003). Because of these difficulties, research on cryptic marine species remains underrepresented, which has significant ramifications from an ecological and conservation perspective (Ackerman & Bellwood 2000; Jones et al. 2002).

Underwater visual census (UVC) has been used as a nondestructive method for measuring the abundance and distribution of reef fishes. However, biases inherent in this method, such as researcher experience and detectability of target species, can cause large interobserver variability and substantially alter survey outcomes (MacNeil et al. 2008; Bernard et al. 2013). Although UVC is a cost-efficient and effective method for larger-bodied, mobile fishes, it is less suitable to detect small and cryptic species (McCormick & Choat 1987; Samoilys & Carlos 2000). Methods that deploy video cameras record less cryptic species than UVC, unless adapted for specific target species (Colton & Swearer 2010; Lowry et al. 2011; Harasti et al. 2014). The popularity of these techniques, designed to enumerate the temporal and spatial patterns of reef fish communities, have led to a poor understanding of cryptic and rare species and their role in the broader ecosystem. Although lethal techniques such as euthanizing with rotenone are an effective way to quantify the diversity of cryptic species (Brock 1982; Kulbicki 1990), their destructive nature means they are unsuitable for threatened species (Ackerman & Bellwood 2000; Smith-Vaniz et al. 2006). Clove oil has been proposed as an alternative to rotenone; however, it is less efficient (Ackerman & Bellwood 2002). Other techniques such as environmental DNA (eDNA) are rapidly gaining popularity, but these only provide data on presence or absence and require sophisticated laboratory facilities and good reference databases (Thomsen et al. 2012). For cryptic reef fishes, gaining a full understanding of whether a species is threatened or simply undersampled can only be achieved through an appropriate census technique. Unfortunately, this is not possible with the current suite of methods.

To sample cryptic coral recruits, researchers are increasingly using a method that capitalizes on the biofluorescent nature of corals (Baird et al. 2005). Newly settled coral polyps that biofluoresce are detected using fluorescent torches, which provides an accurate method for quantifying coral settlement (Piniak et al. 2005). In reef fish, biofluorescence has only recently been discovered and is phylogenetically widespread (Sparks et al. 2014). Although the evolutionary function of fish biofluorescence is unknown, it is particularly common in reef fishes that are cryptically patterned (Sparks et al. 2014). Unlike bioluminescence (an active process whereby light is produced by an organism), biofluorescence is passive and occurs through the absorption of ambient light that is emitted at a different wavelength (Sparks et al. 2014). The work previously done on biofluorescence in fish was mostly descriptive and laboratory based and did not evaluate fish biofluorescence for practical applications (Michiels et al. 2008; Gerlach et al. 2014; Sparks et al. 2014). The recent expansion of fluorescence diving in the recreational dive industry has led to the mass production of fluorescence technology such as torches and camera filters. The availability of this technology, coupled with the discovery of widespread biofluorescence in reef fishes, led us to investigate, for the first time, its use as a survey method for detecting and quantifying cryptic coral reef fish in their natural environment. To do this, we used a fluorescent dive torch to investigate which cryptically patterned fish species emit biofluorescence. We surveyed fishes across multiple locations and multiple individuals within a species to determine spatial and taxonomic variability in biofluorescence. We used 2 seafan-associated pygmy seahorses (*Hippocampus bargibanti* [Whitley 1970], redescribed by Gomon [1997] and *H. denise* [Lourie & Randall 2003]) and 2 coral reef habitat generalists (the triplefins *Ucla xenogrammus* [Holleman 1993] and *Enneapterygius tutuilae* [Jordan et al. 1906]) to test the applicability of this technique as a survey method for estimating densities of cryptobenthic fishes.

## Methods

### Study Sites

We focused on coral reef fishes in Indonesia because this region contains the greatest number of marine fish species in the world and is thus a conservation priority. Biofluorescence surveys of reef fishes in general, and pygmy seahorse in particular, were undertaken at 63 sites in four locations in Indonesia (Bali, Nusa Tenggara, North Sulawesi, Raja Ampat). To determine whether the presence of biofluorescence in fishes varied between regions, we also surveyed two locations outside Indonesia—Christmas Island and the Cocos (Keeling) Islands in the East Indian Ocean. Surveys in Indonesia occurred from July to November 2015, and Christmas and Cocos Islands were surveyed in July 2017. Surveyed habitat varied and included fringing coral reefs, coral rubble, drop offs, and black sand slopes (site details in Supporting Information).

