Calliblepharis rammediorum sp. nov. (Gigartinales, Rhodophyta) from the Israeli Levant Mediterranean Sea

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Abstract – *Calliblepharis rammediorum* sp. nov. (Gigartinales, Rhodophyta) is described from the northern Levant Mediterranean shore of Israel. The new species has a prostrate creeping habit. It usually grows as an epiphyte, strongly attached by several discoidal holdfasts to the base of branches of some seaweed species, mainly on some non-native species and particularly on *Laurencia chondrioides*. Phylogenetic analyses of sequence data indicate that *C. rammediorum* is closely related to an unidentified species from the Cocos (Keeling) Islands, Australia. These two are sister to a complex of two genetic groups both assigned to the morphospecies *Calliblepharis fimbriata*, whose species identity clearly requires further research. *Calliblepharis rammediorum* is the fourth that is newly described for the genus *Calliblepharis* since the beginning of the last decade. Aspects of its biology and ecology are also discussed.

Calliblepharis rammediorum / rbcL / COI-5P / Israel / Levant Mediterranean Sea

INTRODUCTION

Calliblepharis Kützing (1843) is a genus of red seaweeds currently recognized as containing 9 species (Guiry & Guiry, 2017). Three of these: *C. hypneoides* P.Díaz-Tapia, I.Bárbara *et* Hommersand, *C. psammophila* D'Archino *et* W.A.Nelson and *C. saidana* (Holmes) M.Y.Yang *et* M.S.Kim, were added during the past four years to the current species list of the genus. Studies of the morphology of these new species were complemented with molecular analyses (Díaz-Tapia *et al.*, 2013; D'Archino *et al.*, 2015; Yang & Kim, 2017). *Calliblepharis jubata* (Goodenough & Woodward) Kützing is the only species of the genus reported as occurring in the Mediterranean Sea. This species, however, which is characterized by a foliose thallus with marginal proliferations, is distributed only in the western basin and has not been reported from the eastern basin or the Levantine Sea (Guiry & Guiry, 2017).

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Seaweed surveys conducted along the shores of Israel following morphological and molecular studies revealed a new unknown species of *Calliblepharis*. The original specimens were collected at two northern sites in October and November of 2013 at Bat-Galim (Haifa) and Shavei Zion, respectively. The purpose of this paper is to formally describe the new species as *Calliblepharis rammediorum* and to discuss its relationships within the genus and some aspects of its ecology.

MATERIALS AND METHODS

Voucher specimens that have been used in this study are housed in "TAU" (the Herbarium of Tel Aviv University). Investigated specimens are listed in Table 1.

Morphological and ecological study

Specimens examined were collected from the drift, the intertidal and the shallow subtidal (up to 12 meters in depth) of the northern Levantine shore of Israel. Map and tables showing the six collecting sites and their coordinates are represented collectively in Hoffman *et al.* (2014), Hoffman & Wynne (2016), and Table 1. Environmental factors and habitat details were noted.

Pressed and alcohol-preserved specimens of the Mediterranean collections were prepared and deposited in "TAU". Specimens were examined with a Zeiss Axioplan 2 imaging compound microscope (Carl Zeiss, Jena, Germany) and Olympus MVX10 Research Macro Zoom Microscope (Olympus Corp. Tokyo, Japan). Images

Catalogue number			Coordinates (decimal	Year of	Denth of
of specimens	Site	Collector	Degrees)	collecting	distribution
TAU490	Shavei Zion	R. Hoffman	32.983956, 35.081095	2013	1 m
TAU2056	Church Beach, Haifa	R. Hoffman	32.830624, 34.95987	2015	Drift
TAU2142	Achziv	R. Hoffman	33.041992, 35.098706	2015	Drift
TAU2151	Shavei Zion	R. Hoffman	32.983393, 35.081261	2015	Drift
TAU2164 (sequenced)	Church Beach, Haifa	R. Hoffman	32.830624, 34.95987	2015	Drift
TAU2354	Achziv	N. Shankar		2016	12 m
TAU2374	Silent Beach, Haifa	R. Hoffman	32.832736, 34.990865	2016	1 m
TAU2395 (sequenced)	Silent Beach, Haifa	R. Hoffman	32.832661, 34.99079	2017	0.5 m
TAU2459	Silent Beach, Haifa	R. Hoffman	32.832294, 34.989744	2017	Drift
TAU2466	Silent Beach, Haifa	R. Hoffman	32. 832294, 34. 989744	2017	0.5 m
TAU2796	Rosh HaNikra	R. Hoffman	32.085444, 34.105309	2017	1 m
TAU2891	Bat Galim, Haifa	R. Hoffman	32.832416, 34.972586	2017	Drift

