Published in Journal of Eukaryotic Microbiology 53, issue 2, 112-120, 2006
which should be used for any reference to this work

Andalucia (n. gen.)—the Deepest Branch Within Jakobids (Jakobida; Excavata), Based on Morphological and Molecular Study of a New Flagellate from Soil

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ABSTRACT. A new heterotrophic flagellate (*Andalucia godoyi* n. gen. n. sp.) is described from soil. Earlier preliminary 18S rRNA analyses had indicated a relationship with the phylogenetically difficult-to-place jakobid *Jakoba incarcerata*. *Andalucia godoyi* is a small (3–5 μm) biflagellated cell with a ventral feeding groove. It has tubular mitochondrial cristae. There are two major microtubular roots (R1, R2) and a singlet root associated with basal body 1 (posterior). The microtubular root R1 is associated with non-microtubular fibres ''I,'' ''B,'' and ''A,'' and divides in two parts, while R2 is associated with a ''C'' fibre. These structures support the anterior portion of the groove. Several features of *A. godoyi* are characteristic of jakobids: (i) there is a single dorsal vane on flagellum 2; (ii) the C fibre has the jakobid multilaminate substructure; (iii) the dorsal fan of microtubules originates in very close association with basal body 2; and (iv) there is no ''R4'' microtubular root associated with basal body 2. Morphological analyses incorporating the *A. godoyi* data strongly support the monophyly of all jakobids. Our 18S rRNA phylogenies place *A. godoyi* and *J. incarcerata* as a strong clade, which falls separately from other jakobids. Statistical tests do not reject jakobid monophyly, but a specific relationship between *Jakoba libera* and *J. incarcerata* and/or *A. godoyi* is rejected. Therefore, we have established a new genus *Andalucia* n. gen. with the type species *Andalucia godoyi* n. sp., and transfer *Jakoba incarcerata* to *Andalucia* as *Andalucia incarcerata* n. comb.

Key Words. Mitochondrial genome, phylogeny, protist, protozoa, *Reclinomonas*, systematics, ultrastructure, 18S rRNA.

THE jakobids (Jakobida) are a recently circumscribed group of small free-living heterotrophic flagellates comprising half a dozen nominal species in three described genera: *Jakoba*, *Reclinomonas*, and *Histiona* (Flavin and Nerad 1993; O'Kelly 1993; Simpson and Patterson 2001). The group probably also includes the taxa *Stenocodon* and *Stomatochone* (Flavin and Nerad 1993; Patterson et al. 2002), as well as an entity that is not formally described, but is studied under the name ''*Seculamonas ecuadoriensis*'' (e.g. Marx et al. 2003; Gray, Lang, and Burger 2004). Despite their historical obscurity, jakobids are of considerable evolutionary importance. Firstly, jakobid mitochondrial genomes more closely resemble the genome of the ancestral α-proteobacterial symbiont than do any other mtDNAs investigated to date (Gray et al. 2004; Lang et al. 1997). Besides retaining more protein-coding genes than other mitochondria, the mitochondrial genomes of jakobids encode some subunits of a bacterial-type RNA polymerase, while all other eukaryotes employ in its place a non-homologous single-subunit enzyme related to those in T7/T3 phages. The mitochondrial genome data, in isolation, suggest that jakobids may be amongst the earliest-diverging living eukaryotes (Gray et al. 1998; Lang et al. 1999). Secondly, morphological studies place jakobids within the supergroup Excavata, but the Excavata concept itself is highly contentious from a molecular phylogenetic standpoint (Simpson 2003; Simpson and Patterson 1999). Thus, there is considerable interest in determining the phylogenetic position(s) of jakobids, and with comparative evolutionary genomics in mind, understanding their true diversity.

