

First use of acoustic calls to distinguish cryptic members of a fish species complex

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Although molecular methods and bioacoustical analysis have been used to uncover cryptic species, the combination of both methodologies is still rare. The humbug damselfish complex, *Dascyllus aruanus*, is composed of at least two species with *Dascyllus aruanus* in the Pacific Ocean and *Dascyllus abudafur* in the Indian Ocean. However, genetic data suggest that additional species could be found. Therefore, we aimed to determine whether the sounds produced by different populations of fish could help to distinguish cryptic species. Recordings of chase and courtship sounds were made on humbug damselfish populations from Madagascar, Taiwan and French Polynesia. Chase sound features are more variable than courtship sounds, suggesting more constraints on courtship sounds, since they would contribute to premating isolation. Comparison between courtship sounds show the variation in acoustic features between Taiwan and Madagascar align with genetic differences, supporting that sounds could discriminate cryptic species in Teleosts. Moreover, differences in both acoustic features and genetic data are also found between Taiwan and French Polynesia, suggesting two clearly distinct populations. Consequently, the name *D. emamo* can be resurrected for the Polynesian humbug damselfish. External phenotype traits do not allow the distinction between populations, illustrating that only behaviour has been modified.

ADDITIONAL KEYWORDS: bioacoustics – biodiversity – biogeography – communication – Pomacentridae – sibling species.

INTRODUCTION

Although the concept of species is still debated, species can be defined as groups of interbreeding populations reproductively isolated from other such groups (Coyne & Orr, 1989; Mayr, 2001). The main challenge is to identify what kind of isolating barriers can interrupt the gene flow within a population and originate new species (Irwin *et al.*, 2005). The classical mode of speciation concerns geographically isolated lineages resulting from landscape change (Harrison, 2012). A growing number of studies have reported that dispersal events could lead to geographical

isolation and the production of subpopulations called demes (Smith *et al.*, 2014). In large geographic areas, additional phenomena can drive the development of clines where different populations show a gradual variation of some characters (e.g. phenotypic, genetic, ecological and behavioural) along a geographical axis. Clines may be discontinuous when they involve isolated groups and continuous when groups possess contacts (Salomon, 2002). Clines can pre-date speciation since neighbouring populations can interbreed, whereas this is not the case between distant populations. These populations help us to understand the history of divergence of some species from their common ancestor and demonstrate that speciation can occur despite gene flow between the diverging forms (Irwin *et al.*, 2001b, 2005).

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Cryptic species are classified as a single nominal species mainly because individuals share a phenotype (Bickford *et al.*, 2007). Many species with a broad geographic range could actually consist of a series of cryptic species (Borkin *et al.*, 2004). Currently, such cryptic species are often discovered and separated by using molecular or genetic data (Jörger & Schrödl, 2013) but they can also be distinguished on the basis of other traits. In this context, signals used to communicate (Thabah *et al.*, 2006; Funk *et al.*, 2012; Vigoder *et al.*, 2013) are relevant, because differences in these traits might play a central role in reproductive isolation and speciation processes (Irwin *et al.*, 2008). Several studies have shown that mating calls can be used to distinguish between cryptic species in insects (Henry, 1994; Cole, 2008), amphibians (Borkin *et al.*, 2004), birds (Jones, 1997; Irwin *et al.*, 2001a) and mammals (Burton & Nietsch, 2010). The determination of cryptic species using acoustic signals in combination with additional data has never been realized in fishes. However, there are indications it should be possible (Raick *et al.*, 2020). For example, the cusk-eels *Ophidion barbatum* Linnaeus, 1758 and *O. rochei* Müller, 1845 present few differences in their external phenotype (Casadevall *et al.*, 1996), but they show remarkable internal differences between their sound-producing apparatuses, supporting the production of different calls (Casadevall *et al.*, 1996; Parmentier *et al.*, 2006a, 2010a; Picciulin *et al.*, 2019). It is worth mentioning that sounds have been used to locate ‘ecologically cryptic’ fish (Picciulin *et al.*, 2019), the meaning of the word being different in this case because it refers to camouflaged or secretive species (Claridge *et al.*, 2004).

Damselfish are well-known vocal species inhabiting coral reefs worldwide (Lobel *et al.*, 2010). Some species are even able to emit different kinds of sounds in various behavioural contexts (Mann & Lobel, 1995, 1998; Parmentier *et al.*, 2006b, 2010b; Colley & Parmentier, 2012; Parmentier & Frédéricich, 2016). In 2009, a study focusing on sound production in different damselfish species highlighted that the population of the humbug damselfish *Dascyllus aruanus* (Linnaeus, 1758) from Madagascar produces sounds with some features, i.e. pulse period and inter-pulse interval period, that are significantly different from those of the *D. aruanus* population living in French Polynesia (Parmentier *et al.*, 2009). The resulting hypothesis was the existence of a dialect for each population of this species. Interestingly, five years later, studies conducted on mitochondrial and nuclear DNA suggested that populations found on either side of the Indo-Pacific barrier were genetically different and could result from a vicariant event (Borsa *et al.*, 2014; Liu *et al.*, 2014). At the phenotype level, individuals from the Indian Ocean exhibited a blackish blotch at the base of the caudal fin, while individuals

