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On the description of *Tisochrysis lutea* gen. nov. sp. nov. and *Isochrysis nuda* sp. nov. in the Isochrysidales, and the transfer of *Dicrateria* to the Prymnesiales (Haptophyta)

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Abstract The Isochrysidaceae is a family of non-calcifying organisms within the haptophyte order Isochrysidales. *Isochrysis galbana*, a species widely used as a food source in aquaculture, is the best-known representative of this family that contains three genera but only six described species. We sequenced partial nuclear small subunit (SSU) and large subunit rDNA and mitochondrial cytochrome oxidase 1 genes of 34 isochrysidacean culture strains (including authentic strains when available) and compared molecular phylogenetic inferences with cytological and ultrastructural observations. The isochrysidaceaen culture strain *Isochrysis* affinis *galbana* (Tahiti isolate), widely used in aquaculture and commonly known as T-Iso, is clearly genetically distinct from *Isochrysis galbana*, despite seemingly being morphologically identical. A strain with a similar ultrastructure to that of *Isochrysis galbana* except for the lack of body scales had sequences that were more similar to but still distinct from those of *Isochrysis galbana*. *Dicrateria inornata*, a species that lacks body scales, is classified within the Isochrysidaceae, but the SSU rDNA sequence of the authentic strain of this species matches that of *Imantonia rotunda* within another haptophye order, the Prymnesiales. *D. inornata* and *Imantonia rotunda* have similar ultrastructure except for the respective absence/presence of scales. These results lead us to propose the erection of one new genus (*Tisochrysis* gen. nov.) and two new species (*Tisochrysis lutea* sp. nov. and *Isochrysis nuda* sp. nov.). *D. inornata* is reclassified within the Prymnesiales, and *Imantonia rotunda* is transferred to this genus (*Dicrateria rotunda* comb. nov.).

Keywords *Dicrateria* · *Imantonia* · Isochrysidaceae · *Isochrysis galbana* · Phylogeny · Taxonomy · Ultrastructure

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Introduction

The Isochrysidaceae is a family of non-calcifying organisms within the prymnesiophyte order Isochrysidales (Pascher 1910; Edvardsen et al. 2000). The Isochrysidales is a monophyletic group (Edvardsen et al. 2000) that also includes the Noëlaerhadaceae (Jerkovic 1970) (containing the coccolithophore genera *Emiliania*, *Gephyrocapsa* and *Reticulofenestra*) and is one of the four orders belonging to the subclass Calcihaptophycidae (Isochrysidales, Coccolithales, Syracosphaerales and Zygodiscales; de Vargas et al. 2007). Neither coccolith formation nor the existence of a digenetic life cycle has been described in the Isochrysidaceae, whereas the Noëlaerhabdaceae contains taxa that calcify in the diploid phase of a haplo-diplontic life cycle (Green et al. 1996; Houdan et al. 2004).

The Isochrysidales is unique in producing alkenones (Marlowe et al. 1984), a suite of long-chain (C37–C39) unsaturated methyl and ethyl ketones that are resistant enough to be retained in ancient sediments. The ratio of the diunsaturated C37 methyl ketone (C37:2) versus the triunsaturated homologue

(C37:3) (expressed as UK'37=[C37:2]/[C37:3+C37:2]) linearly correlates with growth temperature (Conte et al. 1994) and can be used as a paleothermometer (Brassell et al. 1986; Marlowe et al. 1984). Sedimentary core top UK'37 has been widely adopted by geochemists as a proxy for past sea surface temperature (e.g. Müller et al. 1998). The first appearance of alkenones in the sediment archive extends down to the mid-Cretaceous at ca. 120 Mya (Brassell and Dumitrescu 2004), suggesting that the evolutionary origin of the Isochrysidales occurred at or before this period. Molecular clock studies support this theory and have dated the early divergence of this order in the evolution of the Calcihaptophycideae within the range of 203–119 Mya (Medlin et al. 2008; Liu et al. 2010). In terms of synapomorphic features, the two isochrysidalean families share the existence of a thin membranous sheet in the peripheral ER underneath the plasmalemma which has never been observed in other haptophytes (Van der Wal et al. 1985; Inouye 1997). They also possess relatively simple flagellar roots, a reduced haptonema and organic scales with a unique ornamentation (Billard and Inouye 2004). In certain species currently classified within the Isochrysidales, one or both of the latter two characters (haptonema, scales) may even be absent (e.g. *Emiliania huxleyi* lacks organic scales in the diploid stage and lacks any trace of a haptonema).

Composed of three genera, *Chrysotila* (Anand 1936), *Dicrateria* (Parke 1949) and *Isochrysis* (Parke 1949; Jordan et al. 2005), the Isochrysidaceae contains species that occur as motile biflagellate and/or non-motile mucilage-covered forms. The former state is dominant in the genera *Isochrysis* and *Dicrateria*, and the latter dominates in *Chrysotila*. The evolution and ecology of members of this family have received little attention. Doubt remains over whether coccolith formation was lost or never acquired in the Isochrysidaceae and whether this may be linked to evolution of life cycle type (de Vargas et al. 2007; Liu et al. 2010). An intriguing form of extracellular calcification has been described in *Chrysotila* (Green and Course 1983). Isochrysidaceans have been described almost exclusively from cultures isolated from samples collected in near-shore coastal and estuarine environments, and there is little information on their biogeography or environmental diversity. Culture strains of *Isochrysis galbana*, the type species of the genus, as well as a widespread culture isolate from Tahiti designated as *Isochrysis* affinis *galbana* (commonly named "T-Iso") have been the focus of numerous ecophysiological studies as a consequence of their extensive use as feedstocks in aquaculture (Jeffrey et al. 1994; Bougaran et al. 2003). Lipid content has been broadly characterized in several of these culture strains, revealing profiles suited for the nutrition of larval animals (Brown et al. 1993), with notably the production of high amounts of long-chain polyunsaturated fatty acids such as docosahexaenoic acid (DHA) (Liu and Lin 2001). Other features that make these organisms suitable for use in aquaculture include fast growth rates and wide physico-chemical tolerance ranges (O'Shea et al. 2010). These characteristics also make these strains of potential interest for other biotechnological applications such as biodiesel production (Chisti 2007). Strains of *Isochrysis galbana* typically originate from temperate zones, whereas T-Iso was isolated from a tropical region, and physiological differences have been reported between *Isochrysis galbana* and T-Iso (Ewart and Pruder 1981). However, it is currently not clear whether *Isochrysis galbana* and T-Iso are conspecific or distinct taxa. Several strains designated as *Isochrysis* (often *Isochrysis galbana*) exist in most of the main microalgal culture collections, but most have never been fully characterized and could belong to species with distinct physiological and biochemical characteristics.

