Oleibacter marinus gen. nov., sp. nov., a bacterium that degrades petroleum aliphatic hydrocarbons in a tropical marine environment

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Three Gram-negative, motile, mesophilic, aerobic, rod-shaped bacterial strains, designated 2O1[T], 1O14 and 1O18, were isolated from Indonesian seawater after enrichment with crude oil and a continuous supply of supplemented seawater. The strains exhibited high n-alkane-degrading activity, which indicated that the strains were important degraders of petroleum aliphatic hydrocarbons in tropical marine environments. Phylogenetic analyses based on 16S rRNA gene sequences of members of the *Gammaproteobacteria* showed that the isolates formed a coherent and distinct cluster in a stable lineage containing *Oceanobacter kriegii* IFO 15467[T] (96.4–96.5 % 16S rRNA gene sequence similarity) and *Thalassolituus oleivorans* MIL-1[T]. DNA G + C content was 53.0–53.1 mol%. The major fatty acids were $C_{16:0}$, $C_{16:1}\omega7$ and $C_{18:1}\omega9$ and the hydroxy fatty acids were $C_{12:0}$ 3-OH and $C_{10:0}$ 3-OH. The polar lipids were phosphatidylglycerol, a ninhydrin-positive phospholipid(s) and glycolipids. The major quinone was Q-9 (97–99 %), which distinguished the isolates from *Oceanobacter kriegii* NBRC 15467[T] (Q-8; 91 %). On the basis of phenotypic, genotypic and chemotaxonomic data, including DNA–DNA hybridization, the isolates represent a novel genus and species, for which the name *Oleibacter marinus* gen. nov., sp. nov. is proposed. The type strain of *Oleibacter marinus* is 2O1[T] (=NBRC 105760[T] =BTCC B-675[T]).

A wide variety of micro-organisms are known to degrade petroleum hydrocarbons (Head *et al.*, 2006; Prince, 2005). Among the hydrocarbonoclastic bacteria, members of the genera *Alcanivorax* (Hara *et al.*, 2003; Kasai *et al.*, 2001; Roling *et al.*, 2004; Yakimov *et al.*, 1998, 2005) and *Cycloclasticus* (Dyksterhouse *et al.*, 1995; Kasai *et al.*, 2002; Maruyama *et al.*, 2003) have been identified as key micro-organisms in the degradation of aliphatic and aromatic hydrocarbons, respectively, in marine environments (Harayama *et al.*, 2004). The genus *Alcanivorax* predominates in crude oil-impacted temperate marine environments (Cappello *et al.*, 2007; Hara *et al.*, 2003; Kasai *et al.*, 2001; Roling *et al.*, 2002, 2004; Yakimov *et al.*, 2005).

Members of the genus *Thalassolituus* have recently been shown to dominate in n-alkane-containing temperate seawater microcosms (McKew *et al.*, 2007; Yakimov *et al.*, 2005) and in crude-oil-containing temperate estuarine seawater microcosms (Coulon *et al.*, 2007; McKew *et al.*, 2007). Strains related to the genus *Oceanobacter* dominate in crude-oil-impacted Indonesian seawater microcosms and show high n-alkane-degrading activity, which is comparable to that of the genus *Alcanivorax* and suggests that *Oceanobacter*-related bacteria could be important for the degradation of petroleum aliphatic hydrocarbons in tropical marine environments (Teramoto *et al.*, 2009).

Three strains related to the genus *Oceanobacter*, designated 2O1[T], 1O14 and 1O18, were isolated from seawater collected at Pari Island (5.86° S 106.62° E) located near Jakarta, Indonesia, after enrichment with crude oil and a continuous supply of the seawater supplemented with nitrogen, phosphorus and iron nutrients (Teramoto *et al.*, 2009). The isolates exhibited distinct repetitive extragenic palindromic PCR patterns (Teramoto *et al.*, 2009) and

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 2O1[T], 1O14 and 1O18 are AB435649–AB435651, respectively.