### Focal Species

The reef fish community was surveyed to determine the prevalence of biofluoresence in cryptic and noncryptic species. We defined cryptic fish as species that "closely resemble a part of a substratum, a plant, or a sedentary animal such as a sponge or soft coral" (Randall 2005) or species that are "behaviourally cryptic and are <50 mm total length" (adapted from Depczynski & Bellwood 2003).

To test whether the biofluorescence technique could be used to quantify the abundance of a cryptic fish species, we did dedicated surveys on 4 species. We first focused on 2 pygmy seahorse species (*H. bargibanti* and *H. denise*) because pygmy seahorses are highly cryptic fishes that are listed as data deficient on the IUCN Red List (IUCN 2017), making them of considerable conservation interest. Even though highly valued by the tourism sector (Smith 2010; De Brauwer et al. 2017), their distribution, abundance, and population size is currently unknown. *Hippocampus bargibanti* is a diminutive seahorse species found in the Coral Triangle that reaches a maximum size of 26.9 mm (standard length [SL]) (Gomon 1997). It is an obligate symbiont with gorgonian seafans of the genus *Muricella* (Gomon 1997; Reijnen et al. 2011). Individual *H. bargibanti* occur as either yellow or pink color morphs (Gomon 1997). *Hippocampus denise* reaches a maximum size of 24 mm (SL) (Lourie & Randall 2003) and occupied gorgonian seafans of the genera *Anella* and *Villogorgia* (Lourie & Randall 2003). It also occurs less frequently in *Acanthogorgia* spp., *Echinogorgia* spp. *and Subergorgia* spp. seafans (Reijnen et al. 2011). Host gorgonians are usually found in areas of high current, often in depths >14 m (Reijnen et al. 2011).

To determine whether the UBC method could be used to quantify cryptobenthic species commonly found on coral reefs, we examined 2 habitat generalist species—the largemouth triplefin (*Ucla xenogrammus*) and the highfin triplefin (*Enneapterygius tutuilae* ). Largemouth triplefins reach a maximum size of 47 mm (SL) and are distributed from Christmas Island to the central Pacific. They occur in lagoons and outer reefs and on a range of microhabitats, including corals, sponges, dead reef, and rubble (Allen et al. 2007). The highfin triplefin reaches a maximum size of 40 mm (total length) and is distributed throughout the tropical Indo-Pacific and Red Sea. It occurs on a variety of coral reef microhabitats, including tide pools, reef flats, lagoons, and outer reefs (Allen et al. 2007).

### Biofluorescence in Cryptic Fish Species

To detect fluorescence in as many fish species as possible, we used a Sola (Marina, California) Nightsea fluorescence torch and yellow barrier filter fitted to a dive mask (http://www.lightandmotion.com/sola-nightsea). A widely available and relatively inexpensive (US$550) fluorescence torch was chosen to increase accessibility and decrease costs of this method. Fifty-seven night dives were conducted on scuba or snorkel at 31 sites across the 6 locations to record and photograph fluorescing species. Surveys were conducted from 0 to 30 m and at night to increase the observability of biofluorescence. In some cases, multiple dives were done at the same site. Each fish encountered during a dive was classified as either cryptic or noncryptic, checked for fluorescence, and identified to species where possible. Surveys of multiple individuals per species were necessary to determine whether the presence and pattern of biofluorescence was consistent within a species. In some cases, the same species was surveyed at Indonesia, Christmas Island, and the Cocos Islands to test for regional consistency in biofluorescence. At the Indonesian sites, fluorescent fishes were photographed using a Canon G16 with an Isotta underwater housing and a Fisheye FIX M67 Fluo filter fitted to the lens. A Sola Nightsea torch was used as the sole light source.

### Biofluorescence as a Survey Method

The applicability of biofluorescence as a survey method was tested by comparing density estimates obtained using UBC with those obtained using traditional UVC. Two pygmy seahorses (*H. bargibanti* and *H. denise*) were surveyed during 84 SCUBA dives to a maximum depth of 40 m at 63 sites in all four locations in Indonesia. We did not survey at night because standard UVC reef fish surveys are always undertaken during the day. Daylight surveys were appropriate because biofluorescence can be observed during the day, especially in deeper water, where ambient light levels are low (Mazel 2005; Piniak et al. 2005; Schmidt-Roach et al. 2008). Because each dive was 35–60 minutes, we surveyed only seafans (*Muricella* spp., *Anella* spp., and *Villogorgia* spp.) known to frequently host the pygmy seahorse species (Reijnen et al. 2011; Smith et al. 2012). All visual surveys were done by one observer (M.D.B.), who has more than six years of experience locating pygmy seahorses.