from the Pacific Ocean had an unpigmented caudal fin (Borsa *et al.*, 2014). However, according to the authors, this feature is not fully reliable in the field. The authors suggested the resurrection of the name *Dascyllus abudafur* (Forsskål, 1775) for the Indian Ocean populations and to keep *Dascyllus aruanus* for the Pacific populations. The status of *D. abudafur* is now validated (Eschmeyer *et al.*, 2019; Tang *et al.*, 2021). Because *D. aruanus* in French Polynesia have a distinct genetic composition compared to other Pacific populations (Liu *et al.*, 2014), the population from the Society Islands (French Polynesia) can be separated from the rest of the Pacific populations as a third species. Here, the phenotype could not help as no reported phenotypic difference exists between the Polynesian and the other Pacific populations.

To add to our understanding of the *Dascyllus aruanus* complex, we analysed calls from various sites, covering populations from different localities. We use the term species complex, because the study concerns populations with unclear boundaries and possibly made of more than one species (Derycke *et al.*, 2008; Evin *et al.*, 2008).

We identified and quantified the acoustic features characterizing the calls, because we hypothesized that acoustic signals could help to differentiate different populations and validate the existence of cryptic species. Therefore, we compared acoustic features from distinct populations coming from Madagascar, Taiwan and French Polynesia. Knowing that a single damselfish species can make different kinds of sounds, we first used video recordings to identify the different behaviours and their associated sounds. Then, we focused on the most abundant kind of sounds (signal jump or courtship sounds) to conduct comparisons between groups. Additionally, we compared courtship and chase sounds, because they are produced in different kinds of behaviour, and we briefly investigated meristic data from the three populations to look for useful morphological differences.

MATERIAL AND METHODS

ACOUSTIC DATA

The study was conducted at three different sites. In the lagoon of Tulear (Mozambique Channel, west coast of Madagascar, 23°22'S, 43°40'E), data were first collected during 2-h recording sessions in November 2005. Sea water in the lagoon was on average 26 °C. Sounds were recorded using an Orca hydrophone (sensitivity: -186 dB re 1 V μPa^{-1}) connected via an Orca-made amplifier (ORCA Instrumentation) to a Sony TCD-D8 digital audio tape-recorder (flat frequency response range: 20 Hz–22 kHz, sampling rate of 44.1 kHz, 16-bit resolution). The hydrophone was attached to a buoy so that it floated 0.5 m above the different coral

patches on which *Dascyllus* G. Cuvier, 1829 lived; the water depth was between 1 and 3 m on a flat bottom. All recordings were made with fish approximately at 0.5–2.0 m from the hydrophone. Short video sequences were also recorded with a Digital Olympus video camera (Olympus Corporation, Tokyo, Japan) placed in an underwater housing. A previous study conducted on these sounds (Parmentier *et al.*, 2009) showed they were all produced during courtship behaviour and associated with the stereotyped ‘signal jump’ (Myrberg *et al.*, 1978; Mann & Lobel, 1997). A second session was conducted during May and June 2019 using SNAP underwater acoustic dataloggers set to record for 3 min every 10 min (hydrophone sensitivity: -170 dB re 1 V μPa^{-1} , flat frequency response range: 2 Hz–30 kHz, sampling rate of 44.1 kHz, 16-bit resolution; Loggerhead Instruments, FL, USA) deployed on a coral patch occupied by *Dascyllus*.

In Taiwan, field recordings were made at Dongsha Atoll National Park ($20^{\circ}41'N$, $116^{\circ}43'E$) from April to May 2015 at a depth between 1 and 3 m in a lagoon close to seagrass beds. Sea water in the lagoon was on average 29°C . Recording sessions lasted from 1 to 4 h and were carried out at five different sites that were separated by at least 20 m. Audio and video recordings were made using a SONY HDD video camera placed in a housing (HC3 series, Ocean Images, Cape Coral, FL, USA) and coupled with an external hydrophone HTI96 (High Tech. Inc., Long Beach, MS, USA) (flat frequency response range: 20 Hz–20 kHz, sensitivity: -164 dB re 1 V μPa^{-1} ; Loggerhead Instruments Inc., Sarasota, FL, USA).

In French Polynesia, specimens were recorded in front of Opunohu reef, Moorea Island ($17^{\circ}29'S$, $149^{\circ}51'W$) in June 2017 and from May to June 2019. The sea water in the lagoon was on average 28°C . Recordings were made following the same protocol and using the same materials as in Taiwan. A second session was conducted in June 2019 by using SNAP underwater acoustic dataloggers set to record for 10 min every hour (hydrophone sensitivity: -170 dB re 1 V μPa^{-1} , flat frequency response range: 2 Hz–30 kHz, sampling rate of 44.1 kHz, 16-bit resolution; Loggerhead Instruments, FL, USA)