A supplementary table and three supplementary figures are available with the online version of this paper.

highest 16S rRNA gene sequence similarity with *Oceanobacter kriegii* IFO 15467[T] (96.4–96.5 %). However, *Oceanobacter kriegii* NBRC 15467[T] did not show any petroleum hydrocarbon-degrading activity (Teramoto *et al.*, 2009) and had a different spectrum of growth substrates from the isolates (Bowditch *et al.*, 1984; Teramoto *et al.*, 2009). The strains were deposited in the NBRC and the BTCC (Biotechnology Culture Collection, LIPI, Cibinong, Indonesia).

Strains 2O1[T], 1O14 and 1O18 were cultivated on dMBPyr agar [containing (l^{-1}) 3.74 g marine broth 2216 (Difco), 5 g sodium pyruvate, 1.5 % (w/v) agar, 0.1 l distilled water and 0.9 l seawater; marine broth 2216 was dissolved in distilled water by boiling for 1 min and filtered through a 0.45 µm membrane (JHWPO 4700; Nihon Millipore) to remove insoluble components]. Phase-contrast microscopy showed that cells of the isolates were motile. Transmission electron microscopy (H7600; Hitachi) with freshly grown cells (25 °C) negatively stained with phosphotungstic acid showed rod-shaped (0.3–0.6 × 1.5–2.8 µm) and monoflagellated cells (Supplementary Fig. S1, available in IJSEM Online). Gram-staining tests with 3 % (w/v) KOH (Buck, 1982) and the staining procedure described by Gerhardt *et al.* (1994) showed that the isolates were Gram-negative. Tests for anaerobic growth were conducted on dMBPyr agar at 25 °C in a GasPak anaerobic jar (Becton Dickinson) for 3 weeks. The isolates did not grow under anaerobic conditions.

Growth of strains 2O1[T], 1O14 and 1O18 was tested on dMBPyr agar at 4, 10, 15, 20, 25, 30, 35, 37 and 40 °C for 25 days. Growth with 0, 1, 3, 5, 7, 10, 12, 15, 17 and 20 % (w/v) NaCl was tested on R2A agar (Difco) at 25 °C for 2 weeks. Growth at pH 2–11 (at intervals of 1 pH unit) was tested in filter-sterilized dMBPyr broth inoculated with a small amount of freshly grown cells transferred from dMBPyr agar using a toothpick and incubated with shaking (125 r.p.m.) at 25 °C for 7 weeks. The isolates grew at 10–40 °C (optimum 25–30 °C), but not at 4 °C, with 1–7 % (w/v) NaCl and at pH 6–10 (optimum pH 6–9 for strains 2O1[T] and 1O14 and pH 7–9 for strain 1O18).

Conditions for growth of *Oceanobacter kriegii* NBRC 15467[T] were also tested. *Oceanobacter kriegii* NBRC 15467[T] grew at 10–40 °C (optimum 25–30 °C); previously, this strain was reported to be unable to grow at 40 °C (Bowditch *et al.*, 1984). The NaCl concentration range and optimum for *Oceanobacter kriegii* NBRC 15467[T] were the same as those of the isolates, which was consistent with the report that this strain requires >100 mM (>0.58 %, w/v) NaCl for optimal growth (Bowditch *et al.*, 1984). The pH range for growth of *Oceanobacter kriegii* NBRC 15467[T] was tested for 3 days and was pH 5–9.

Catalase and oxidase activities of the isolates were assayed by dropping 3 % (v/v) hydrogen peroxide onto fresh cells on a glass slide and using oxidase reagent (bioMérieux), respectively. For physiological tests using the API 20 E, API 20 NE and API ZYM systems (bioMérieux), cells freshly grown on dMBPyr agar at 25 °C were suspended in artificial seawater [38.39 g artificial sea salt l^{-1} (Marine Art SF-1; Tomita Pharmaceutical)] and the cell density was adjusted to an OD$_{550}$ of 0.125 for API 20 E and API 20 NE and an OD$_{550}$ of 1.5 for API ZYM. The plates were incubated at 30 °C and results were recorded after 2 days for API 20 E and API 20 NE and 4.5 h for API ZYM. Each isolate was tested in duplicate. The physiological properties of the isolates were identical. Strains 2O1[T], 1O14 and 1O18 were catalase- and oxidase-positive. With API 20 NE, the isolates were positive for urease, gelatinase and reduction of nitrate to nitrite and negative for indole production from tryptophan, fermentation of glucose, arginine dihydrolase, aesculin hydrolase, *β*-galactosidase and assimilation of L-arabinose, glucose, D-mannose, maltose, D-mannitol, *N*-acetyl-D-glucosamine, potassium gluconate, adipate, n-caprate, DL-malate, sodium citrate and phenylacetate. With API 20 E, the isolates were negative for all tests: urease, gelatinase, *β*-galactosidase, arginine dihydrolase, lysine and ornithine decarboxylases, tryptophan deaminase, citrate utilization, H$_2$S production, indole production from tryptophan, acetoin production and fermentation/oxidation of arabinose, glucose, melibiose, rhamnose, sucrose, inositol, mannitol, sorbitol and amygdalin. The reason for the differences in results for urease and gelatinase from API 20 NE and API 20 E is unknown. The results with API ZYM are given in the species description.