Table 1. Collecting details of specimens of Calliblepharis rammediorum examined

were acquired with an Olympus DP71 microscope digital camera, Olympus SP820UZ digital camera (Olympus Corp. Tokyo, Japan) and the digital camera of the smartphone LGG4 (LG Electronics Inc. Seoul, South Korea). Morphological characteristics of the specimens were compared to the features of currently recognized taxa of the genus *Calliblepharis*.

Molecular study

Subsamples of specimens TAU2164 and TAU2395 (GWS038670 and GWS039490; Table 2) were dried in silica gel with the DNA extracted following Saunders & McDevit (2012). The *rbcL* sequence was generated for TAU2164 (1335 bp: TAU2395 only had *rbcL*-3P data generated for identification – 800 bp) following the protocols of Saunders & Moore (2013; attempts to generate COI-5P data failed). Additional data for COI-5P and *rbcL* were similarly generated for representative Cystocloniaceae to facilitate phylogenetic analyses of the genus *Calliblepharis* (Table 2) again following Saunders & Moore (2013). Sequencing was performed at Genome Quebec, and the data were uploaded to BOLD (http://www.barcodinglife.org; Dataset DS-ISCALL1) and GenBank (http://www.ncbi.nlm.nih.gov/; Table 2).

The new data generated here were added to published COI-5P and *rbcL* data of representative Cystocloniaceae to assess the phylogenetic affinities of our new species within the *Calliblepharis/Hypnea* complex (Table 2; considerable care was taken to restrict the analyses to species for which the two genes were clearly from the same genetic species, i.e. generated from the same specimen or in the same study) including *Cystoclonium purpureum* (Hudson) Batters and two species of *Rhodophyllis* as the outgroup (Díaz-Tapia *et al.*, 2103; D'Archino *et al.*, 2015). Our alignments were 78% complete for COI-5P (data missing for four genetic groups; Table 2) and 100% complete for *rbcL*. The single (partitioned by codon) and multigene alignments (partitioned by gene and then by codon) were analyzed using maximum likelihood (ML) methods in RAxML employing a GTR+I+G model in Geneious Pro 10.2.2 (Drummond *et al.*, 2009). Node support was estimated using nonparametric bootstrapping (1000 replicates). As no strong conflicts were uncovered in the single gene analyses, only the combined result is presented.

RESULTS

Morphology and ecology

Calliblepharis rammediorum R.Hoffman, M.J.Wynne, & G.W.Saunders, sp. nov. Figs 1-16

Diagnosis: Thalli forming a bit stiff but also flexible entangled clump, up to 2 cm high and 4 cm wide, pink to crimson red, epiphytic, consisting of terete to complanate axes, 1.5 mm broad and 200-500 μ m in diameter, branched, prostrate, creeping, rarely anastomosing, strongly attached to other seaweeds, and rarely also to small rocks, by many small discoidal holdfasts (200-400 μ m in diameter) growing from each branch and branchlet; branches mostly distichously arranged in an irregular pattern, spiny and arcuate; cortex composed of smaller outer cells forming