After locating a suitable gorgonian host, a 2-minute survey was completed covering the entire area of the seafan. This was done either without fluorescence (UVC) or with a fluorescence torch and yellow barrier filter (UBC). The method was assigned randomly to each seafan, resulting in 65 fans surveyed using UBC and 81 using UVC. For biofluorescence surveys, the Sola Nightsea torch was used with the focused beam on the highest light intensity (1700 Mw), held no more than 20 cm from the gorgonian seafan. After each observation, we photographed the entire seafan and took a close-up of the polyps to allow for identification to genus.

The suitability of the biofluorescence method was then tested on two cryptobenthic species that are habitat

generalists found on coral reefs; the largemouth triplefin (*U. xenogrammus*) and the highfin triplefin (*E. tutuilae*). The largemouth triplefin was surveyed at two sites at Christmas Island and the highfin triplefin at three sites at the Cocos Islands. At Christmas Island, surveys were done while scuba diving at 15 m on the outer coral reef slope or wall where the largemouth triplefin was abundant. This species was not present at Cocos Islands, so we surveyed the highfin triplefin at 1 m while snorkelling over coral reef in the lagoon. At each site (both locations), we completed 8 replicate 20 × 2 m belt transects. Four transects were surveyed either without fluorescence (UVC) or with a fluorescence torch and yellow barrier filter (UBC). The order of the eight transects was randomised. For the biofluorescence surveys, the same Sola Nightsea torch was used with the narrow beam on the highest light intensity (1700 Mw) with a yellow barrier filter placed in front of the observer's mask. All surveys in Cocos and Christmas Island were done before sunset (17:00 and 18:15), when the light levels were suitable for UVC and UBC.

### Analyses

To test whether observed patterns in fluorescence were independent from crypsis, a Pearson's chi-square test of independence with Yates' continuity correction was conducted. Sample odds ratio was calculated, following Quinn and Keough (2002), to compare probabilities of biofluorescence in cryptic versus noncryptic species.

The number of *H. bargibanti* individuals per host seafan were recorded for each method (survey of seafan with [UBC] and without fluorescence torch [UVC]). One-tailed, *t* tests were conducted to compare numbers of *H. bargibanti* individuals detected using the different methods. One tailed *t* tests were used because it was hypothesized that the UBC method would result in an increase in detections. Pearson's chi-square and one-tailed *t*-tests were conducted using R (R Development Core Team 2010). Data on *H. denise* were not subjected to analysis because of very low sample size ($n = 7$ across all locations). The mean number of *U. xenogrammus* detected per transect for each method was compared with one-tailed *t* tests in R (R Development Core Team 2010). Data for *E. tutuilae* were square-root transformed to meet assumptions of normality and then compared with one-tailed *t* tests in R (R Development Core Team 2010).

## Results

### Biofluorescence of Cryptic Fish Species

During the night surveys, 1528 individuals from 230 species were observed. Ninety-five fish species (887 individuals) showed obvious biofluorescence, of which 73 species were previously unknown to fluoresce (full list in Supporting Information). Of the 95 cryptic species encountered, 83 (87.3%) fluoresced (Fig. 1). In contrast, only 12 (8.9 %) of the 135 noncryptic species encountered during the night surveys fluoresced (Fig. 1). The probability of exhibiting biofluorescence was 70.9 times greater for cryptic species than for non-cryptic species ($\hat{\theta} = 70.9$; $\chi^2 = 138.44$, df $= 1$, $p < 2.2 \times e^{-16}$). A cryptic species was 6.9 times more likely to be fluorescent than nonfluorescent relative to a noncryptic species, which was 10.3 times less likely to be fluorescent than nonfluorescent.