The use of different recorders between and within locations should not affect the different features we have measured. Also, this kind of method (Passive Acoustic Recording) cannot provide the exact number of specimens that produced sounds. Recordings were sub-sampled at 4000 Hz and analysed using Avisoft-SASLabPro 5.2.13 software (Avisoft Bioacoustics, Glienicke/Nordbahn, Germany). Sound types attributed to *Dascyllus* were visually and aurally detected by inspecting the oscillograms (representation of the amplitude as a function of time) and spectrograms (representation of the frequency

composition as a function of time) of the recordings. Only the sounds with a high signal-to-noise ratio were used in the analysis. Temporal features were measured from oscillograms, and frequency features were obtained from power spectra [Fast Fourier Transform (FFT), 256 points, Hann window]. The sound features measured from the oscillograms were: sound duration (in ms); number of pulses in a sound; pulse period (measured as the average peak-to-peak interval between consecutive pulses in a sound, in ms); interpulse interval (IPI, measured as the time from the end of one pulse to the beginning of the next, in ms); and pulse duration (measured as the time from the beginning of one pulse and its end, in ms). Spectral characteristics of sounds, obtained from power spectra [Hamming window, fast Fourier transform (FFT): 512 points] allowed measurement of dominant frequency, i.e. frequency component with the most energy (Hz). The dominant frequency was measured for each individual pulse within a pulse train.

MORPHOLOGICAL DATA

In each location, damselfish specimens were collected by scuba-diving in the lagoon in water from 1.0 to 3.5 m depth using hand nets. In this part of the study, we used a total of 32 specimens: 11 specimens from Madagascar, nine specimens from Taiwan and 12 specimens from French Polynesia.

Five specimens from each location were randomly selected to look for morphological differences. Selected meristic features are classically used in taxonomic studies; these include the number of spines and rays in the anal, dorsal, ventral, caudal, pelvic and pectoral fins, the number of the vertebra that bears the last abdominal rib, total number of vertebrae and number of abdominal vertebrae, lateral line scale count (includes only scales with tubes) and supraneural bones. All 15 specimens were cleared and stained with Alizarin red S using the method of Taylor and Van Dyke (1985) to reveal the skeletal structures.

Six specimens from Madagascar, four specimens from Taiwan and seven specimens from French Polynesia were added to the first lot of cleared and stained specimens to measure morphometric data. According to the study of Pyle and colleagues (Pyle *et al.*, 2008), multiple measurements were made from each specimen. Total length is the distance between the tip of the upper lip and caudal tip of the caudal fin; standard length is the distance between the tip of the upper lip and the posterior edge of the hypural plate; head length is between the tip of the upper lip and the posterior edge of the opercle; depth is the maximum vertical distance between belly and base of dorsal spines; snout length is the distance from the tip of the upper lip to the anterior edge of the orbit; orbit

diameter is eye diameter; interorbital width is the least bony width between the eyes; upper jaw length is the distance from the tip of the upper lip and the posterior tip of the maxilla; caudal peduncle is least depth; length of dorsal spine 1, 2 and 3 and of anal spines 1 and 2; length of the longest pectoral-fin ray.

All the fish were examined using a Wild M10 binocular microscope with a camera lucida attached and measurements were taken with a digital calliper. Measurement values were recorded to the nearest hundredth (0.01) of a mm. *Dascyllus aruanus* is a species with protogynous sex change (female to male), so the sex does not affect the skeletal meristics (Hattori & Casadevall, 2016).

Different specimens were deposited in the Royal Belgian Institute of Natural Sciences in Belgium. *Dascyllus aruanus* from Dongsha Atoll (Taiwan): IRSNB 25994 – 25995 – 25999 and from Moorea (French Polynesia): 25996 – 25997 – 25998 – 26537 – 26539. *Dascyllus abudafur* from Tulear (Madagascar): IRSNB 25936 – 25937 – 25938.

STATISTICS

The Shapiro–Wilk normality test was used to test the normality of the data. The homoscedasticity of the variances was checked with the Bartlett test. Wilcoxon–Mann–Whitney or Student *t*-tests were used to compare sound characteristics between sound types, and Kruskal–Wallis tests were used to compare sounds between populations. Kruskal–Wallis tests were followed by Dunn’s multiple comparison tests for pairwise comparisons. A principal component analysis (PCA) was run on the individual means of the six acoustic features to give an overview of the potential acoustic differences between populations. Spearman’s rank correlation coefficient (*r*_S) was used to quantify the relation between principal components and acoustic features. The *F*-test of equality of variances was used to compare the variances of the PCs between the different sound types. A cross-validated linear discriminant analysis (LDA) was then carried out on the standardized acoustic features to discriminate the groups. We used LDA to study group differences by using the sonic variables simultaneously. The LDA is a multivariate method that is frequently used in bioacoustic research to examine whether calls from different populations can be distinguished by their acoustic properties (Burton & Nietsch, 2010). The LDA calculates several linear functions that we used in a classification procedure to assign each group to its appropriate population (correct assignment) or to another population (incorrect assignment). The cross-validated LDA was realized with a hold-out cross-validation. The fraction of data used for training was

66.6% and 50 random permutations were carried out. The LDA also permits an assessment of how well sounds can be assigned to the correct population. All the statistics were carried out with R 3.5-1 (R Core Team, 2018) and the significance level was $\alpha = 0.05$.