Four jakobids—*Jakoba libera*, *Reclinomonas americana*, *Histiona aroides*, and *Jakoba incarcerata*—have been examined by electron microscopy (Flavin and Nerad 1993; Mylnikov 1989; O'Kelly 1993, 1997; Patterson 1990; Simpson and Patterson 2001). These taxa have a relatively similar organization—all have a ventral groove and attendant cytoskeleton of the typical ''excavate'' type, a single vane in a dorsal position on the posterior flagellum (F1), and a similar arrangement of the flagellar apparatus, including a distinctive organization of the non-microtubular ''C fibre'' (Simpson and Patterson 1999, 2001). Some differences between jakobid taxa have been noted, e.g. the mitochondrial cristae are either flattened or tubular (O'Kelly 1993), but both intuitive accounts and formal cladistic analyses suggest the monophyly of jakobids among excavate eukaryotes (Simpson 2003; Simpson and Patterson 2001). By contrast, molecular phylogenetic studies do not support jakobid monophyly. *Jakoba libera* and *R. americana* do form a strong clade in 18S rRNA trees of eukaryotes, but *Jakoba incarcerata* always falls as a separate branch (Cavalier-Smith 2003; Nikolaev et al. 2004a; Simpson et al. 2002b). Alpha tubulin and beta tubulin phylogenies also place *J. incarcerata* as a branch separate from both *J. libera* and *R. americana* (Edgcomb et al. 2001; Simpson et al. 2002b). Thus, the monophyly of jakobids as a whole and the monophyly of the genus *Jakoba* in particular are highly uncertain (Cavalier-Smith 2003; Simpson 2003).

Taxonomic undersampling is still a major concern in determining the phylogenetic relationships of excavate groups (Simpson 2003). This is especially true of jakobids, as all ultrastructural and molecular data from the phylogenetically difficult-to-place *J. incarcerata* derives from a single isolate, which is no longer in culture. Recently one of us has isolated jakobid-like flagellates on two occasions from clay-rich soils in southern Spain (EL., unpubl. data). A general 18S rRNA gene phylogeny indicates that these new isolates are close relatives of *J. incarcerata* (EL., unpubl. data). Here, we present morphological data from one of these isolates, and report a more focused molecular phylogenetic study. We confirm the current non-monophyly of the taxon *Jakoba*, and propose a new genus, *Andalucia* n. gen. We employ the newly cultured organism as the type (*Andalucia godoyi* n. sp.), and transfer to *Andalucia* the organism formerly called *Jakoba incarcerata*, as *Andalucia incarcerata* n. comb.

MATERIALS AND METHODS

Isolation and culturing. *Andalucia godoyi* isolate ''And28'' was isolated from a clay soil from Andújar (38°16′N, 46°16′W). Soil was resuspended with Neff's amoeba saline non-nutrient medium in a blender, and serial dilutions were made on a 96-well

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microtitre plate containing 1:300 tryptone soy broth-enriched amoeba saline. Growing populations of protists were transferred to culture flasks containing the same medium, and monoprotistan cultures were obtained by serial subculturing. For light and electron microscopy, encysted flagellates were transferred to 15-ml polypropylene tubes containing 5 ml of 1:300 LB broth-enriched amoeba saline, and grown at 20 °C for three days.

Light microscopy. Cells were examined with a Zeiss Axiophot compound microscope with differential interference contrast (DIC) optics, and a Zeiss Axiovert 200 M inverted microscope with phase contrast optics. Both systems mounted Zeiss Axiocam HR digital cameras. For phase contrast, cells were fixed with glutaraldehyde (final concentration 1.25% v/v) for 5 min immediately before observation.

Electron microscopy. Cells were pelleted by centrifugation (2,300 *g*, 5 min), then swamped in a fixation cocktail containing 2% (v/v) glutaraldehyde in 50 mM cacodylate buffer (pH 7.2), for 30 min. Cells were rinsed several times in the same cacodylate buffer, then post-fixed in 0.2% (w/v) OsO_4 in the same cacodylate buffer for 60 min. Cells were rinsed in a descending series of cacodylate solutions, then water, then trapped in agar, dehydrated through an acetone series, and embedded in Epon resin. Cured blocks were serially sectioned with a diamond knife on a Leica Ultracut UCT ultramicrotome. Series were mounted on pioloform-coated slot grids, stained with lead citrate and uranyl acetate, and examined using a FEI/Philips Technai-12 transmission electron microscope.

Morphological phylogenetic analysis. The morphological analysis was based on the matrix for excavate groups introduced by Simpson (2003). This was updated by combining our new data from *A. godoyi* with that for *A. incarcerata* in a single taxon (*Andalucia*). The annotated matrix is available by request to AGBS. We analysed two taxon sets—''excavate taxa only'' (i.e. Euglenozoa, oxymonads and parabasalids excluded), with 71 informative characters, and ''all excavates,'' with 73 characters. Maximum parsimony trees were searched for via 100 random addition sequences plus TBR rearrangements using PAUP* 4b10 (Swofford 2003). Bremer support values (decay indices) were calculated using Autodecay 5.04 (Eriksson 2001) in concert with PAUP*, with the same tree searching.