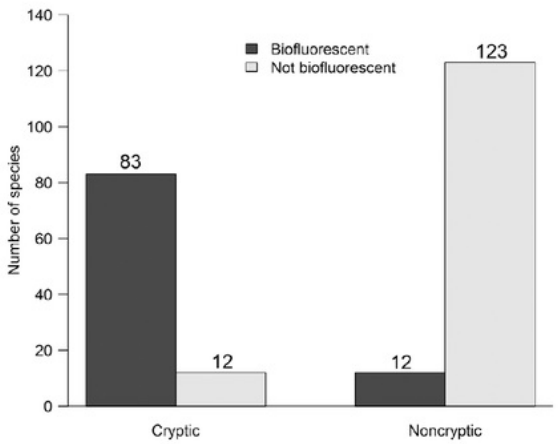


*Figure 1. Number of cryptic and noncryptic species observed to exhibit biofluorescence.*

We observed 49 families of fishes, of which 27 families had at least one biofluorescent species, whereas 22 families did not have any fluorescing species (complete list in Supporting Information). Reliable identification to species level was not always possible for *Gobiidae* and *Tripterygiidae*; therefore, these were excluded from further analyses, although more than four different fluorescing species were found in each family (details in Supporting Information). In the 13 families for which found more than five species, the families with the highest percentage of cryptic species also contained the highest percentage of fluorescent species (Fig. 2). From this subgroup, three families had only cryptic species, all of which exhibited biofluorescence. In contrast, five families lacked cryptic species and had no fluorescent species. The *Nemipteridae* family was an exception to this trend, none of the species observed were cryptic, but all showed biofluorescence ($n = 5$) (Fig. 2, photos in Supporting Information).

### Biofluorescence as a Survey Method

Pygmy seahorses were rarely encountered. Thirty-two *H. bargibanti* and 7 *H. denise* were found during the seafan

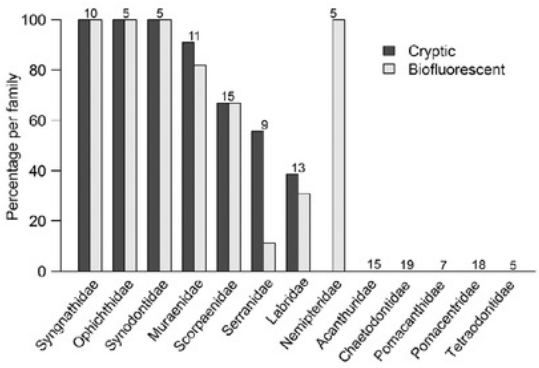


*Figure 2. Comparison of percentage of species per family exhibiting biofluorescence and crypsis (numbers above bars: number of species surveyed per family).*

surveys. All individuals fluoresced in the red and green spectrum. Red fluorescence was invariably strongest in the tail of the seahorse, and only the eyes emitted green fluorescence (Fig. 3). The host gorgonian seafans (*Muricella* sp., *Anella* sp., *Villogorgia* sp.) were never observed to fluoresce.

For *H. bargibanti*, 146 *Muricella* sp. seafans were surveyed, 65 with UBC and 81 with UVC. On average, nearly double the number of *H. bargibanti* were detected using the fluorescence method compared with the non-fluorescent surveys (Fig. 4), although the difference between survey methods was not statistically significant at $\alpha = 0.05$ for this species ($t = -1.28$; $p = 0.13$). Of the 32 individual *H. bargibanti* observed, 20 were found using the UBC method and 12 individuals were located using only visual surveys. Using the UBC method, an average of 0.31 (SE 0.07) seahorses were detected per seafan, compared with 0.15 (SE 0.05) with the UVC method (Fig. 4). Because of the extremely low abundance, there were insufficient data to statistically analyze *H. denise*.

Nearly 3 times more largemouth triplefin were detected in UBC transects ($n = 23$) than in UVC transects ($n = 8$). Per transect significantly higher numbers (mean = 2.9 individuals/40 $m^2$ [SE 0.5]) were found in UBC surveys than in UVC surveys (mean = 1.0 individuals/40 $m^2$ [SE 0.2]; $t = -2.6112$; $p = 0.03$) (Fig. 4). At the Cocos Islands, 139 highfin triplefins were encountered in 24 transects, and the mean density detected in UBC transects (mean = 8.8/40 $m^2$ [SE 1.1]) was more than triple that recorded in UVC transects (mean = 2.8 individuals/40 $m^2$ [SE 0.4]) ($t = -4.258$; $p < 0.001$) (Fig. 4).