In this context, we investigated a set of Isochrysidaceae strains in order to assess the taxonomic diversity and phylogenetic relationships within the family. Molecular phylogenetic analyses were based on sequences of the nuclear small subunit (SSU) and large subunit (LSU) rDNA and the mitochondrial cytochrome oxidase 1 (cox1) genes, and morphostructural studies were conducted using light microscopy (LM) and transmission electron microscopy (TEM). Where possible, the taxonomic study was based on the use of authentic (i.e. representative of type material) culture strains. The study revealed new diversity within this family, and one new genus and two new species are described here. The proposed taxonomic and phylogenetic schemes provide a framework for the identification of strains that have potential for use in various biotechnological applications and for future studies on the environmental biodiversity, biogeography and microevolution of members of this family.

Materials and methods

Origin of analysed strains

Isochrysidales strains (Table 1) from the Algobank Culture Collection (AC), the Plymouth Culture Collection (PLY), the Provasoli-Guillard Center for Culture of Marine Phytoplankton (CCMP) and the Roscoff Culture Collection (RCC) were maintained in natural seawater-based (salinity 35) K/2(-Si,-Tris,-Cu) medium (Keller et al. 1987) at 17 °C with 50 μmol photons m^{-2} s^{-1} illumination provided by daylight neon tubes with a 14:10 h L/D cycle.

DNA sequencing

Genomic DNA was extracted using the DNeasy Plant mini kit (Qiagen). SSU, LSU and cox1 genes were PCR amplified using the primer pairs listed in Table 2. PCRs were performed

Table 1 List and sequence accession numbers of the Isochrysidales strains used in this study

Original strain name	Synonym strain name	Revised taxonomic name	Strain code	Equivalent strain code	Origin	Isolator	Date of Isolation	Genbank accession number (SSU+ LSU + cox1)		
Chrysotila lamellosa			AC17	RCC1195	English Channel; coastal	Billard	1968	KC888096	KC888131	KC888164
Chrysotila lamellosa			RCC1195	AC17	English Channel; coastal	Billard	1968	KC888095	KC888130	KC888163
Chrysotila lamellosa			PLY353		UK; estuarine	Parke	1963	KC888098	KC888133	KC888166
Chrysotila lamellosa			PLY408	CCAP918/1, CS272	UK; terrestrial	Jowett	1966	KC888099	KC888134	KC888167
Chrysotila lamellosa			PLY475		Austria, Freshwater	Parke	1971	KC888100	KC888135	KC888168
Chrysotila lamellosa			PLY489		UK; terrestrial	Hibberd	1972	KC888129	KC888162	KC888195
Chrysotila sp.		*Chrysotila lamellosa*	PLY509		UK; coastal	Jocelyn	1973	KC888101	KC888136	KC888169
Chrysotila sp.			PLY510A		UK; coastal	Green	1974	KC888102	KC888137	KC888170
Dicrateria inornata[a]			PLY564	CS267	English Channel; coastal	Gross	1935	KC888128		
Dicrateria sp.		*Isochrysis nuda*	AC49	RCC1207	Atlantic (Morocco), coastal	Fresnel	1978	KC888114	KC888149	KC888182
Dicrateria sp.		*Isochrysis nuda*	RCC1207	AC49	Atlantic (Morocco), coastal	Fresnel	1978	KC888115	KC888150	KC888183
Gephyrocapsa oceanica			RCC1281	THAU1	Indian Ocean	Probert	2000	KC404144	KC404190	KC404334
Gephyrocapsa oceanica			RCC1286	AS62E	Mediterranean Sea	Probert	1999	KC404147	KC404194	KC404398
Isochrysis aff. galbana	*T-Iso*	*Tisochrysis lutea*	AC102	T.ISO, HAP34T, CCAP927/14, CCMP1324, NEPCC 601, CS-177, RCC1349	Pacific (Tropical), coastal	Haines	1977	KC888118	KC888152	KC888185
Isochrysis aff. galbana	*T-Iso*	*Tisochrysis lutea*	RCC1349	AC102, T.ISO, HAP34T, CCAP927/14, CCMP1324, NEPCC 601, CS-177	Pacific (Tropical), coastal	Haines	1977	KC888119	KC888153	KC888186
Isochrysis galbana[a]			PLY565	AC34, AC101, CCAP927/1, UTEX987, RCC1347, RCC1348, CCMP1323, NEPCC2,	Irish Sea; coastal	Parke	1938	KC888106	KC888141	KC888174
Isochrysis galbana			AC34	AC101, CCAP927/1, UTEX987, CCMP1323, RCC1347, NEPCC2,	Irish Sea; coastal	Parke	1938	KC888107	KC888142	KC888175
Isochrysis galbana			AC101	AC34, CCAP927/1, UTEX987, RCC1348, CCMP1323, NEPCC2,	Irish Sea; coastal	Parke	1938	KC888108	KC888143	KC888176
Isochrysis galbana			RCC1348	AC34, AC101, CCAP927/1, UTEX987, NEPCC633, PLY565, CCMP1323, NEPCC2,	Irish Sea; coastal	Parke	1938	KC888110	KC888145	KC888178
Isochrysis litoralis[a]			AC18	RCC1346	English Channel; coastal	Lepailleur	1965	KC888113	KC888148	KC888181
Isochrysis litoralis			RCC1346	AC18	English Channel; coastal	Lepailleur	1965	KC888112	KC888147	KC888180
Isochrysis sp.		*Tisochrysis lutea*	CCMP463	RCC1350	Atlantic ocean	Glazer	1985	KC888120	KC888154	KC888187
Isochrysis sp.		*Tisochrysis lutea*	RCC1350	CCMP463	Atlantic ocean	Glazer	1985	KC888121	KC888155	KC888188
Isochrysis sp.		*Tisochrysis lutea*	RCC1344	AC620, S-1	Atlantic (spain); coastal	Probert		KC888122	KC888156	KC888189
Isochrysis sp.		*Isochrysis nuda*	RCC2477		English Channel; coastal	Ota	2009	KC888116		

Table 1 (continued)

Original strain name	Synonym strain name	Revised taxonomic name	Strain code	Equivalent strain code	Origin	Isolator	Date of Isolation	Genbank accession number (SSU+ LSU + cox1)		
Isochrysis sp.		*Isochrysis galbana*	PLY8		Irish Sea; coastal	Knight-Jones	1948	KC888103	KC888138	KC888171
Isochrysis sp.		*Isochrysis galbana*	PLY240		Irish Sea; coastal	Butcher	1964	KC888104	KC888139	KC888172
Isochrysis sp.		*Chrysotila lamellosa*	PLY352		UK, estuarine	Parke	1963	KC888097	KC888132	KC888165
Isochrysis sp.		*Isochrysis nuda*	PLY401B		English Channel; coastal	Jowett	1966	KC888117	KC888151	KC888184
Isochrysis sp.		*Tisochrysis lutea*	PLY506A		Pacific (Tropical), coastal	Martin	1978	KC888125	KC888159	KC888192
Isochrysis sp.		*Tisochrysis lutea*	PLY506B		Pacific (Tropical), coastal	Martin	1978	KC888126	KC888160	KC888193
Isochrysis sp.		*Tisochrysis lutea*	PLY506C		Pacific (Tropical), coastal	Martin	1978	KC888127	KC888161	KC888194
Isochrysis sp.	*Pseudosichrysis paradoxa*	*Isochrysis galbana*	PLY507	AC80, RCC1353, CCAP 949/1, CCMP715, UTEX 1988	North Atlantic USA; estuarine	Ott	1980	KC888105	KC888140	KC888173
Isochrysis sp.		*Tisochrysis lutea*	PLY562		North Sea; coastal	Amundsen	1962	KC888124	KC888158	KC888191
Pseudoisochrysis paradoxa		*Isochrysis galbana*	AC80	CCAP949/1, CCMP715, RCC1353, UTEX1988	North Atlantic USA; estuarine	Ott	1980	KC888109	KC888144	KC888177
Pseudoisochrysis paradoxa		*Isochrysis galbana*	RCC1353	AC80, CCAP949/1, CCMP715, UTEX1989	North Atlantic USA; estuarine	Ott	1980	KC888111	KC888146	KC888179