Table 2. Collection details and GenE	3ank accession	numbers for the specimens used in our molecular phyloger	netic analyses	
Name	Voucher	Collection details	$COI-5P^2$	$rbcL^2$
Calliblepharis ciliata (Hudson) Kützing	GWS014644	Smerwick Harbour, Kerry, Ireland; M.D. Guiry, 12.8.2010	MG462770	MG462775
Calliblepharis fimbriata (Greville) Kützing	GWS036376	Preekstoel, Stilbaai, Western Cape,w South Africa; K. Dixon & J. Ferreira Costa, 25.11.2014	MG462769	MG462773
	GenBank ¹		ND	AF385654
<i>Calliblepharis hypneoides</i> P. Díaz-Tapia, I. Bárbara <i>et</i> M.H. Hommersand	GenBank ¹		KC121086	KC121124
Calliblepharis jubata (Goodenough et Woodward) Kützing	GWS014653	Smerwick Harbour, Kerry, Ireland; M.D. Guiry, 12.8.2010	KF026474	KF026494
Calliblepharis psammophilus D'Archino et W.A. Nelson	GenBank ¹		ND	KT439336
Calliblepharis rammediorum sp. nov.	GWS038670	Church Beach, Haifa, Israel; R. Hoffman, 7.12.2015 (TAU2164)	ND	MG462776
	GWS039490	Silent Beach, Haifa, Israel: R. Hoffman, 24.1.2017 (TAU2395)	ND	MG462774 ³
Calliblepharis saidana (Holmes) M.Y. Yang et M.S. Kim	GenBank ¹		ND	MF083583
Calliblepharis sp1Cocos	GWS037835	Inner lagoon, Cocos (Keeling) Islands, Australia: K. Dixon & G.W. Saunders, 9.12.2013	MG462771	MG462777
Cystoclonium purpureum (Hudson) Batters	G0421	Lepreau, New Brunswick, Canada; G.W. Saunders, 7.10.1995	ND	KC130217
	GWS017885	Woods Hole, Massachusetts, U.S.A.; B. Clarkston, D. McDevit, M. Bruce, A. Savoie, C. Longtin, 14.4.2010	HM915113	ND
Hypnea asiatica P.J.L. Geraldino, E.C. Yang et S.M. Boo	GenBank ¹		EU240798	EU240837
Hypnea caespitosa P.J.L. Geraldino et S.M. Boo	GenBank ¹		FJ694901	FJ694936
Hypnea charoides J.V. Lamouroux	G0358	Leeman, Western Australia, Australia; G.T. Kraft & G.W. Saunders, 7.11.1995	HM915818	KC130220
Hypnea musciformis (Wulfen) J.V. Lamouroux	GenBank ¹		KT428779	KT428787
Hypnea nidulans Setchell	GenBank ¹		FJ694900	FJ694946
<i>Hypnea pannosa</i> J. Agardh	GenBank ¹		FJ694892	FJ694958
Rhodophyllis multipartita Harvey	GWS015211	Burying Ground Point, Tasmania, Australia; G.W. Saunders & K. Dixon, 21.1.2010	HM917635	KF026495
Rhodophyllis spMAR2	GWS040848	Stora Lauvøyna, Norway; G.W. Saunders & T. Bringloe, 11.6.2016	MG462772	MG462778

¹ These data were acquired from records on GenBank for which C01-5P and *rbcL* were from the same specimen or genetic group. ² ND indicates that no data were generated for that marker for that specimen. ³ Sequence for the *rbcL*-3P region only (Saunders & Moore 2013).

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rosettes around inner larger cells; as seen in longitudinal section, medulla consisting of long elliptic axial cells surrounded by one layer of large rounded to elongate cells; fertile gametophytes not observed; tetrasporangia immersed in the cortex, scattered over the thallus, ellipsoidal, 15-25 μ m in width and 25-40 μ m in length.

Found growing in the shallow-deep subtidal and in intertidal pools.

Holotype: TAU 2395 (GWS039490) (Fig. 1), collected at the Silent Beach (32.8327°N, 34.9908°E) in Haifa (Israel), growing as epiphyte on *Laurencia chondrioides* Børgesen on 24 January, 2017, collected by R. Hoffman.

Paratypes: listed in Table 1.



Figs 1-6. *Calliblepharis rammediorum*: **1.** Part of the Holotype. **2.** *In situ* specimen. **3.** Terete axis with adventitious attachment disc. **4.** Face view of attachment disc. **5 & 6.** Thalli with compressed axes and irregular, distichous branching. Scale bars = 2 cm (Fig. 1); 10 mm (Fig. 2); 200 μ m (Fig. 3); 100 μ m (Fig. 4); 2 mm (Figs 5 & 6).