## Discussion

Our results demonstrate that the UBC method is effective at finding cryptic species that are otherwise hard to detect and quantify. We found that biofluorescence was ubiquitous in cryptic species within and outside of the center of reef fish biodiversity (the Coral Triangle), which means our method is applicable across a wide range of species and geographic locations. We also found that UBC could be used to gather data on abundance, distribution, and habitat use of common (triplefins) and vulnerable (pygmy seahorses) cryptic species. The use of efficient survey methods like UBC could help assess species' extinction risk, based on IUCN criteria, to ensure adequate protection for rare species, shed light on the ecological roles of cryptic species, and potentially answer the long-standing question of whether cryptic species are indeed rare or merely under sampled (Jones et al. 2002).

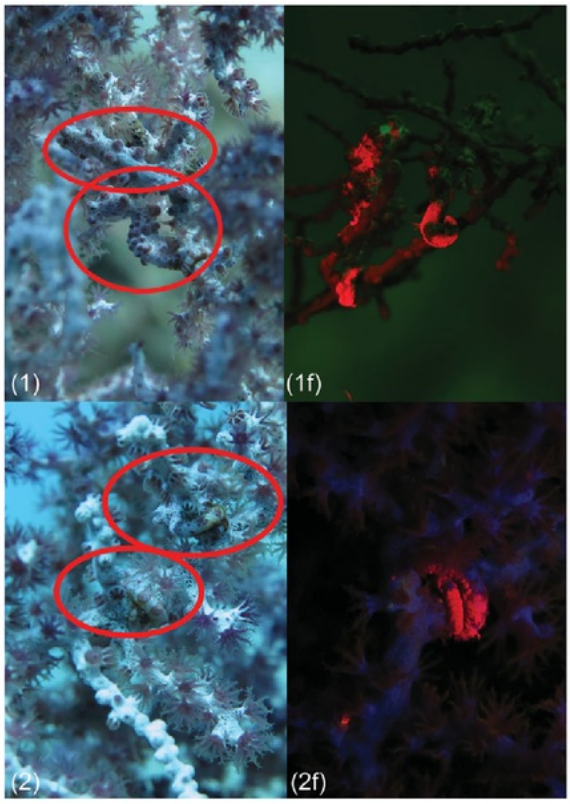


*Figure 3. Bargibant's pygmy seahorse (*Hippocampus bargibanti*) (1, 2) under ambient light and (1f, 2f) in the underwater biofluorescence census (1 and 1f, entire body of the seahorses; 2 and 2f, close-up of the tails ; 1 and 2, same 2 animals by day; red circles, individual seahorses; 1f, taken by day with high-intensity blue LED torch and yellow filter, different animals; 2f, taken at night with high-intensity blue LED torch and yellow filter, same animal as in 1 and 2).*

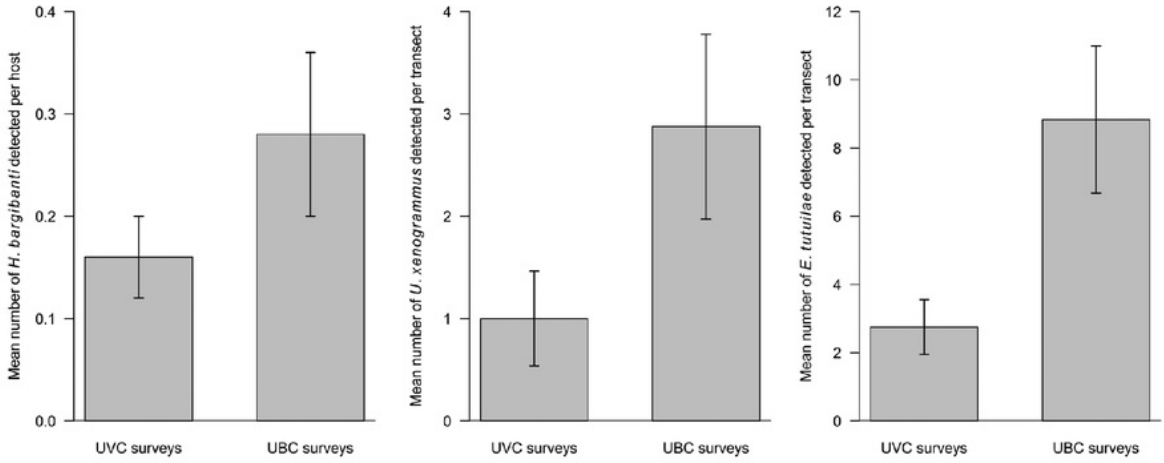


*Figure 4. Number of detected individuals of* Hippocampus bargibanti *(Indonesia),* Ucla xenogrammus *(Christmas Island), and* Enneapterygius tutuilae *(Cocos Island) with normal visual surveys (UVC) compared with underwater biofluorescence census (UBC) surveys. Detections of H. bargibanti are the mean number of individuals found per gorgonian seafan host, and detection of* U. xenogrammus *and* E. tutuilae *are the mean number of individuals found per 40-m$^2$ transect.*