AC Algobank-Caen, *CCAP* Culture Collection of Algae and Protozoa, *CCMP* Provasoli-Guillard Center for Culture of Marine Phytoplankton, *CS* Commonwealth Scientific and Industrial Research Organisation, *NEPCC* North East Pacific Culture Collection, *PLY* Plymouth Culture Collection, *RCC* Roscoff Culture Collection, *UTEX* University of Texas

[a] Authentic strains

Table 2 Primers used in this study

Target gene	Primer name	Primer sequences 5′–3′	Direction	Reference	Couple used in this study
SSU	A18Dir	AACCTGGTTGATCCTGCCAGT	Forward	Sogin 1990	Prym887r
	A18Rev	TCCTTCTGCAGGTTCACCTAC	Reverse		Prym429f
	Prym429f	GCGCGTAAATTGCCCGAA	Forward	Coolen et al. 2004	A18Rev
	Prym887r	GGAATACGAGTGCCCCTGAC	Reverse		A18Dir
LSU	LHapto4	ATGGCGAATGAAGCGGGC	Forward	Liu et al. 2010	Couple
	IspLR2	CTTCACCCTACCCAGGCATA	Reverse	In this study	
cox1	igA	GCAATATCTAGTCCTGAATTTGA	Forward	Hayashi-Ishimaru et al. 1997	Couple
	igB	ACCAGCATTTGGAATAGTTTCAC	Reverse		

in a total reaction volume of 25 μL using the Phusion high-fidelity PCR master mix with GC buffer (Finnzymes). The PCR protocol employed was as follows: 30-s initial denaturation at 98 °C, followed by 32 cycles of 10 s at 98 °C, 30-s annealing at 55 °C and 1-min extension at 72 °C. A final 5-min extension step at 72 °C was conducted to complete the amplification. Amplification products were controlled by electrophoresis on a 1 % agarose gel. The PCR products were sequenced directly on an ABI PRISM 3100 xl DNA autosequencer (Perkin-Elmer) using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer).

Phylogenetic reconstruction

For both ribosomal genes, an alignment including GenBank sequences from other haptophytes was first performed with the online version of the SILVA aligner program (http://www.arb-silva.de/; Quast et al. 2013). For cox1, the alignment was performed using MAFFT (Katoh et al. 2007). Alignments were double checked *de visu* in the sequence editor BIOEDIT (Hall 1999). For the cox1 gene, a fragment of around 400 nucleotides was sequenced, and 330 nucleotides were retained for sequence alignment that was manually checked after codon translation.

Appropriate models of DNA substitution were determined with TREEFINDER (Jobb et al. 2004), using the three proposed statistics (AIC, AICc and BIC) and the partition-wise option. For SSU and LSU rDNA, a GTR substitution model was selected, taking into account a shaped distribution of the rates of substitution among sites. For cox1, a J1 substitution model (Jobb et al. 2004) was selected, taking into account a gamma-shaped distribution of the rates of substitution among sites. The selected models and parameters were used to perform phylogenetic analyses using two methods: maximum likelihood (ML) as implemented in TREEFINDER and Bayesian statistics with Mr.BAYES v3.1.2 (Ronquist and Huelsenbeck 2003). The robustness of the topology of ML trees was tested by bootstrapping with 1,000 replicates. The Bayesian analysis was conducted with two runs of four Markov chains, for at least three million generations, sampling every 100th generation. The burn-in option was set for discarding 25 % of the 30,000 trees found.

Morphological characterization

Living cells were observed with an Olympus BX51 (Olympus Corporation, Japan) light microscope equipped with differential interference contrast optics. Polysaccharide excretions from cultured cells were stained with 1 % toluidine blue. Whole mounts were prepared for TEM from a drop of culture fixed with 1 % osmium vapour on a Formvar-coated grid and negative stained with 1 % uranyl acetate diluted in water/ethanol (1:1). For thin sections, cells were collected by gentle centrifugation and fixed with a 2.5 % glutaraldehyde solution with 0.25 M sucrose in 0.1 M sodium cacodylate buffer at pH 7.2 for 2 h. After three rinses in 0.1 M cacodylate buffer with decreasing sucrose concentration (0.25 M, 0.1 M, 0 M), cells were postfixed for 1 h with 1 % osmium tetroxide and washed once in 0.1 M cacodylate buffer. Dehydration was performed by transfer through a graded ethanol series (30, 50, 70, 85, 90, 95 and 100 %) for 10 min each. The dehydrated cells were suspended in a 1:1 (*v*/*v*) mixture of Epon resin and ethanol for 1 h and then embedded in 100 % Epon resin. The embedded samples were polymerized at 60 °C for 24 h and sectioned using a Leica ultramicrotome with a diamond knife. Thin sections were placed on formvar-covered copper grids. Sections were stained with uranyl acetate followed by poststaining with lead citrate according to (Reynolds 1963). Whole mounts and sections were examined under a JEOL JEM 1400 transmission electron microscope at an accelerating voltage of 80 kV.

Results

Phylogenetic relationships

The topology of the ML tree inferred from a concatenated sequence of the three genes (SSU and LSU rDNA, cox1)

(Fig. 1) was very similar to the phylogenetic reconstructions inferred from each individual gene (e.g. SSU rDNA, Fig. 2). Bayesian analyses yielded the same overall topology.