Oceanobacter kriegii NBRC 15467[T] was also analysed with the API ZYM, API 20 NE and API 20 E systems. The following results were different from those of the novel isolates: with API ZYM, negative for lipase (C14) and cystine arylamidase; with API 20 NE, negative for urease; and with API 20 E, positive for arginine dihydrolase, citrate utilization, gelatinase and fermentation/oxidation of glucose. *Oceanobacter kriegii* NBRC 15467[T] was positive for gelatinase and reduction of nitrate to nitrite with API 20 NE and API 20 E, which differs from the negative result reported by Bowditch *et al.* (1984).

The analysis of fatty acid methyl esters used the Microbial Identification System (MIDI) with cells freshly grown on dMBPyr agar at 25 °C for 20 days (strains 2O1[T], 1O14 and 1O18) or 3 days (*Oceanobacter kriegii* NBRC 15467[T]). Fatty acids not identified by the system were further analysed using GC-MS (Agilent). The cellular fatty acid profiles are shown in Table 1 with that of the next closest phylogenetic neighbour with a validly published name, *Thalassolituus oleivorans* MIL-1[T] (Yakimov *et al.* 2004). The predominant fatty acids of the isolates were C$_{16:0}$, C$_{16:1}$ω7 and C$_{18:1}$ω9 and the hydroxy fatty acids were C$_{12:0}$ 3-OH and C$_{10:0}$ 3-OH. *Oceanobacter kriegii* NBRC 15467[T] contained C$_{18:1}$ω7 as a major fatty acid and did not contain C$_{18:1}$ω9. *T. oleivorans* MIL-1[T] and the novel isolates did not contain C$_{18:1}$ω7 as a major fatty acid.

Isoprenoid quinones were extracted from cells grown in dMBPyr broth with shaking (125 r.p.m.) at 25 °C to early

Table 1. Cellular fatty acid compositions of strains 2O1[T], 1O14 and 1O18 and their closest culturable phylogenetic neighbours

Strains: 1–3, *Oleibacter marinus* gen. nov., sp. nov. strains 2O1[T] (1), 1O14 (2) and 1O18 (3); 4, *Oceanobacter kriegii* NBRC 15467[T]; 5, *Thalassolituus oleivorans* MIL-1[T] (data from Yakimov *et al.*, 2004). Values are percentages of total fatty acids. −, Not detected; ND, no data available.

Fatty acid	1	2	3	4	5
$C_{10:0}$	2.6	4.6	3.1	0.2	ND
$C_{10:0}$ 3-OH	1.8	2.5	1.7	2.0	ND
$C_{11:0}$	0.3	0.5	−	−	ND
$C_{12:0}$	7.7	8.3	7.9	7.7	2.0
$C_{12:0}$ 3-OH	2.8	3.4	3.4	1.9	1.4
$C_{13:1}$	−	−	−	0.2	ND
$C_{14:1}\omega9$	−	−	−	−	1.2
$C_{14:0}$	6.9	2.8	4.4	0.3	10.2
$C_{16:1}\omega5$	0.6	0.8	−	−	ND
$C_{16:1}\omega7$	22.8	17.1	38.3	25.7	33.5
$C_{16:1}\omega9$	0.6	1.3	1.3	−	ND
$C_{16:0}$	26.6	21.0	21.7	28.0	29.5
$C_{17:1}\omega8$	1.4	1.7	0.9	−	ND
$C_{18:1}\omega7$	2.9	2.7	5.4	28.6	ND
$C_{18:1}\omega6$ and/or $\omega9$	18.8*	27.4*	9.0*	−	10.9
$C_{18:0}$	2.9	4.6	0.8	1.1	7.7
Unidentified	1.0	1.0	2.1	1.7	3.6
Summed features†					
2	0.2	0.3	−	−	ND
3	−	−	−	1.7	ND
7	−	−	−	0.9	ND