According to the availability of the data, two PCA were realized on the correlation matrices on the morphometric features that were previously divided per the SL. The first PCA ($N = 22$ specimens) concerned the features that were measured on both cleared and intact specimens: body depth, head length, interorbital width, longest pectoral ray, orbit diameter, peduncle height and snout length. The second PCA ($N = 12$ specimens) has been realized on morphometric features measured on cleared and stained specimens. The features were lower jaw length, upper jaw length, lengths of anal spines 1 and 2 and lengths of dorsal spines 1, 2 and 3. In each case, spearman correlation (*r*_S), and associated *P*-values corrected by the Holm method, between meristic features, PC1 and PC2, were calculated with a correlation matrix for each PCA. Kruskal–Wallis tests were used to compare PCs between populations. Kruskal–Wallis tests were followed by Dunn’s multiple comparison tests for pairwise comparisons (with Benjamini & Hochberg corrections).

RESULTS

DIFFERENT TYPES OF SOUNDS

Damselfish can produce sounds during six different behaviours: conspecific fighting, heterospecific fighting, conspecific chasing, heterospecific chasing, signal jumps and mating/visiting, (Luh & Mok, 1986; Lobel & Mann, 1995; Mann & Lobel, 1998; Parmentier *et al.*, 2009, 2010b). Thanks to videos recorded in Taiwan and French Polynesia, all these behaviours, except mating/visiting, were observed. Tulear recordings did not include videos, but signal jumps were visually identified by the investigators. All sounds consisted of pulses that were isolated or emitted in trains. The low amount of conspecific fighting ($N = 1$) and heterospecific chasing ($N = 11$) recorded did not allow statistical comparison. Consequently, these results were not analysed in the present study. Heterospecific fighting occurred between *D. aruanus* and *D. flavicaudus* Randall & Allen, 1977, but it was not possible to identify the emitter. Sound types emitted during signal jumps and conspecific chasing differ (Table 1). In both Taiwan and French Polynesia, conspecific chasing sounds were significantly shorter (Wilcoxon–Mann–Whitney; French Polynesia: $W = 412$, $P < 0.001$; Taiwan: $W = 1108$, $P < 0.01$) and made of fewer pulses (Wilcoxon–Mann–Whitney; French Polynesia: $W = 178$,

$P < 0.001$; Taiwan: $W = 777, P < 0.001$) than signal jump sounds. On the other hand, pulse duration (French Polynesia: Wilcoxon–Mann–Whitney, $W = 1455, P < 0.001$; Taiwan: Student t -test, $t = 11, P < 0.001$) and pulse period (Wilcoxon–Mann–Whitney; French Polynesia: $W = 1041, P < 0.001$; Taiwan: $W = 2265, P < 0.001$) were significantly longer during conspecific chasing than during signal jumps. The interpulse interval was longer in French Polynesia for the conspecific chasing sounds in comparison to the signal jumps (Wilcoxon–Mann–Whitney, $W = 826, P < 0.05$) but no statistical difference was found between both types of sounds in Taiwan (Wilcoxon–Mann–Whitney, $W = 1404, P = 0.74$). The dominant frequency of sounds was significantly higher during conspecific chasing than during signal jumps in both locations (Wilcoxon–Mann–Whitney; French Polynesia: $W = 1347, P < 0.01$; Taiwan: $W = 2157, P < 0.001$).

SIGNAL JUMPS AND GEOGRAPHY

The sound duration, number of pulses, interpulse interval and dominant frequency were significantly different among all the three fish populations (Kruskal–Wallis, $\chi^2 = 36.225, d.f. = 2, all P < 0.001$). Duration and number of pulses were higher in the Madagascar population than in French Polynesia, whereas dominant frequency was lower; signal jump for the Taiwan population showed intermediate positioning. The interpulse interval was longer in Taiwanese fish than in Madagascar, and shorter in French Polynesia (Table 1). The pulse period did not differ between Madagascar and Taiwan (Kruskal–Wallis, $\chi^2 = 42, d.f. = 2, P < 0.001$; Dunn, $Z = -0.94, P = 0.35$) and the pulse duration was not different between Madagascar and French Polynesia (Kruskal–Wallis, $\chi^2 = 36, d.f. = 2, P < 0.001$; Dunn, $Z = -1.83, P = 0.07$).

The acoustic diversity associated with signal jumps was summarized by a PCA based on the six acoustic features (Fig. 1). The first two principal