Within the Isochrysidaceae, three main clades are statistically strongly supported. The *Chrysotila* clade is composed of the uncharacterized strain PLY510A and a subclade grouping sequences from several strains designated as *Chrysotila lamellosa* or *Chrysotila* sp. The *Isochrysis* clade contains all *Isochrysis galbana* strains, *Pseudoisochrysis paradoxa* AC80 and RCC1353, *Isochrysis litoralis* AC18 and RCC1346, and three strains with distinct but identical sequences, AC49 and RCC1207 (designated as *Dicrateria* sp.) and PLY401B (*Isochrysis* sp.). The *Tisochrysis* clade consists of the *Isochrysis* affinis *galbana* Tahiti isolate (AC102 and RCC1349) which had sequences identical to three other T-Iso-like strains (PLY562, RCC1344 and CCMP463) and almost identical to three undescribed strains (PLY506A, PLY506B, PLY506C). Of the three genes sequenced, these latter three strains differed from the T-Iso strains by a single nucleotide substitution in the SSU rDNA sequence (Fig. 2). The SSU rDNA sequence of the authentic strain of *Dicrateria inornata* (PLY564) is identical to sequences of the Prymnesiales species *Imantonia rotunda* (Fig. 2).

Morphological descriptions

Dicrateria sp. AC49 and RCC1207, Isochrysis sp. PLY401b

Cells are predominantly non-motile, mostly spherical (3.5–6 μm in diameter) with a single golden yellow plastid (Fig. 3a). Motile cells exhibit two slightly unequal flagella measuring between 7.5 and 9 μm (Fig. 3b). Thin sections of the basal apparatus revealed two flagellar basal bodies between which a reduced haptonema without scales is inserted (Fig. 3c); one flagellar basal body exhibits a typical haptophycean flagellar transition. The flagella were often abbreviated suggesting their release during fixation (Fig. 3c, d). Thin sections revealed that the cell membrane is not surrounded by organic scales. The cell ultrastructure shows the presence of a mitochondrion, a Golgi apparatus with "peculiar" cisternae (dictyosome) (Manton 1966, 1967) and a single plastid per cell enclosed within a nucleoplastidial membrane and with an immersed pyrenoid traversed by thylakoid lamella (Fig. 3d).

Isochrysis affinis galbana (T-Iso) AC102 and RCC1349

Cells of this species are predominately motile, golden brown in colour, with cell shape varying from spherical to ovate or oblong (3.5–6 μm) with an apical depression into which the

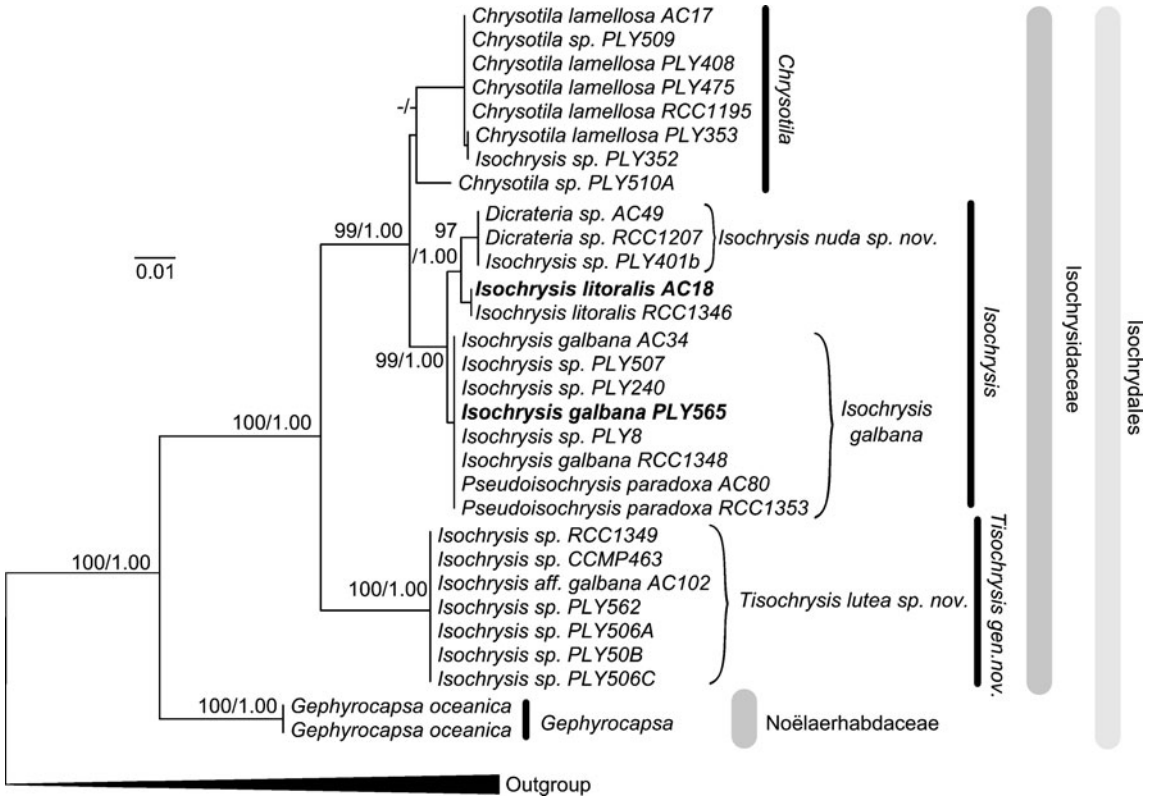


Fig. 1 Culture-based molecular phylogeny of the Isochrysidales inferred from a concatenated matrix from nuclear SSU, LSU rDNA and cox1 sequences. Maximum likelihood topology is shown with bootstrap values (BVs), and posterior probabilities (PPs) are indicated on the nodes; nodes with low supports for both methods (BV values under 50 % and PP under 0.80) are left blank