*Identified as $C_{18:1}\omega9$.

†Summed features represent groups of two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 2 consisted of $C_{12:0}$ aldehyde and/or an unidentified fatty acid with equivalent chain-length 10.928, summed feature 3 consisted of $C_{16:1}\omega7$ and/or iso-$C_{15:0}$ 2-OH and summed feature 7 consisted of one or more of an unidentified fatty acid with equivalent chain-length 18.846, $C_{19:1}\omega6$ and $C_{19:0}$ cyclo $\omega10$.

stationary phase (5–7 days for the novel isolates, 2–3 days for *Oceanobacter kriegii* NBRC 15467[T] and 4 days for *T. oleivorans* DSM 14913[T]) and were analysed as described previously (Fukunaga *et al.*, 2008). The isoprenoid quinones of the isolates were Q-9 (97–99 %) and Q-8 (1–3 %) and the isoprenoid quinones of *Oceanobacter kriegii* NBRC 15467[T] were Q-8 (91 %), Q-7 (7 %) and Q-9 (2 %), which confirmed the report by Satomi *et al.* (2002). The isoprenoid quinones of *T. oleivorans* DSM 14913[T] were Q-9 (97 %) and Q-8 (3 %). The major quinone in the novel isolates, Q-9, was clearly different from the major quinone in their closest phylogenetic neighbour with a validly published name, *Oceanobacter kriegii* NBRC 15467[T], Q-8.

For polar lipid analysis, cells were grown in dMBPyr broth with shaking (125 r.p.m.) at 28 °C to stationary phase (10 days for strain 2O1[T] and 3 days for *Oceanobacter kriegii*

NBRC 15467[T]). Polar lipids were extracted from 200–250 mg freeze-dried cells (Minnikin *et al.*, 1979) and separated by two-dimensional TLC on Kieselgel 60 F$_{254}$ plates (Merck) using chloroform/methanol/water (65 : 25 : 4, by vol.) in the first dimension and chloroform/acetic acid/methanol/water (80 : 18 : 12 : 5, by vol.) in the second dimension. Total lipid content was revealed by spraying with 10 % (w/v) molybdophosphoric acid in ethanol and incubating at 140 °C for 1 h. Specific functional groups were revealed by spraying with ninhydrin (Wako) (free amino groups), Dittmer and Lester reagent (Dittmer & Lester, 1964) (phosphate groups), anisaldehyde/sulfuric acid/ethanol (1 : 1 : 18, by vol.) (glycolipids) and periodic acid–Schiff stain (Shaw, 1968) (vicinal hydroxyl groups). The polar lipid profiles are shown in Supplementary Fig. S2. Strain 2O1[T] and *Oceanobacter kriegii* NBRC 15467[T] contained phosphatidylglycerol, a ninhydrin-positive phospholipid(s) and a few glycolipids. *T. oleivorans* DSM 14913[T] has also been shown to contain phosphatidylglycerol, phosphatidylethylamine (a ninhydrin-positive phospholipid) and glycolipids (Yakimov *et al.*, 2004). Thus, no differences were detected in the polar lipid compositions of strain 2O1[T], *Oceanobacter kriegii* NBRC 15467[T] and *T. oleivorans* DSM 14913[T] by the method used in this study.