components of the PCA explained cumulatively 74.1% of the variation, with PC1 and PC2 explaining, respectively, 45.1% and 29% of the variation. The duration ($rS = 0.93, P < 0.001$), the pulse number ($rS = 0.86, P < 0.001$) and the dominant frequency ($rS = -0.83, P < 0.001$) mostly contributed to the first principal component, whereas the interpulse duration ($rS = -0.84, P < 0.001$) and the pulse period ($rS = -0.68, P < 0.001$) were principally associated with the second principal component. The populations from the three sites are easily distinguished within this acoustic space, especially along PC1. PC1 supports significant differences between the signal jumps of the three sites (Kruskal–Wallis, $\chi^2 = 197, d.f. = 2, P < 0.001$; Dunn, $Z = 13.95, 6.91 \text{ \& } -5.73, all P < 0.001$) whereas PC2 isolates Taiwan (Kruskal–Wallis, $\chi^2 = 49, d.f. = 2, P < 0.001$; Dunn, $Z = 0.29$ and $P = 0.77$ between Madagascar and French Polynesia, $Z = 6.55$ and $P < 0.001$ between Madagascar and Taiwan, $Z = 5.93$ and $P < 0.001$ between French Polynesia and Taiwan). An LDA was then performed on the six standardized features of the signal jumps of the three sites to complement the PCA. The contingency table supports that sounds were highly correctly assigned to their group: the classification rates were 98.6% in Madagascar, 68.8% in Taiwan and 81.3% in French Polynesia (Table 2). Among misidentifications, 71.4% were made between French Polynesia and Taiwan, while the sounds of French Polynesia were almost never classified with sounds from Madagascar and vice versa (0.4% of the misidentifications).

CONSPECIFIC CHASE COMPARISON

Acoustic features of conspecific chasing were compared between Taiwan and French Polynesia. Madagascan sounds could not be included as only signal jump sounds were recorded at the time of the field mission in 2005. There was no significant difference in the pulse duration (Student t -test; $t = -0.2, P = 0.82$),

Table 1. Comparison of the different call characters in different populations from the *Dascyllus aruanus* complex. CC = conspecific chase; SJ = signal jump; numbers between brackets correspond to the number of analysed sounds

	<i>D. abudafur</i> (Madagascar)	<i>D. aruanus</i> (Taiwan)	<i>D. aruanus</i> (French Polynesia)	<i>D. aruanus</i> (Taiwan)	<i>D. aruanus</i> (Tahiti)
Behaviour	SJ ($N = 123$)	SJ ($N = 73$)	SJ ($N = 93$)	CC ($N = 43$)	CC ($N = 21$)
Duration (ms)	181 ± 35	131 ± 48	85 ± 30	113 ± 84	48 ± 31
# pulses	7 ± 1	5 ± 2	4 ± 1	4 ± 2	2 ± 1
Pulse duration (ms)	12 ± 2	10 ± 2	12 ± 3	17 ± 4	17 ± 6
Pulse period (ms)	28 ± 2	29 ± 3	25 ± 5	36 ± 9	38 ± 12
Interpulse interval (ms)	17 ± 2	19 ± 36	14 ± 5	19 ± 8	21 ± 12
Dominant frequency (Hz)	472 ± 37	638 ± 93	746 ± 84	709 ± 96	923 ± 305

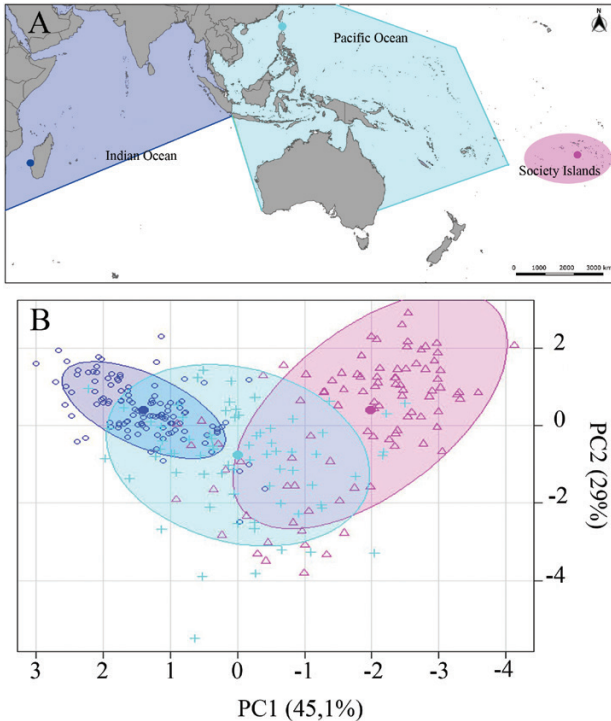


Figure 1. Background shading indicates the geographical distribution of the three populations (Liu *et al.*, 2014) from the *Dascyllus aruanus* complex. A, in each geographical area, dots correspond to sampling locations. B, scatterplot of principal component PC1 versus PC2, performed with individual mean values of the six acoustic properties of calls corresponding to the signal jump for the three suggested species: *D. abudafur* (blue ●), *D. aruanus* (turquoise +) and *D. emamo* (pink▲). Ellipses correspond to 90% of the observations, dots correspond to the ellipse centra.