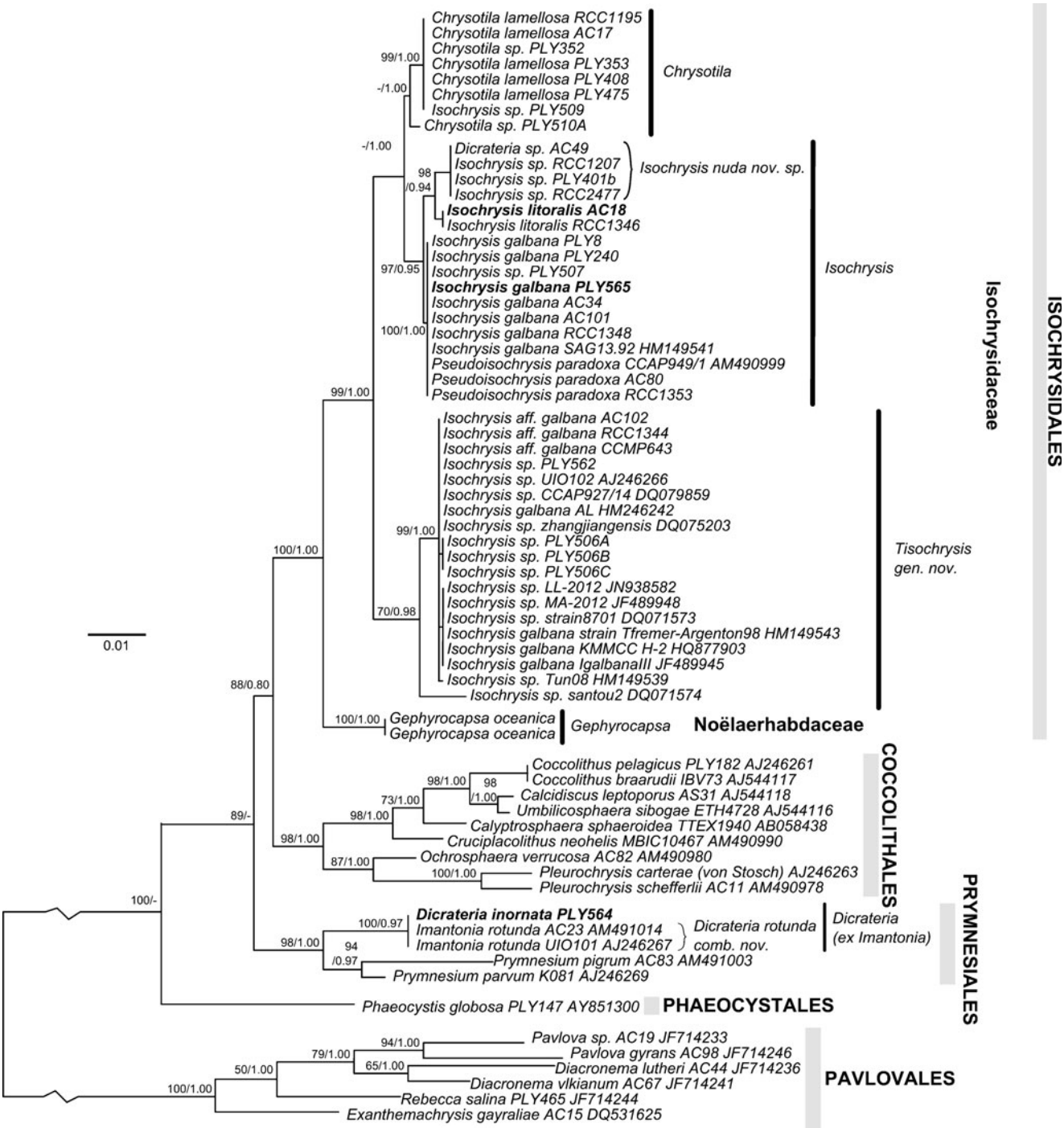


Fig. 2 Culture-based molecular phylogeny of the Isochrysidales inferred from nuclear SSU rDNA sequences. Maximum likelihood topology for 43 Isochrysidales representatives with the position of *D. inornata* PLY564 (type strain) in *bold* (Coccolithales, Prymnesiales, Phaeocystales and Pavlovales are shown as outgroup). Bootstrap values (BVs) and posterior probabilities (PPs) are indicated on the nodes; nodes with low supports for both methods (BV values under 50 % and PP under 0.80) are left blank

flagella are inserted (Fig. 4a). The two flagella are equal in length (around 7 μm), and a short haptonema is present (around 100 nm; Fig. 4a, d). The cell is covered by a dense layer of thin organic scales identical to those of *Isochrysis galbana*, measuring around 100 nm with a superficial pattern of around 40 radial ridges and a central swelling (Fig. 4e). Smaller scales measuring around 10 nm with a superficial pattern of 12 radial ridges are abundantly present on the haptonema and on the cell membrane in the vicinity of the flagellar insertion (Fig. 4c, d, f). The emergent part of the