Molecular sequencing. 18S rRNA sequences from *A. godoyi* have been reported previously (Genbank nos. AY965865 and AY965870; EL. et al., unpubl. data). To obtain an 18S rRNA sequence from ''*Seculamonas ecuadorensis*'' (ATCC 50688), a dense culture was grown in WCL medium (http://megasun.bch.umontreal.ca/People/lang/FMGP/methods/wcl.htm) enriched with *Enterobacter aerogenes*. Cells were concentrated by centrifugation, and genomic DNA extracted using a CTAB-based protocol (Clark and Diamond 1991). A near-complete 18S rRNA gene sequence was amplified by PCR using primers ''EukA'' and ''EukB'' (Medlin et al. 1988), Amplicons were cloned into a TA vector (TOPO 2.1, Invitrogen, Carlsbad, CA), and a positive clone was completely sequenced in both directions. This sequence has the Genbank Accession number DQ190541.

Molecular phylogenetic analysis. Sequences were aligned by eye to a large alignment of 18S rRNA sequences (based on a seed alignment kindly provided by C. Berney, University of Geneva). Representing broad eukaryotic diversity, 51 (near-) complete sequences were retained for analysis. Only ''short branching'' groups were included, plus Euglenozoa and Heterolobosea (these groups often separate jakobids in 18S rRNA trees). All available near-complete sequences from jakobids were included, except one peculiarly divergent *R. americana* sequence (Genbank no. AF053089). A conservative 1,198 positions were considered ''unambiguously aligned'' and retained for analysis. The alignment is available by request to AGBS.

Sequences were analysed by maximum likelihood (ML) using PAUP*. A general time-reversible model of nucleotide substitution was used, with a ''gamma distribution plus invariable sites'' model for among-site rate variation (GTR+Γ+*I* model), with the gamma distribution approximated by four equi-probable discrete categories, and empirical base frequencies. This model was selected over simpler plausible models (GTR+Γ, TrN+Γ+*I*, TrN+Γ) by likelihood ratio tests. Parameter values were estimated from the data under a Jukes-Cantor BioNJ tree. The ML tree was searched for using 20 random taxon addition sequences, with TBR rearrangements. A 375-replicate ML bootstrap analysis was performed using the same model (neighbour-joining starting trees, with TBR rearrangements).

To test some alternative hypotheses of relationships, we searched for ML trees where the following groupings were constrained to be monophyletic: (i) all jakobids, including the two *Andalucia* species; (ii) *J. libera* plus *Andalucia*; (iii) *J. libera* and *A. incarcerata*; (iv) *J. libera*, ''*Seculamonas*,'' and *Andalucia*; and (v) *J. libera*, ''*Seculamonas*,'' and *A. incarcerata*. Constraints (ii)—(v) generated reasonable topologies in which the two *Andalucia* species formed a clade with *J. libera* to the exclusion of *Reclinomonas* (under such topologies, it may have been possible to assign *Andalucia* spp. to the genus *Jakoba*). Model and search strategies were identical to the original ML search. We also generated a set of ''reasonable trees'' by allowing TBR rearrangements on the 20 bootstrap trees that conferred the highest likelihood on the original data, and saving the 100 best trees encountered (all saved trees were $< 5 \ln L$ units less likely than the ML tree). The constraint trees, plus the ML tree, plus the 0, 10, 35 or 100 most likely of the ''reasonable trees'' set were compared by ''approximately unbiased'' tests using Consel 0.1 (Shimodaira and Hasegawa 2001) with default parameter settings. The inclusion of different numbers of ''reasonable trees'' only slightly affected the *P* values for the test trees.

RESULTS

Light microscopy of *Andalucia godoyi* n. gen., n. sp. Live cells measure 3–5 μm. A groove extends down the ''ventral'' side of the cell (Fig. 1, 2). Seen from the side, the cell is bean-shaped, but usually with a slightly pointed anterior end; starved cells are more elongate. The two flagella are twice the length of the cell body. They insert at the top of the groove, near the apex of the cell, usually at a markedly obtuse angle. Cells were observed freely swimming (i.e. not adhering in place on either flagellum), usually rotating about their longitudinal axis. The anterior flagellum (F2) beats around the anterior end of the cell in a circular movement, while the posterior flagellum (F1) beats in the vicinity of the groove, often in a wave-like manner (Fig. 3). Cysts were readily formed in culture (Fig. 4). Stressed cells usually rounded up such that the groove was ablated, and often lost their flagella.