Table 2. Classification accuracy of signal jump sounds based on the linear discriminant analysis in *Dascyllus aruanus* complex

Class	Madagascar	Taiwan	French Polynesia	N
Site				
Madagascar	98.6%	1.4%	0%	73
Taiwan	6.8%	82.2%	11%	73
French Polynesia	0%	21.9%	78.1%	43

pulse period (Wilcoxon–Mann–Whitney; $W = 295$, $P = 0.48$) and interpulse duration (Wilcoxon–Mann–Whitney; $W = 272$, $P = 0.81$) between populations from Taiwan and French Polynesia (Table 1). However, sound duration and number of pulses were significantly higher in Taiwan than in the French Polynesian population, whereas dominant frequency

Table 3. PC1 and PC2 variations of conspecific chases and signal jumps in *Dascyllus aruanus* from Taiwan and French Polynesia

Sound type	Site	σ^2 PC1	σ^2 PC2
Conspecific chase	Taiwan	2.43	4.69
	French Polynesia	2.12	6.95
Signal jump	Taiwan	0.87	0.67
	French Polynesia	0.48	1.36

was significantly higher in the French Polynesian population (Wilcoxon–Mann–Whitney, $W = 903$, 0 and 0 ; all $P < 0.001$).

The first principal components of the PCA explained 35.7% of the variation and is based on mainly the sound duration ($rS = 0.96$) and the number of pulses ($rS = 0.83$). Both features allowed separation between the two sites. The second principal component explained 34.9% and is correlated with the interpulse duration ($rS = -0.8$), the dominant frequency ($rS = -0.56$) and the pulse period ($rS = -0.90$). The second component does not allow separation of the geographical regions (Wilcoxon–Mann–Whitney; $W = 173$, $P = 0.07$).

A last PCA was performed to compare the geographic variations according to the behaviour (signal jump vs. conspecific chase). Interestingly, variances of PC1 (41.2%) and PC2 (32.2%) were more important for sounds related to conspecific chases than for signal jumps (F-test, $F = 5$ & 2 , both $P < 0.001$) (Table 3). In other words, features of sounds related to conspecific chases were more variable than sounds from signal jumps recorded at the same place.

MORPHOLOGICAL ANALYSIS

We did not find any difference in meristic (Table 4) and morphometric (Supporting Information, Table S1) characters between the specimens from the three sites. The first PCA analysis did not indicate any difference in morphometric characters between the three populations. The measures cannot be used to distinguish specimens from the three locations. There is no difference in PC1 and PC2s values between the three studied populations (PC1: Kruskal–Wallis; $\chi^2 = 6.44$, d.f. = 2, $P = 0.04$, Dunn: $Z = 1.96, -0.32, -2.36$; $P = 0.08, 0.75$ and 0.055 ; PC2: Kruskal–Wallis; $\chi^2 = 5.14$, d.f. = 2, $P = 0.08$). Again, the second PCA analysis did not allow to find any difference in morphometric characters between the three populations (Kruskal–Wallis; $\chi^2 = 5.12$ and 2.00 ; d.f. = 2, $P = 0.08$ and 0.37 , respectively). Consequently, we were unable to distinguish the three populations based on morphological data we used.

Table 4. Meristic data in specimens (Spec.) of *Dascyllus abudafur* (Madagascar) and *Dascyllus aruanus* (Taiwan and Polynesia). Number of spines and rays (X + Y) in the anal fin, dorsal (Caud. dors.) and ventral (Caud. vent.) part of the caudal fin, pelvic fin (Pelv.) and pectoral fin (Pect.); number of the vertebrae having the last abdominal rib (rib), total number of vertebrae (Vert T.), number of abdominal vertebrae (Vert. A.); number of scales involved in the lateral line (Lat), number of supraneural bones (Sup N.)

	Anal	Caud. dors.	Caud. vent.	Dorsale	Pelv.	Pect.	Rib	Vert T.	Vert. A	Lat	Sup N.
Madagascar											
Spec. #1	2 + 12	2 + 10	2 + 9	12 + 12	1 + 5	1 + 17	16	25	11		3
Spec. #2	2 + 12	2 + 10	2 + 10	12 + 12	1 + 5	1 + 17	15	25	11	17	3
Spec. #3	2 + 13			12 + 13	1 + 5	1 + 16	17	25	11	17	3
Spec. #4	2 + 13	2 + 10	2 + 9	12 + 13	1 + 5	1 + 18	16	25	11	17	3
Spec. #5	2 + 13	2 + 10	2 + 9	12 + 13	1 + 5	1 + 16	16	25	11	17	3
Taiwan											
Spec. #1	2 + 12	2 + 10	2 + 9	12 + 12	1 + 5	1 + 18	16	24	11	17	3
Spec. #2	2 + 13	2 + 10	2 + 9	11 + 13	1 + 5	1 + 17	16	24	11	17	3
Spec. #3	2 + 13	2 + 10	2 + 9	12 + 13	1 + 5	1 + 17	15	25	11		3
Spec. #4	2 + 13	2 + 10	2 + 9	12 + 12	1 + 5	1 + 18	16	25	11	17	3
Spec. #5		2 + 10	2 + 9		1 + 5			25	11	17	3
French Polynesia											
Spec. #1	2 + 13	2 + 10	2 + 9	12 + 13	1 + 5	1 + 17	16	25	11	17	3
Spec. #2	2 + 13	2 + 10	2 + 9	12 + 13	1 + 5	1 + 17	16	25	11	17	3
Spec. #3	2 + 13	2 + 10	2 + 9	12 + 13	1 + 5	1 + 17	16	25	11	17	3
Spec. #4	2 + 13	2 + 10	2 + 9	12 + 12	1 + 5	1 + 17	15	25	11	17	3
Spec. #5	2 + 13	2 + 10	2 + 9	13 + 13	1 + 5	1 + 17	16	25	11		3