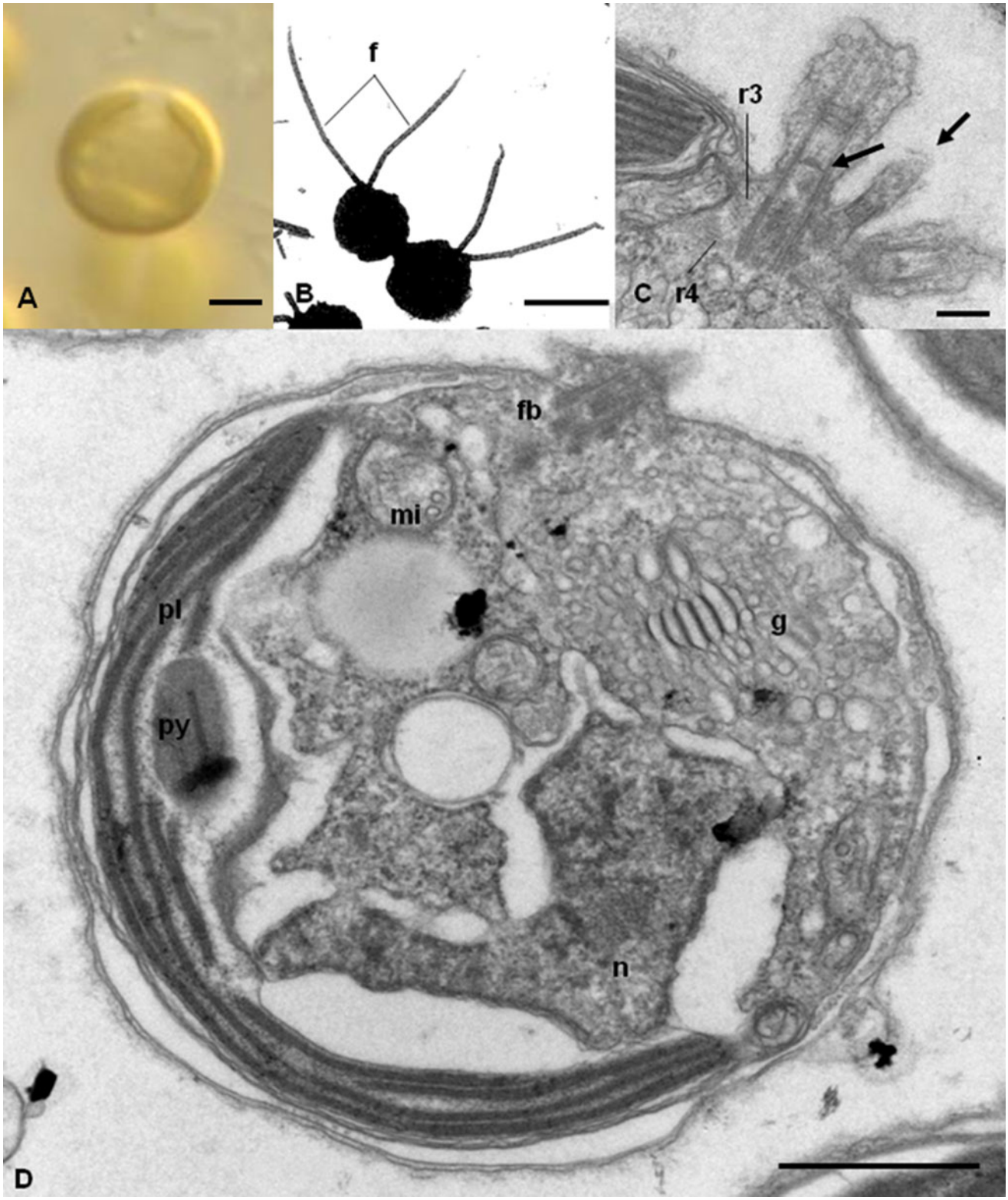


Fig. 3 Light and transmission electron micrographs (respectively LM and TEM) of *Isochrysis nuda* sp. nov. (*Dicrateria sp.* AC49 and RCC1207, and *Isochrysis sp.* PLY401b). **a** LM of a non-flagellated cell with one single plastid. **b**. TEM of whole mounted flagellated cells. **c**. TEM of basal body in transverse section showing flagellar base with a transition zone (*left arrow*) and abbreviated haptonema (*right arrow*). **d** TEM of a cell displaying a flagellar base, a Golgi apparatus, a mitochondrion, a nucleus, a parietal plastid and a pyrenoid travresed by a thylakoid lamella in longitudinal section. Scale bars: **a** 1 μm, **b** 5 μm, **c** 200 μm, **d** 1 μm. Abbreviation: *f* flagellum, *fb* flagellar base, *g* Golgi apparatus, *mi* mitochondrion, *n* nucleus, *pl* plastid, *py* pyrenoid, *r3* and *r4* microtubular roots 3 and 4

haptonema is composed of three or four microtubules within a sheath of endoplasmic reticulum (Fig. 4d, f). Thin sections reveal the presence of one or two parietal plastids linked to the nucleus within a nucleoplastidial membrane (Fig. 4e). A prism-shaped pyrenoid traversed by a single thylakoid lamella is present in the plastid. The flagellar apparatus includes root R1 adjacent to the left flagellum and its accessory component (CR1), root R2 seemingly composed of two microtubules, and roots 3 and 4 originating on either side of the right flagellar base (Fig. 4f–h). In the late stationary phase of culture, most cells lose their flagella and become non-motile. These cells are mostly spherical, ranging in diameter between 4 and 6 μm, and sometimes produce a mucilage covering. In such old cultures, the colour of cells typically becomes brownish orange to orange.

Discussion

Three clades were distinguished within the Isochrysidaceae in this study, and these can be assimilated to three genera: *Chrysotila*, *Isochrysis* and the new genus *Tisochrysis*. The *Chrysotila* clade is composed of several strains, most with identical sequences including the two *C. lamellosa* strains considered representative of the reference material by Green and Parke (1975). The *Isochrysis* clade consists of eight strains of *Isochrysis galbana* (including the authentic strain PLY565 and two strains previously named *Pseudoisochrysis paradoxa*) with identical sequences, the authentic strain of *Isochrysis litoralis* (AC18) and the derived strain RCC1346, and three strains with identical sequences previously designated either as *Dicrateria* sp. (AC49 and RCC1207) or *Isochrysis* sp. (PLY401B). The *Tisochrysis* clade is composed of the strain informally known as *Isochrysis* aff. *galbana* "Tahiti isolate" (T-Iso), together with other genetically identical or quasi-identical strains.

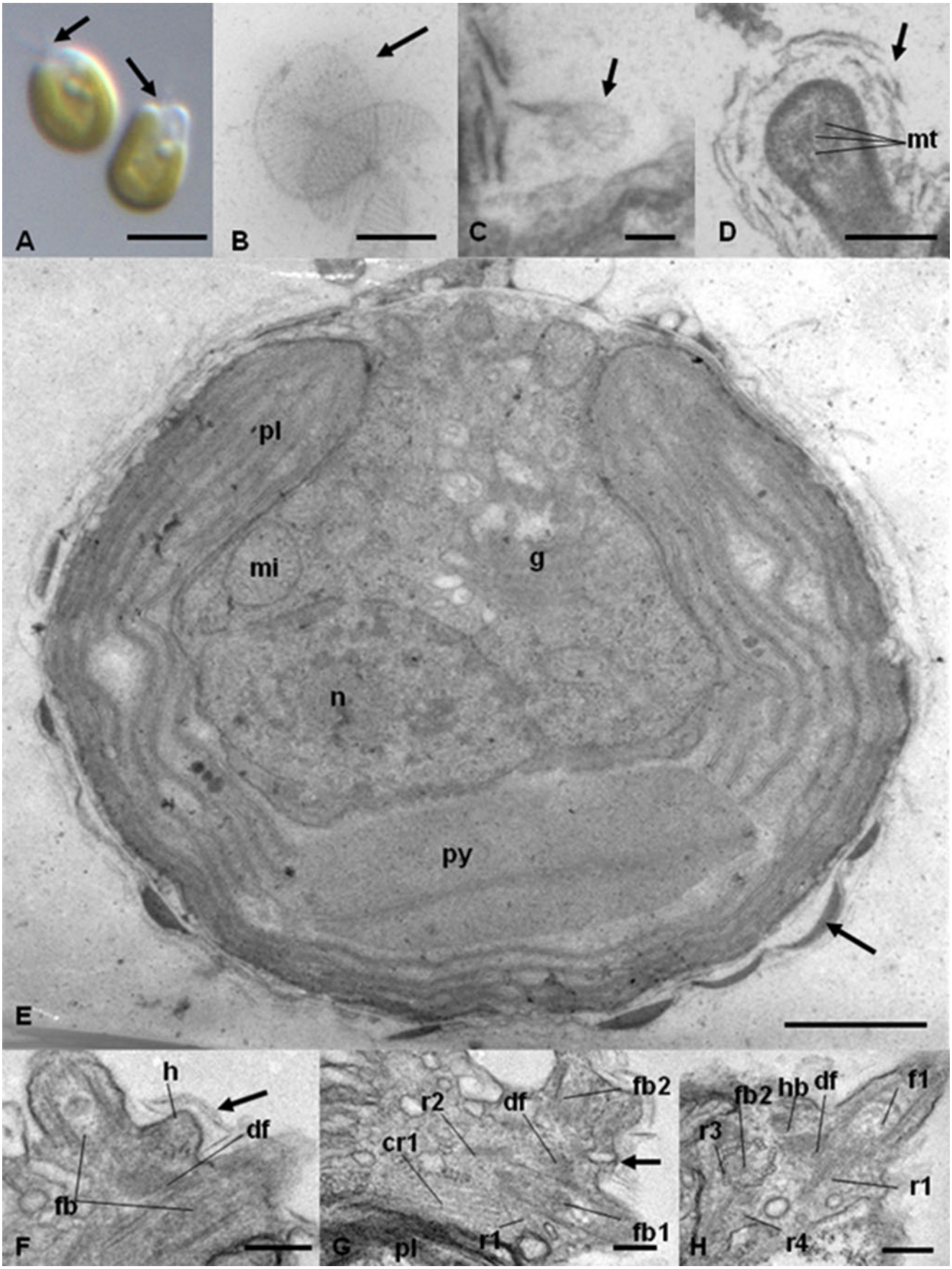
Chrysotila clade

The two known *Chrysotila* species (*Chrysotila stipitata*, *C. lamellosa*) were described by Anand (1936, 1937) from samples collected from the supra-tidal zone of chalk platforms in southern England, and therefore, no authentic cultures exist. Green and Parke (1975) isolated and analysed the ultrastructure of cultures that they considered representative of each of these two species. In our study, several culture strains were found to be morphologically and genetically identical to the two strains that Green and Parke (1975) designated as *C. lamellosa* (PLY 353, PLY408), but we were not able to access any strains that corresponded morphologically to the type species of the genus, *C. stipitata*. One strain, PLY510A, was genetically distinct from but morphologically identical to the *C. lamellosa* strains. Despite the fact that this strain does not exhibit the morphological characters characteristic of *C. stipitata* (i.e. cells embedded in smooth mucilaginous stalks), we consider that it cannot be unambiguously described as a new taxon (cryptic with *C. lamellosa*) in the absence of sequences of *C. stipitata*.