Currently, methods to detect cryptic species are often destructive or inefficient. Destructive methods such as the use of rotenone are efficient at detecting cryptic species, but in the case of rare or threatened species it is counterproductive to kill individuals while surveying them (Ackerman & Bellwood 2000; Smith-Vaniz et al. 2006). Standard UVCs have repeatedly been shown to underestimate the abundance of cryptic fish species and are affected by species detectability and observer experience (McCormick & Choat 1987; Samoilys & Carlos 2000; Bernard et al. 2013), which may be why cryptic species are rarely included in marine diversity assessments (e.g., Sandin et al. 2008; Osborne et al. 2013; Go et al. 2015). In our study, the UBC method consistently detected more individuals of 3 reef fish species than traditional UVCs, a result consistent across different locations and depths. Our UBC method is nondestructive, which makes it more suitable for monitoring potentially rare species. The increased detection probability with the UBC method is likely to decrease interobserver variability and therefore increase the accuracy of surveys (MacNeil et al. 2008). The high incidence of biofluorescence in other cryptic fish means the UBC method could be applicable to a wide host of other cryptic species.

The UBC method was effective for habitat specialists (pygmy seahorses) and habitat generalists (triplefins). Furthermore, we found other cryptic and highly abundant species such as Gobies (*Gobiidae*) displayed stronger fluorescence and would therefore be even more suitable to survey with this method. This is an important discovery because these families are often abundant and ubiquitous on coral reefs yet are nearly always ignored during UVC surveys such that their importance to reef ecosystems is grossly underestimated (Depczynksi & Bellwood 2003; Lefèvre et al. 2016). Our surveys across multiple individuals and locations consistently showed which species exhibited biofluorescence (and which did not), highlighting the utility of the UBC approach. Variation in the body location and strength of the biofluorescence was apparent between families of cryptic species. Determining the cause of this variation and why biofluorescence is more prevalent in cryptic than noncryptic species are pertinent questions for future research. Our observations of biofluorescence in numerous invertebrates (*Decapoda*, *Polychaeta*, *Cephalopoda*) suggests the UBC method has considerable potential to be expanded beyond fish taxa when surveying for diversity and conservation purposes.

The UBC works best when natural light is low or absent. Thus, we recommend surveys be conducted during times of low light (e.g., overcast, around dawn or dusk) or at night. The UBC surveys can still be effective during the middle of the day in low-light habitats (e.g., shaded walls, deeper water). Biofluorescence is also more obvious particularly against backgrounds that provide maximum contrast. For example, in pygmy seahorses, the contrast between the nonfluorescent host gorgonians and the fluorescent nature of their tails proved an effective method to locate and quantify individuals. Thus, the optimal approach for surveying cryptic species with UBC will need to consider the light and contrast conditions given the ecology (habitat, depth range) and behavior (nocturnal, diurnal, crepuscular) of the study species. Given species' variability in strength of fluorescence, we

advise assessment of target species' suitability prior to commencing UBC surveys.

The biggest hurdle to assessing the conservation status of cryptic species is establishing their population size. Many reef fishes are easily observed with UVC, as a result conservation strategies to protect these species are well advanced (Duarte et al. 2008; McClenachan et al. 2012). Conversely, cryptic species are difficult to locate and require experienced observers or complex methods to quantify their abundance (Ackerman & Bellwood 2000; Smith et al. 2012). The lack of efficient survey methods for cryptic species has resulted in a significant data shortage on their extinction. Consequently, the marine environment has double the number of species listed as data deficient compared with terrestrial species (Webb & Mindel 2015). Most of those assessed to date belong to a few well-studied taxonomic groups or commercially important fish (McClenachan et al. 2012).