DISCUSSION

A cline refers to a spatial gradient in a specific trait (Huxley, 1938). This variation could correspond to an evolutionary divergence probably related to the emergence of new species. Several studies have reported that differences in advertisement calls in frogs and birds corresponded to a geographic cline (Tubaro *et al.*, 1993; Pröhl *et al.*, 2007). The concept of cline, based on acoustic communication, has not been clearly evoked in Teleosts. However, geographical variations in the calls have been reported for a small number of species. For example, the boatwhistle advertisement calls of the toadfish *Opsanus tau* (Linnaeus, 1766) show variations in call duration and fundamental frequency on opposite sides of the Chesapeake Bay (Fine, 1978a, b). In the minnow *Cyprinella galactura* (Cope, 1868), agonistic and courtship signals between populations from Ozark and Appalachian Mountains also showed geographical acoustic signal divergence (Phillips & Johnston, 2008).

If we consider the humbug damselfish as a single, main evolutionary lineage across the entire Indo-Pacific Ocean, we do not observe longitudinal variation of meristic and morphometric characters, but we highlight differences in the acoustic features of the signal jumps among various populations. From Madagascar to French Polynesia, there is a decrease in the duration and the pulse number, whereas there is an increase in the dominant frequency of the call. These differences in dominant frequency could be related to fish size (Myrberg *et al.*, 1993; Lobel & Mann, 1995; Colleye *et al.*, 2009; Parmentier & Fine, 2016). Even if we could not identify and measure the recorded individuals, we performed random recordings at the three sites, which should have assured representative sampling. Therefore, dominant frequency suggests that fish from Madagascar would overall be bigger than humbug damselfish from Taiwan, which would also be bigger than the population from French Polynesia, supporting phenotype differences.

Differences in call features suggest that the existing cline could be related to the presence of dialects along the east–west axis (Parmentier *et al.*, 2009). However, phyletic comparisons of humbug damselfish metapopulations between Indian and Pacific Oceans support that the two forms are reciprocally monophyletic (Borsa *et al.*, 2014; Liu *et al.*, 2014; Tang *et al.*, 2021). According to our hypothesis, the cline would have pre-dated speciation. No location with mitotype polymorphism (Indian vs. Pacific) was observed, except at the western shore of Lombok Strait (Borsa *et al.*, 2014), supporting the existence of a common ancestor before vicariance on either side of the Indo-Pacific barrier. The two forms, which are genetically distinct (both mitochondrial

and nuclear loci), were also characterized by distinct pigmentation patterns, although this feature was not completely reliable (Borsa *et al.*, 2014). As a result, the Indian Ocean humbug damselfish is now called *D. abudafur*, whereas *D. aruanus* is maintained for the Pacific Ocean humbug damselfish population. Our acoustic data supports this distinction because the Indian Ocean population shows specific acoustic features, suggesting that the speciation process is already completed, with emerging species that show differences in their calling behaviours even before any differences in morphology could be observed (Price *et al.*, 2003; Tinghitella *et al.*, 2009).

There were also differences between calls from Taiwanese and French Polynesian populations. Genetically, the population of French Polynesia is separated from the rest of the Pacific populations as an evolutionary significant unit, presumably as a consequence of the geographical isolation of the Society Islands (Liu *et al.*, 2014). According to the unified species concept of De Queiroz (2007), species can be defined as separately evolving metapopulations (i.e. inclusive population made up of connected subpopulations). The two Pacific forms (Taiwan and French Polynesia) show distinct genetic (Liu *et al.*, 2014) and behavioural criteria that support the hypothesis of the coexistence of two species in the Pacific Ocean. In other words, the ancestral Indo-Pacific lineage could be now split into three populations. Two populations currently possess the status of species (*Dascyllus aruanus* and *Dascyllus abudafur*), but this is not the case for the population from Society Islands. The humbug damselfish in French Polynesia was originally called *Pomacentrus emamo* Lesson, 1831 in his natural history expedition to the Society Islands (Lesson, 1831). The name ‘emamo’ was the original name given by the inhabitants of Bora Bora (French Polynesia) to this fish. *Pomacentrus emamo* was then synonymized with *D. aruanus* in subsequent studies (Allen, 1991). We observed an equivalent difference between the sounds of the population from Taiwan and those of Madagascar (now *D. abudafur*) and French Polynesia. According to both acoustic observation and genetic data, we suggest resurrecting the epithet ‘emamo’ to name the population from the Society Islands ***Dascyllus emamo* (Lesson, 1831) comb. nov.**, maintaining *Dascyllus aruanus* for the rest of the Pacific Ocean and *Dascyllus abudafur* for the Indian Ocean. Acoustically, the difference between Taiwan and Madagascar is similar to the difference between Taiwan and the Society Islands. Further studies on the humbug damselfish complex should be carried out over a wider range to confirm our hypothesis and to seek distinct morphological features that would support the species status. Without additional

morphological data, the diagnosis of the phenotype of *Dascyllus emamo* corresponds to the one of *Dascyllus aruanus*, differences being at the level of the geographical location and acoustic features since the population from French Polynesia possesses sounds that are associated with signal jumps that show low number of pulses (4 ± 1) produced at a rate of 25 ± 5 ms.