Isochrysis clade

Several culture isolates have sequences identical to the authentic culture of *Isochrysis galbana*. Two strains, AC80 and RCC1353 (equivalent to UTEX1988 and CCAP949/1), were designated as *Pseudoisochrysis paradoxa*, a *nomen nudum* applied provisionally to a culture by the isolator F. Ott, the paradox being that the organism resembled *Isochrysis* in morphology but was greenish in colour (Jordan et al. 2005). The culture was subsequently studied in detail by Pennick (1977) and shown to be a prasinophyte and formally described as *Pyramimonas virginica*. However, the original name was still used for copies of this culture maintained in certain culture collections, and Farmer (1993) reported this strain to be an alkenone producer; to our knowledge, all copies of this strain are brownish yellow in colour like *Isochrysis* cultures. Our results, as well as those of Sàez et al. (2004), confirm that AC80 and RCC1353 are morphologically (data not shown) and genetically identical to *Isochrysis galbana*. On the one hand, an explanation could be that the original culture was a mixture of *Isochrysis galbana* and the prasinophyte *Pyramimonas virginica* and that one or the other organism came to dominate different copies of the strain. On the other hand, we cannot exclude the possibility that mislabelling occurred with the strain that Pennick described as *Pyramimonas virginica*. As there is no evidence for *Pseudoisochrysis paradoxa* strains being contaminated, the paradoxical situation remains unresolved. The name *Pseudoisochrysis paradoxa* was never formally proposed and so is invalid, and we consider that strains attached to this name should logically be named *Isochrysis galbana*.

Strains AC49, RCC1207 and PLY401B differ from *Isochrysis galbana* and *Isochrysis litoralis* in not possessing body scales, a character that links them to *D. inornata.* However, the SSU rDNA sequence of the authentic strain of *D. inornata* (PLY564) does not match those of AC49, RCC1207 and PLY401B, but rather is almost identical to that of the prymnesialian species *Imantonia rotunda*. This genetic similarity corroborates published information that shows that *D. inornata* and *Imantonia rotunda* are very similar in ultrastructure (Green and Pienaar 1977; Edvardsen et al. 2000) and pigment signatures (Jeffrey and Wright 1994; Zapata et al. 2004), and the fact that neither produces alkenones (Marlowe et al 1984). The only clear morphological distinction between these two taxa is the presence (*Imantonia rotunda*) or absence (*D. inornata*) of body scales. Based on these results, we transfer *Dicrateria* to the Prymnesiales and include *D. rotunda* comb. nov. in this genus (*Dicrateria* having priority over *Imantonia*), the diagnosis of which is emended to include organisms with or without scales. A new species, *Isochrysis*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◀ **Fig. 4** Light and transmission electron micrographs (respectively LM and TEM) of *T. lutea* sp. nov. (*Isochrysis* aff. *galbana* AC102 and RCC1349). **a** LM of two motile cells with apical depression (*arrows*). **b** TEM of body scales (*arrow*) in transverse section. **c** TEM of haptonematal scale (*arrow*) in glancing section. **d** TEM of the haptonema in longitudinal section, showing scales (*arrow*) and three constitutive microtubules. **e** TEM of a cell surrounded by osmiophilic muciferous bodies (*arrow*) and displaying a nucleus, a Golgi apparatus, a parietal plastid and a pyrenoid crossed by a thylakoid lamella in longitudinal section. **f** TEM of basal body in transverse section showing flagellar base and distal fibre, abbreviated haptonema composed of four microtubules and adjacent haptonematal scales (*arrow*) **g** TEM of basal body in longitudinal section, showing flagellar base, a distal fibre, roots 1 and 2 and the accessory compound component of root 1, haptonematal scales (*arrow*). **h** TEM of basal body in transverse section, showing flagellar and haptonematal bases, a distal fibre and roots 1, 3 and 4. Scale bars: **a** 5 μm, **b** 200 nm, **c**, **d** 10 nm, **e** 1 μm, **f**–**h** 100 nm. Abbreviation: *cr1* accessory compound component of root 1, *df* distal fibre, *fl* flagella, *fb* flagellar base, *fb1* and *fb2* left and right flagellar base, *g* Golgi apparatus, *h* haptonema, *mi* mitochondrion, *mt* microtubule, *n* nucleus, *pl* plastid, *py* pyrenoid, *r1*, *r2*, *r3* and *r4* microtubular root 1, 2, 3 and 4

nuda sp. nov., is described for the taxon represented by the culture strains AC49, RCC1207 and PLY401B. In contrast to *Isochrysis galbana*, the palmelloid phase is prominent in cultures of both *Isochrysis litoralis* and *Isochrysis nuda*, and the haptonema is reduced to such an extent that it is only visible in electron microscopy in the latter two species that cluster together in the molecular phylogeny. *Isochrysis nuda* is distinguished from *Isochrysis litoralis* by the lack of body scales.

Tisochrysis clade

To our knowledge, this is the first time that the phylogenetic relationships of T-Iso with other members of the Isochrysidales have been presented. The morphological and ultrastructural characters of T-Iso are extremely similar to those of *Isochrysis galbana*, even for characters that are often phylogenetically discriminant such as scale morphology and the structure of the flagellar/haptonematal basal body (Table 3). We noted a difference in plastid coloration (orange for T-Iso vs brown for *Isochrysis galbana*) that is most marked in stationary-phase cultures. One other character defining both T-Iso and *Isochrysis* is excretion of a thin mucilage layer by non-motile cells, well characterised by toluidine blue staining. Despite this morphostructural similarity, the genetic distance between the *Isochrysis* and T-Iso clusters is in excess of 1 % in SSU rDNA sequences, i.e. equal to or greater than the genetic distance between most closely related genera for which data for this gene exist within the haptophytes (Edvardsen et al. 2000; Medlin et al. 2008). Should the clade containing T-Iso be retained within the genus *Isochrysis*, the genus would clearly be polyphyletic. Thus, we conclude that the definition of a new genus for this clade is warranted, and we propose the name *Tisochrysis* for the new genus in reference to the well-known T-Iso strain. Within this new genus, we describe the species represented by the T-Iso strain as *Tisochrysis lutea* sp. nov., in reference to the distinctive coloration of the cells.