For three large, cryptic families (*Gobiidae*, *Scorpaenidae*, *Syngnathidae*), representing over 2000 species globally, <44% have been assessed for their extinction risk (Nelson 1994; IUCN 2017). Twenty-one percent of the species in these families that have been assessed are considered data deficient (IUCN 2017). Because cryptic species represent more than 60% of fish numbers on coral reefs and are crucial for a well-functioning marine ecosystem (Depczynski & Bellwood 2003; Depczynski et al. 2007), it is critical this issue be resolved. The UBC survey method provides a nondestructive way to obtain the abundance and distribution data needed when applying IUCN criteria B (extent of occurrence and area of occupancy) and D (small or restricted population). Furthermore, repeated UBC surveys can provide data pertinent to IUCN criteria A (population declines) when assessing the conservation status of hundreds of cryptic species.

A sound knowledge of the abundance and distribution of species is crucial to implementation of effective protective measures. We demonstrated that biofluorescence is common in cryptically patterned marine species in the global center of reef fish biodiversity and provide a much-needed, nondestructive survey method to identify, quantify, and ultimately protect cryptic species for which data are currently lacking. Fluorescent diving torches are widely available, easy to use, and inexpensive, opening the way for the UBC survey method to be adopted globally by underresourced marine conservation groups and for conducting IUCN assessments of extinction risk. Similarly, there is great potential for the UBC method in citizen science for collecting much-needed data on rare and endangered species due to the low cost and user-friendly nature of the method (Louv et al. 2012; Edgar et al. 2017). We propose that the use of the UBC survey method is a cost-effective tool to detect and count rare and cryptic species that will facilitate future research and further much-needed conservation initiatives globally.

## Acknowledgments

Logistical support was provided by Black Sand Dive Retreat, Critters@Lembeh, Froggies Divers, Nomad Divers Bangka, Safari Bali, and Sea Safari. We thank D. Dumitrescu and L. Gordon for assistance during fieldwork and help with photography. We also thank B. Saunders for useful statistical insights and M. Bunce for useful comments and inspiration. Research within Indonesia was permitted through a RISTEK research permit granted to M.D.B. in collaboration with J.J. and R.A.R. Research in Christmas Island was conducted by J.P.H. under the appropriate permits from Christmas Island National Park. A Society for Conservation Biology Marine Section Small Grant provided funding for surveys in Bangka Island, Indonesia.

## Supporting Information

A map of survey locations (Appendix S1), full list of all species surveyed (Appendix S2), examples of biofluorescence in the same individuals of 4 different species (Appendix S3), pictures taken at night with a normal torch for lighting and a picture taken by day in only ambient light (Appendix S4); and pictures taken at night with high intensity blue LED torch and yellow filter (Appendix S5) are available online. The authors are solely responsible for the content and functionality of these materials. Queries (other than absence of the material) should be directed to the corresponding author.

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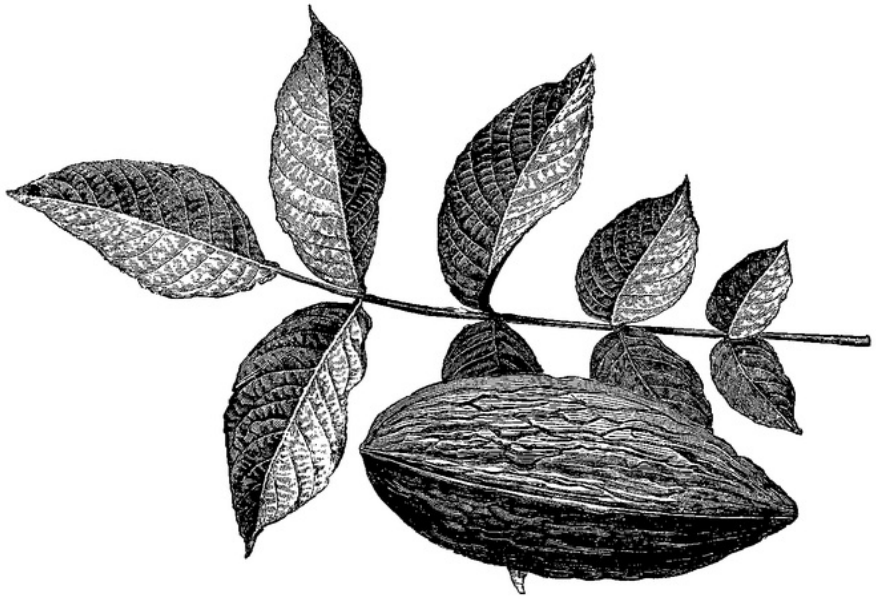

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