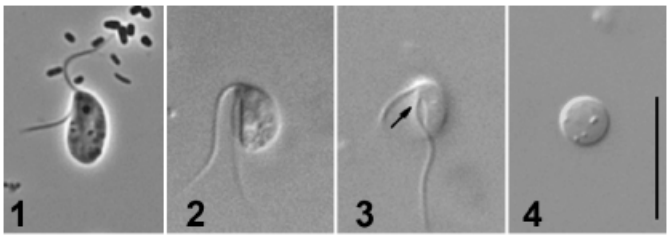

Fig. **1–4.** Light micrographs of *Andalucia godoyi* n. gen., n. sp. **1.** Fixed cell (phase contrast). **2–4.** Pictures in vivo (DIC). **2.** Active cell. **3.** Active cell, with arrow indicating the position of the groove. **4.** Cyst. Scale bar represents 10 μm.

This occurred rapidly when cells were observed under high magnification, presumably due to hypoxia and/or temperature stress.

Electron microscopy. The rather elongated nucleus (ca $0.5 \times 1\,\mu m$) is located anteriorly, and has a central nucleolus (Fig. 5). A moderately dense spherical organelle, the paranuclear body, is associated with the posterior end of the nucleus. It is ca 300 nm across, with a single bounding membrane and no obvious internal structure (Fig. 10). The single mitochondrion has tubular cristae (Fig. 6, 9, 10). The mitochondrion lies alongside the nucleus, with its anterior end near the basal bodies. A single Golgi dictyosome with three to five cisternae is located anteriorly, ventrally and to the right of the flagellar apparatus (Fig. 17). The posterior half of the cell contains food vacuoles with ingested bacteria (Fig. 5).

Both flagella have a standard ''9+2'' axoneme. The posterior flagellum (F1) has a single vane, which is located on the dorsal side of the axoneme and originates some way posterior to the flagellar emergence (Fig. 9). The vane has a maximum breadth of at least 170 nm (Fig. 8) and has a striated appearance in grazing section (period ca 30 nm, Fig. 8). Both basal bodies are ca 330 nm long. They are normally observed at an angle of 135°, and separated by ca 70 nm, with the anterior basal body (2) offset dorsally and to the right relative to the posterior basal body (1, Fig. 12). The basal bodies are connected by a crescent-shaped structure, the striated connecting fibre (StC). A thin (smooth) crescent (SmC) is associated with the dorsal side of basal body 2 (Fig. 12).

The dorsal fan of peripheral microtubules originates in association with the anterior side of basal body 2 (Fig. 7). A dense sheet up to 170 nm broad, the fan-associated sheet (FA), lies between the dorsal fan and basal body 2 (Fig. 6, 7, 12). The dorsal fan includes ca 12 microtubules, which diverge to support the dorsal side of the cell. There are no discrete microtubular roots associated with basal body 2.

There are two major microtubular roots, R1 and R2, associated with basal body 1; R1 originates against the right edge of basal body 1, is directed posteriorly, and consists of a flat row of