Concluding remarks

This study reveals new diversity within this non-calcifying calcihaptophyte lineage. With current knowledge, *Isochrysis galbana* and *T. lutea* would be considered conspecific according to a morphological species concept, which for practical reasons is the concept still most commonly applied. However, the genetic separation of these two taxa is clear, and there is some chemotaxonomic evidence that supports the distinction. For example, a number of studies have described significant differences in their lipid contents. Patterson et al. (1994) reported differences in sterol content (epibrassicasterol in *Isochrysis galbana* vs brassicasterol in *Tisochrysis* strains) and alkenone production (differences in the ratio of alken-2-ones (37:4, 37:3, 37:2) and alken-3-ones (38:3, 38:2)). Liu and Lin (2001) described the presence of DHA in both species, but they reported the lack of eicosapentaenoic acid in *T. lutea* (CCMP1324), whereas it is present in *Isochrysis galbana* (CCMP1323, originating from the authentic strain isolated by Parke in 1938). Whilst we acknowledge that the current definition of a new cryptic taxon based essentially on genetic grounds will be difficult to apply in certain contexts (e.g. time-series based on microscope observations, use of un-characterized strains in aquaculture hatcheries), we feel that it will prove useful not only for informing the choice of culture strains for future biotechnological applications, but also as a framework for interpreting future environmental monitoring by gene sequencing efforts. As in many other protistan lineages, there have been an increasing number of reports of similar patterns of cryptic or pseudo-cryptic speciation for haptophytes in recent years, such as within the pavlovophyceaen genus *Pavlova* (Bendif et al. 2011). Despite this discovery of new diversity within the Isochrysidaceae, the known diversity within this family remains low compared to most other haptophyte families. Discovery of further cryptic or pseudo-cryptic diversity within the Isochrysidaceae would not be surprising given the morphostructural conservation between genetically distinct taxa demonstrated here. Significant levels of microdiversity would be predicted to occur within the Isochrysidaceae given the heterogeneous nature of the near-coastal environment in which they are found and thus increased likelihood of geographical isolation of populations.

Taxonomic treatments

Isochrysis nuda Bendif et Probert, sp. nov.

Diagnosis: Cells solitary, non-motile, spherical (3.5–6 μm in diameter), without scales. Flagella two, slightly unequal (7.5–

Table 3 Summary of morphological and ultrastructural characters within *Isochrysis* and *Tisochrysis*

		Morphological and ultrastructural characters			
Genus		*Isochrysis*			*Tisochrysis*
Species		*I. galbana*	*I. litoralis*	*I. nuda sp. nov.*	*T. lutea*
Habitats	Zonation	Coastal	Littoral	Coastal	Coastal
	Climate (or latitude)	Temperate	Temperate	Temperate/tropical	Tropical
Motile stage	Description	Dominant stage, ellipsoid, variable morphology	Elongated with subequal flagella	Subovate, not metabolic, with slightly unequal flagella	Dominant stage, ovate, oblong, round (variable morphology)
	Cell size (μm)	5–6×2–4× 2.5–3	4×7	3–5.5	4.5–7.5×3–6
Non-motile stage	Description	Round cell with homogenous mucilage	Dominant stage hemispherical or round, no sarcinochrysis condition	Dominant stage, spherical	Various morphology, mostly round or ovate cell with homogenous thin mucilage or coccoid
	Cell size (μm)	5–6	4–6×6–8; 5	3.5–6	4–6
Body scales	Size (μm)	0.3–0.4×0.2–0.3	0.3×0.18	–	0.35×0.25
Insertion of Appendages		Apical	Apical	Apical	Apical
Flagella	Length (μm)	Approx. 7	7–8×8–9	7–9	Approx. 7
	Flagellar action	Homodynamic	Homodynamic (Billard and Gayral 1972)	Homodynamic	Homodynamic
Haptonema	Presence/absence	+	–	+	+
	Microtubule composition	5 MT	?	3MT	3MT
	Organisation	4 MT base	?	–	3–4MT
	Special feature	Scales (0.08–0.10×0.05–0.08)	?	–	Scales (0.10×0.07)
Plastid	Characteristics	1 yellow brown parietal	1–2 golden yellow parietal	1 yellow brown	1–2 golden yellow parietal
	Pyrenoid	1	1/plastid	1/plastid	1/plastid

The data, from previous studies and the current work, support the novel, morpho-genetic framework presented herein

9 μm) inserted apically with abbreviated haptonema (100 nm) between; latter with three to four microtubules in the emergent part. Plastid single, parietal, yellow-brown with immersed pyrenoid. Asexual reproduction by division in motile stages. Nucleotide sequences of nuclear SSU and LSU rDNA and cox1 gene are distinctive. Substitutions in nuclear SSU rDNA sequence at positions 216 (C/T), 706 (T/C), 1329 (C/T), 1348 (T/C), 1667 (T/C) and 1668 (C/T) are diagnostic compared to other species members of *Isochrysis*.

Holotype: Cryopreserved strain RCC1207 in the Roscoff Culture Collection

Etymology: *nuda* to highlight the lack of body scales

Tisochrysis Bendif et Probert, gen. nov.

Diagnosis: Cells solitary, motile, covered by several layers of scales with ten radiating ribs arranged in each of four quadrants. Flagella two, equal, inserted apically with abbreviated haptonema between, bearing small round scales with 12 radial ridges. Plastid single, parietal, yellow-brown with immersed pyrenoid. Asexual reproduction by division in motile and non-motile stages. Nucleotide sequences of nuclear SSU and LSU rDNA and cox1 gene are distinctive from previously described isochrysidalean taxa.

Etymology: combination of T from Tahiti and *Isochrysis*

Type species: *Tisochrysis lutea* Bendif et Probert, sp. nov.

Diagnosis: Cells solitary, motile, 3-7.5 μm in diameter, metabolic, covered by several layers of scales with superficial pattern of around 40 radial ridges. Flagella two, equal (around 7 μm) inserted apically with abbreviated haptonema (100 nm) between with three to four microtubules in emergent part and bearing small oval scales with central swelling and around 12 radial ridges. Plastid single, parietal, yellow-brown with immersed pyrenoid. Asexual reproduction by division in motile and non-motile stages. Non-motile cells embedded in thin layer of mucilage

Holotype: Cryopreserved strain RCC1349 in the Roscoff Culture Collection

Etymology: *lutea* refers to the orange colour of cells, particularly noticeable in old cultures of the species

Dicrateria Parke emend. Bendif et Probert

Synonym: Imantonia Reynolds 1974

Type species: *Dicrateria inornata* Parke

Dicrateria rotunda (Reynolds) Bendif et Probert, comb. nov.

Basionym: *Imantonia rotunda* Reynolds (Reynolds 1974)

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