Figs 7-12. *Calliblepharis rammediorum*: 7 & 8. Axis with unilateral branching and spinose tips. 9. Cross-section of a compressed axis. Ax: axial cell; P: periaxial cell. 10. Surface view of thallus showing rosette arrangement of smaller cortical cells around larger cortical cells. 11. Apex of an axis with a single apical cell (arrow). 12. Apical region of an axis with small hair cells (arrows). Scale bars = 1 mm (Fig 7); 200 μ m (Fig. 8); 100 μ m (Fig. 9); 50 μ m (Figs 10 & 11); 25 μ m (Fig. 12).

Etymology: rammediorum, a combination of the first names of the parents of Dr. Hoffman, Rami (his father) and Medi (his mother).

Distribution: the northern Levantine Mediterranean shore of Israel, along ca. 45 km, from Haifa in the south northward up to the northern border with Lebanon at Rosh HaNikra.

Vegetative morphology and habit: Thalli pink to crimson red, branched, prostrate, creeping and entangled (Figs 1, 2), a bit stiff but also flexible, 1-2 cm tall,



Figs 13-16. *Calliblepharis rammediorum*: **13.** Longitudinal section of an axis showing uniaxial row. **14.** Surface view of thallus showing sporangia pit-connected to surrounding cells (arrows). **15.** Crosssection of an axis showing zonately divided tetrasporangia (arrows). **16.** Regeneration of an axis after being grazed. Scale bars = $100 \mu m$ (Fig. 13); $50 \mu m$ (Figs 14-15); $200 \mu m$ (Fig. 16).

1-4 cm wide, mostly epiphytic, rarely epilithic and epiphytic at the same time, attached to the substratum by scattered tiny multicellular discoidal holdfasts (Fig. 3), 100-400 µm in diameter (Fig. 4). Branching is irregularly distichous (Figs 5, 6), alternate (Fig. 6) or occasionally dichotomous, mostly in single plane (Figs 5, 6) but sometimes in three-dimensional space (Fig. 7). Branches often arcuate and tapered to points (Figs 7, 8), terete mostly at the lower part of the plant, becoming sub-terete to compressed in cross section at the middle and apices of plant (Figs 6, 9), up to 1.5 mm wide and 200-500 µm in diameter. Branchlets spiny (Figs 7, 8). Cortex composed of a mixture of slightly sunken large angular to rounded cells, 30-65 um in diameter, surrounded by smaller rounded surface cells, 10-24 µm in diameter, usually forming distinct rosettes (Fig. 10). The thallus grows uniaxially initiated by a distinct apical cell (Fig. 11). Occasionally, hairs develop from cortical cells of branchlets at apical parts (Figure 12). Longitudinal and cross sections of the midfrond shows single elliptic cylindrical elongate axial cells, each $23-30 \times 40-50 \times$ 90-300 µm mostly surrounded by a single layer of (4-) 5-6 (-7) large, rounded to elliptic cylindrical medullary cells, $40-70 \times 50-130 \times 120-200 \ \mu m$ (Fig. 13).

Reproductive structures: Tetrasporangia immersed in the cortex, scattered over the thallus surface, not in modified branches, initiated from modified cortical cells that bear lateral cells connected through pit connections (Fig. 14). Mature tetrasporangia are elongate, 15-25 µm in width and 25-40 µm in length (Fig. 15).



Fig. 17. Maximum likelihood phylogeny for the concatenated COI-5P and *rbcL* alignment with bootstrap support > 70% indicated along branches. Type species for genera are indicated with an asterisk while the new species is presented in bold type.

Habitat: All collected specimens of Calliblepharis rammediorum were found growing as epiphytes on other seaweeds. The vast majority were attached to lower parts of the main branches of Laurencia chondrioides by discoidal holdfasts. However, specimens were also observed to be attached and creeping around stipes and lower branches of Galaxaura rugosa (J.Ellis & Solander) J.V.Lamouroux, Amphiroa rigida J.V.Lamouroux, Ellisolandia elongata (J.Ellis & Solander) K.R.Hind et G.W.Saunders and Lychaete pellucida (Hudson) M.J.Wynne. Two specimens found growing on L. chondrioides were also attached by several holdfasts to gravel located below this red nonnative species.

Although significant numbers of specimens were collected throughout the year, fertile gametophytes were not observed. The winter season showed most specimens cast ashore. Most specimens collected at spring and summertime were found with marks consistent with heavy grazing by fish, but also often with regeneration of branchlets (Fig. 16).