To determine G+C content, genomic DNA was extracted according to the protocol of Marmur (1961) from cells grown to early stationary phase in dMBPyr broth at 25 °C and analysed using HPLC (Mesbah *et al.*, 1989). The DNA G+C content of the isolates was 53.0–53.1 mol%, which was similar to the G+C contents of *Oceanobacter kriegii* NBRC 15467[T] (54–56 mol%; Bowditch *et al.*, 1984) and *T. oleivorans* MIL-1[T] (53.2 mol%; Yakimov *et al.*, 2004).

Quantitative DNA–DNA hybridization, using genomic DNA extracted as described above, was performed using the fluorometric hybridization method described by Ezaki *et al.* (1989), using 25 % formamide at 56.8 °C for the hybridization reactions. DNA–DNA relatedness between each of the isolates was ⩾75 %, which indicated that the isolates are representatives of the same species. The isolates showed ⩽29 % DNA–DNA relatedness with *Oceanobacter kriegii* NBRC 15467[T] (Supplementary Table S1).

Almost-complete 16S rRNA gene sequences of strains 2O1[T], 1O14 and 1O18 (Teramoto *et al.*, 2009) showed 99.8–99.9 % sequence similarity. The 16S rRNA gene sequences were aligned with related sequences of members of the *Gammaproteobacteria* available in public databases using CLUSTAL X (Thompson *et al.*, 1997). Phylogenetic trees were inferred from the aligned sequences (1335 bp) using neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Swofford, 2000) algorithms and analysed using bootstrapping (Felsenstein, 1985) based on 1000 resamplings. The neighbour-joining tree is shown in Fig. 1. The isolates formed a coherent and distinct cluster that was supported by a high bootstrap value (100 %) within a stable phyletic group that contained *Oceanobacter kriegii* IFO 15467[T] and *T. oleivorans* MIL-1[T]. 16S rRNA
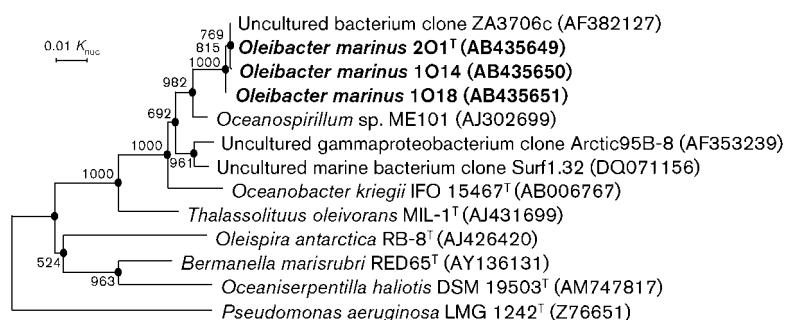
Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequence showing the phylogenetic positions of strains 2O1[T], 1O14 and 1O18 and related taxa. Bootstrap values (>500) based on 1000 resamplings are shown at branch nodes. Filled circles indicate that the corresponding nodes were recovered in a tree generated with the maximum-likelihood method with bootstrap values >60 %. Bar, 0.01 substitutions per nucleotide position.

gene sequence similarity between the isolates and *Oceanobacter kriegii* IFO 15467[T] was 96.4–96.5 %.

In summary, despite having high 16S rRNA gene sequence similarity, the isolates were different from *Oceanobacter kriegii* NBRC 15467[T] by having a different spectrum of substrates for growth and other phenotypic dissimilarities, especially the traits listed in Table 2, a different major quinone and $C_{18:1}\omega9$ as a major fatty acid. The isolates were different from *T. oleivorans* DSM 14913[T] in their hydroxy fatty acid composition. In addition, the isolates showed high n-alkane-degrading activity (Supplementary Fig. S3); *Oceanobacter kriegii* NBRC 15467[T] did not degrade alkanes (Teramoto *et al.*, 2009) and *T. oleivorans* DSM 14913[T] degraded alkanes significantly more slowly than the isolates when tested with the method described by Teramoto *et al.* (2009) (Teramoto & Harayama, 2011). Also, a gene similar to *alkB* (alkane mono-oxygenase) is present in the isolates and absent from *Oceanobacter kriegii* NBRC 15467[T] (Bowditch *et al.*, 1984; Teramoto *et al.*, 2009; Yakimov *et al.*, 2004).