Although there is an overlap in the PCA, this would not be related to hybridization. *Dascyllus aruanus* larvae have a pelagic life for 16–24 days prior to settlement on reefs (Wellington & Victor, 1989). They preferentially recruit near settled congeners by using chemical cues (Sweatman, 1988). This allows the pre-recruits to recognize their natal areas, thus potentially enhancing self-recruitment and hampering dispersal (Liu et al., 2014). These life-history characteristics indicate that late *D. aruanus* larvae are unlikely to drift in surface currents, and hence larvae may not be dispersed over distances as long as their pelagic duration would potentially allow. Moreover, the use of a common sonic mechanism (Parmentier et al., 2007; Colleye et al., 2011; Olivier et al., 2015) can be important in having similar sonic characteristics (Phillips & Johnston, 2009). Overlap in PCA has been found between distinct species (Parmentier et al., 2009; Colleye et al., 2011; Boyle et al., 2015; Mélotte et al., 2016) and should consequently not be used to signal hybridization.

Interestingly, differences were previously found between calls of the three-spot damselfish *Dascyllus trimaculatus* (Rüppell, 1829) from Madagascar and French Polynesia, and these sounds were also first considered to be dialects (Parmentier et al., 2009). Genetic studies have divided the *D. trimaculatus* complex into two major groups, the Indian Ocean and the Pacific (Bernardi et al., 2002; Salas et al., 2020), but distinct specific names were not attributed. As in the humbug damselfish complex, further studies on both mitochondrial and nuclear markers have now divided the widespread *D. trimaculatus* into three deeply divergent genetic partitions that correspond to three distinct zones: Indian Ocean, Central-West Pacific and southern French Polynesia (Leray et al., 2010). We hypothesize that populations from these zones should possess different acoustic features that could support the existence of different species.

According to our results, the number of pulses and the pulse period distinguishes signal jump sounds from conspecific chasing sounds. Signal jump sounds have more pulses with shorter pulse periods. The signal jump sound has functions in courtship behaviour and as a territorial signal (Mann & Lobel, 1995). Sounds are produced by the male in front of a female, but male damselfish can also start a signal jump when exposed to other males (Holzberg, 1973; Kenyon,

1994). In *Dascyllus albisella* Gill, 1862, sounds with more pulses are easier to detect than sounds with fewer pulses, making this feature particularly useful to attract females from outside the territory of the male (Mann & Lobel, 1995). Moreover, variances of PC1 and PC2 are much lower in signal jumps than in conspecific chases showing that sounds associated to signal jumps are more stereotyped and conserved within a species (Table 3). This difference could be related to the message delivered. Constraints related to the required conspecific identification for mating are probably more important than constraints related to chases since signal jumps serve as indicators for species identity and mate quality. These kinds of advertisement calls are also important for mate recognition and hence contribute to premating isolation (Funk et al., 2012).

Differences in genetic data and call features among the three studied populations obviously support growing evolutionary divergence between them. Differences were, however, not found at the level of morphology. This could be due to the small sample size and to conservative body plan in relation to the use of the same kind of habitat in the different geographical regions. This suggests that calls may evolve faster than morphology, perhaps owing to strong selection on calls (Funk et al., 2012). Genetic drift represents another potential driver of acoustic divergence (Fine, 1978b, a; Phillips & Johnston, 2009). Different studies on avian and mammal vocalizations have also reported the importance of genetic drift in causing signal evolution, meaning that signal divergence might occur in the absence of any kind of selection. Under the drift scenarios, a positive correlation can be expected between acoustic and genetic distance (Irwin et al., 2008; Campbell et al., 2010). Additional data on a larger number of humbug damselfish populations are required to elucidate relationship between acoustic and genetic distances (Irwin et al., 2008; Campbell et al., 2010). In birds and mammals, acoustic features could also evolve as a result of culture with the accumulation of errors in vocal learning among diverging populations (Lin et al., 2015). This learning behaviour is, however, currently not known in fish (Longrie et al., 2008).

From a practical point of view, as has been shown for some frog (Padial et al., 2008; Funk et al., 2012) and bird species (Irwin et al., 2001a, 2008), this study supports the theory that sounds can be used to distinguish fish populations that have similar phenotypes. Complimentary to other behavioural features, calls seem to be valuable for the determination of species identity and can help in the identification of cryptic species since the calls can reflect genetic differences. These species require special consideration in conservation planning, because species that are

already considered endangered or threatened might be composed of multiple species, which might require different conservation strategies (Bickford *et al.*, 2007).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Proportional measurements (ratio of standard length, mm) of specimens from the *Dascyllus aruanus* complex. A, measurements are from the cleared and stained specimens. B, measurements are from specimens that were not cleared and stained and kept in 70% alcohol. Mada = Madagascar, Tahiti = French Polynesia.