Molecular results

The maximum likelihood phylogeny for the combined COI-5P and *rbcL* alignment is presented (Fig. 17). As with previous studies, *Hypnea* was solidly resolved as monophyletic whereas *Calliblepharis*, although monophyletic here, lacked support (Fig. 17). *Calliblepharis rammediorum* sp. nov. was tightly associated with an unnamed species from the Cocos (Keeling) Islands, Australia, but these species nonetheless had seven differences in *rbcL* (0.5% divergent), which is consistent with distinct species. These two taxa were sister to two (presumably overlooked; 4.3% divergent) species identified as *Calliblepharis fimbriata* (Greville) Kützing from southern Africa [data generated here and GenBank AF385654 from Hommersand & Fredericq (2001)]. Not surprisingly, more species await description in the genus *Calliblepharis*. The previous taxa are moderately sister to *C. saidana*, and these to a lineage with the generitype *Calliblepharis ciliata* (Hudson) Kützing, but this latter relationship has no support leaving uncertainty regarding monophyly of the genus *Calliblepharis* (Fig. 17).

DISCUSSION

Calliblepharis rammediorum is a common red algal species found along the northern Levant Mediterranean shore of the Israel. This species prefers growing attached to other seaweeds and almost exclusively on the non-native species *Laurencia chondrioides*. Specimens collected during spring and summer were found with marks consistent with heavy grazing by fish over their entire thalli. The remaining thalli were entangled and regenerating around the main branches of individuals of *L. chondrioides*. Hence, the proliferation of *C. rammediorum* may depend on the success of *L. chondrioides* and the fact that, for some reason, it is not being grazed by the common rabbitfish grazers *Siganus rivulatus* Forsskål and *S. luridus* (Rüppell). These very common invasive fish, which are becoming very abundant during spring and summer, graze in the shallow subtidal of the eastern Levantine Mediterranean Sea (Hoffman & Wynne, 2015). The hidden niche around the main branches of *L. chondrioides* is beyond the reach of these grazers, giving *C. rammediorum* a chance to survive and establish populations along the Levantine Sea.

The generic placement of the new species based on molecular data remains equivocal as it joins the type of *Calliblepharis*, *C. ciliata*, but with no support in the current analysis (Fig. 17). The situation is further complicated by the lack of monophyly for *Calliblepharis* in every study that includes more than a few species, the species assigned to this genus typically forming a paraphyletic grade leading to species assigned to *Hypnea* (e.g. Díaz-Tapia *et al.*, 2013; D'Archino *et al.*, 2015; Yang & Kim, 2017). Although further work is needed, more detailed analyses indicate that *C. rammediorum* is correctly assigned to *Calliblepharis*, while *C. planicaulis* (Harvey) Kylin and some six new species from Australia will form the core of a new genus sister to *Hypnea* (Saunders, unpublished results). Recent molecular and morphological study led Yang & Kim (2017) to transfer *Hypnea* saidana to the genus *Calliblepharis*. Because there are ca. 70 species in *Hypnea*, some known only for their vegetative attributes, it is quite possible that other species

of *Calliblepharis* (and the new genus required for *C. planicaulis*) are hiding among them, especially among the other *Hypnea* species having compressed to flattened axes and more or less secund to alternate branches in a single plane, such as *H. pannosa* J.Agardh, *H. variabilis* Okamura and *H. yamadae* Tanaka (e.g., see Yamagishi & Masuda 1997).

The suite of morphological characters that separates the new species from its congeners is the following: its prostrate habit of entangled, intricate axes, alternately branched, the sub-terete to compressed axes, only up to 1.5 mm. in breadth, the cortical cells forming rosettes, the tetrasporangia produced not in discrete sori or nemathecia but immersed among cortical cells (not deeply sunken or "hidden").

Small hairs observed near apices in *Calliblepharis rammediorum* have also been described in other species of the genus, as in *C. planicaulis* by Min-Thein & Womersley (1976) and Womersley (1994) and in *C. psammophila* by D'Archino *et al.* (2005). An "indistinct" formation of rosettes in the cortex has also been described for *C. celatospora* by Kraft in Chiovitti *et al.* (1998). *Calliblepharis rammediorum* is most similar to *C. hypneoides* in its intricately branched, entangled, prostrate habit, forming cushions, and in the production of secondary peg-like protrusions that anchor the thallus to its host algal species or to itself and occasionally to rock (Díaz-Tapia *et al.*, 2013). The features that separate this pair of species is that the axes in *C. hypneoides* are clearly terete unlike the compressed axes in the new species, and that the tetrasporangia occur in sori in *C. hypneoides* but are produced scattered in the cortex in *C. rammediorum*.