On the basis of phenotypic, genotypic and chemotaxonomic data, it is proposed that strains 2O1[T], 1O14 and 1O18 are

representatives of a novel genus and species, for which the name *Oleibacter marinus* gen. nov., sp. nov. is proposed.

Description of *Oleibacter* gen. nov.

Oleibacter [O.le′i.bac′ter. L. n. *oleum* oil; N.L. masc. n. *bacter* rod; N.L. masc. n. *Oleibacter* an oil (-degrading) rod].

According to 16S rRNA gene sequence analysis, belongs to the *Gammaproteobacteria*. Cells are Gram-negative, motile, aerobic rods. n-Alkane-degrading activity is observed. Predominant cellular fatty acids are $C_{16:0}$, $C_{16:1}\omega7$ and $C_{18:1}\omega9$ and hydroxy fatty acids are $C_{12:0}$ 3-OH and $C_{10:0}$ 3-OH. The major isoprenoid quinone is Q-9 and minor amounts of Q-8 are present. Polar lipids are phosphatidylglycerol, a ninhydrin-positive phospholipid(s) and glycolipids. The DNA G+C content of known strains of the type species is 53.0–53.1 mol%. The type species is *Oleibacter marinus*.

Description of *Oleibacter marinus* sp. nov.

Oleibacter marinus (ma.ri′nus. L. masc. adj. *marinus* of the sea, marine).

Table 2. Selected physiological and biochemical characteristics that differentiate strains of *Oleibacter marinus* gen. nov., sp. nov. from their closest culturable phylogenetic neighbours

Strains: 1, *Oleibacter marinus* gen. nov., sp. nov. (*n*=3); 2, *Oceanobacter kriegii* NBRC 15467[T]; 3, *Thalassolituus oleivorans* DSM 14913[T]. Data were obtained in this study unless indicated. +, Positive; −, negative; ND, no data available.

Characteristic	1	2	3
Monopolar flagella	1	1[a]*	1–4[b]
Growth at 4 °C	−	−[a]	+[b]
Optimum temperature (°C)	25–30	25–30	20–25[b]
pH range	6–10	5–9	7.5–9[b]
Reduction of nitrate	+	+ [−[a]]	−[b]
Fatty acids (%)			
$\quad C_{18:1}\omega6$ and/or $\omega9$	9.0–27.4†	0	10.9[b]
$\quad C_{18:1}\omega7$	2.7–5.4	28.6	ND
Major 3-OH fatty acid (% of total 3-OH)	$C_{12:0}$ (58–67)	$C_{10:0}$ (51) [$C_{12:0}$ (54)[b]]	$C_{12:0}$ (100)[b]
Major isoprenoid quinone (%)	Q-9 (97–99)	Q-8 (91)	Q-9 (97)

*Data obtained from: *a*, Bowditch *et al.* (1984); *b*, Yakimov *et al.* (2004).
†$C_{18:1}\omega9$.

Displays the following properties in addition to those given in the genus description. Cells are 0.3–0.6 × 1.5–2.8 µm and monoflagellated. Growth occurs at 10–40 °C (optimum 25–30 °C), at pH 6–10 (optimum from pH 6–7 to pH 9) and with 1–7 % (w/v) NaCl. Catalase- and oxidase-positive. As single carbon and energy sources, acetate, propionate and pyruvate are utilized, but L-arabinose, D-fructose, D-glucose, ribose, glycolate, citrate, formate, DL-lactate, aspartate, L-asparagine and L-proline are not. With GN2 MicroPlates, β-hydroxybutyric acid, γ-hydroxybutyric acid, L-glutamic acid and Tween 80 are oxidized and Tween 40 is often oxidized. Exhibits high n-alkane-degrading activity. With API 20 NE, positive for urease, gelatinase and reduction of nitrate to nitrite. With API ZYM, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase, but negative for trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-fucosidase and α-mannosidase.

The type strain is 2O1[T] (=NBRC 105760[T] =BTCC B-675[T]), isolated from seawater at Pari Island, located off Jakarta, Indonesia. Strains 1O14 (=NBRC 105758 =BTCC B-676) and 1O18 (=NBRC 105759 =BTCC B-677) were isolated from the same source and belong to the species.

Acknowledgements

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