Key to the currently recognized species of Calliblepharis Kützing:

1a.	Thallus consisting of percurrent, erect main axis, with branches, habit not intricate or prostrate
1b.	Thallus intricately caespitose, with a sprawling or prostrate, not percurrent habit, with branches mostly distichously arranged in an irregular pattern, final branches often spinose
	2a. Thallus consisting of a primary blade, usually 30-70 mm in width
3a.	Primary blade up to 70 mm in width, 30 cm in length; blade margin plane, fringed, with short, simple or branched proliferations up to 5 mm long
3b.	Primary blade broad and linear, forking once palmately or sometimes dichotomously; margins frilled or proliferatingC. fimbriata (Greville) Kützing
	4a. Axes terete, lower branches 2-5 mm in width <i>C. planicaulis</i> (Harvey) Kylin 4b. Axes not terete
5a. 5b.	Tetrasporangia sunken in surface, hidden
	 6a. Primary blade up to 300 mm in length, usually less than 7 mm in width, simple, dichotomously or irregularly branched, with proliferations up to 30 mm long, which arise both from blade margin and blade surface
	op primary place up to 12 mm in width proliterations also arising from place

- 7b. Tetrasporangia scattered in cortical region, not in defined sori or nemathecia ...9
- 9a. Thalli forming an entangled mass 5-12 cm high, usually epilithic; cortical cells not forming rosettes *C. psammophila* D'Archino & W.A.Nelson
- 9b. Thalli forming a prostrate, entangled mass, to 2 cm high and to 4 cm wide, usually epiphytic; cortical cells forming rosettes*C. rammediorum* R.Hoffman *et al.*

Characters used in the key are based on the following published accounts:

C. celatospora: Chiovitti et al. 1998

C. ciliata: Dixon & Irvine 1977; Loiseaux-de Goër & Noailles 2008; Bárbara 2012; Díaz-Tapia *et al.* 2013

C. fimbriata: Kützing 1868; Børgesen 1932; Anand 1943; Seagrief 1973; Simons 1976

C. hypneoides: Díaz-Tapia et al. 2013

C. jubata: Dixon & Irvine 1977; Loiseaux-de Goër & Noailles 2008; Bárbara 2012; Díaz-Tapia *et al.* 2013

C. occidentalis: Joly et al. 1965; Guimarães & Pereira 1993, as C. fimbriata; Guimarães 2006

C. planicaulis: Harvey 1859, 1862, both as *Hypnea planicaulis*; Kylin 1932; Min-Thein & Womersley (1976); Womersley 1994

C. psammophila: D'Archino et al. 2015

C. rammediorum: this paper

C. saidana: Holmes 1896 and Okamura 1909, as *Hypnea saidana*; Tanaka 1941, as *H. saidana*; Millar 1990, as *H. saidana*; Yang & Kim 2017

Seaweed surveys conducted along the Israeli Levant Mediterranean Sea during the past four years indicated that *C. rammediorum* has the same distribution reported previously for some non-native algal species such as the red seaweeds *Laurencia chondrioides* (Hoffman *et al.*, 2014) and *Monosporus indicus* Børgesen (Hoffman & Wynne, 2016). Both *C. rammediorum* and *L. chondrioides* are currently limited to less than 50 Km of shoreline between Haifa and Rosh HaNikra (see map and coordinates in Hoffman *et al.*, 2014).

Our phylogenetic analyses show that *C. rammediorum* is tightly associated with an unnamed species of *Calliblepharis* from the Cocos Keeling Islands, Australia (Fig. 17). Because of the above and the fact that the northern shores of Israel, particularly the Haifa Bay, are an acknowledged a hot spot for alien seaweeds, we assume that there is a chance that the new species is exotic and that its occurrence in Israel is the result of a new introduction event. Since the north site at Rosh HaNikra is by the border with Lebanon, we also speculate that there is a chance that this species is found there as well.

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