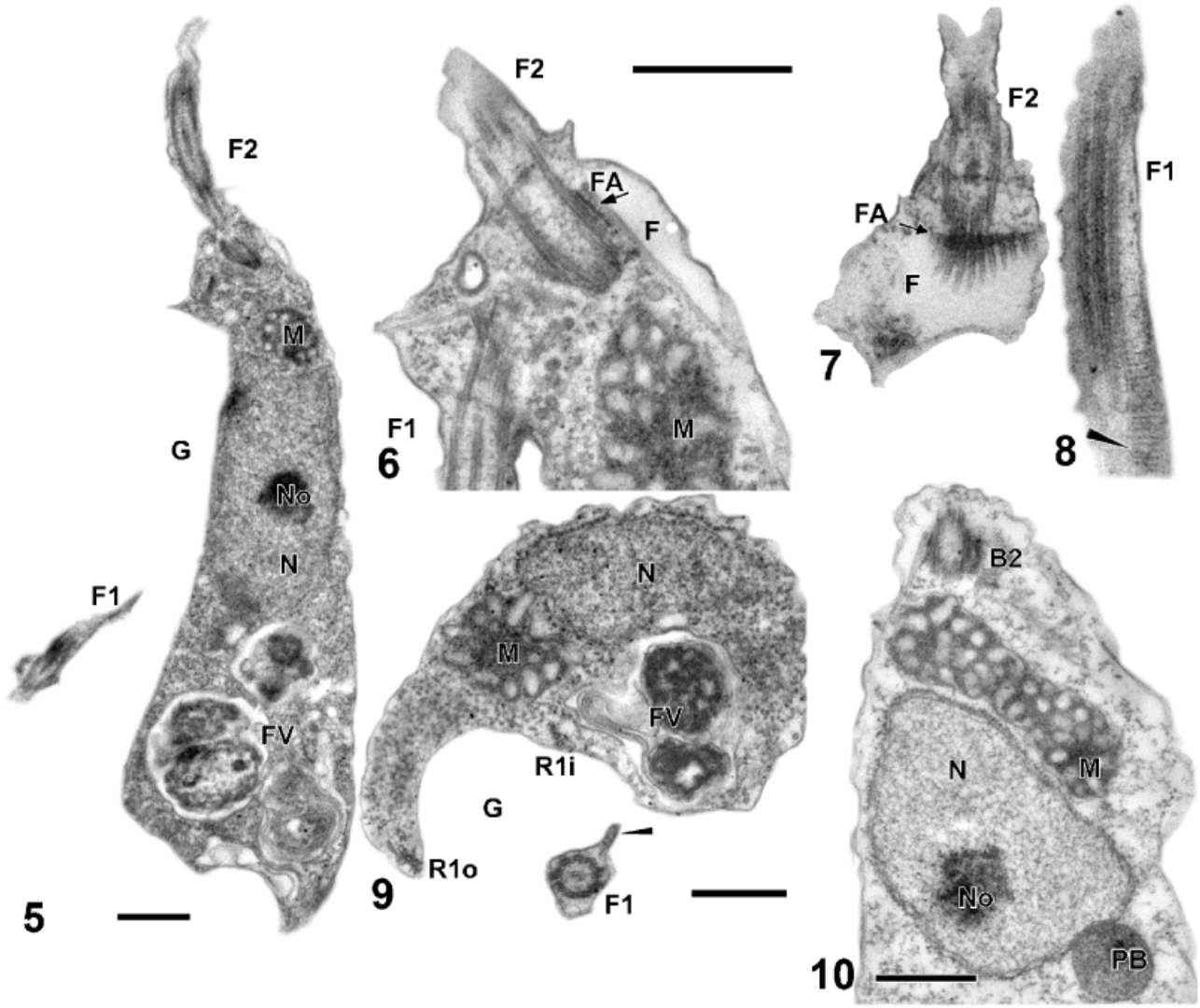

Fig. **5–10.** Transmission electron micrographs of *Andalucia godoyi* n. gen., n. sp. **5.** General view of the cell in longitudinal section. F1, posterior flagellum; F2, anterior flagellum; FV, food vacuole; G, groove; M, mitochondrion; N, nucleus; No, nucleolus. **6.** Flagellar apparatus in longitudinal section. F, dorsal fan; FA, fan-associated dense sheet. **7.** Oblique section of the cell apex showing the origin of the dorsal fan. **8.** Longitudinal section of F1, showing the flagellar vane in grazing section (arrow). **9.** Transverse section of the groove. R1i, inner portion of the right root; R1o, outer portion of the right root. The arrow indicates the vane. **10.** View of the mitochondrion, nucleus and paranuclear body (PB). B2, anterior basal body. All scale bars represent 500 nm (Scale bar in Fig. 6 applies for Fig. 6, 7; Scale bar in Fig. 9 applies for Fig. 8, 9).

microtubules, staggered such that the outermost microtubules originate more posteriorly than the innermost (Fig. 14–16). There is a non-microtubular ''I'' fibre associated with the ventral face of R1 (Fig. 14, 15). A dense ''B'' fibre originates against the right-ventral side of basal body 1 (Fig. 14), and continues posteriorly, to the right of basal body 1, converging on the outer portion of R1 (Fig. 18). A non-microtubular ''A'' fibre originates on the dorsal side of basal body 1, and associates with the dorsal side of R1 (Fig. 14–16). The A fibre has a striated appearance in some sections (Fig. 11, 14). A singlet microtubule (S) originates in the ''corner'' formed by the dorsal side of R1, and the right side of basal body 1 (Fig. 15). This singlet microtubule is initially connected to the dorsal face of R1 by a singlet-associated fibre (SA) (Fig. 15), and is directed posteriorly.

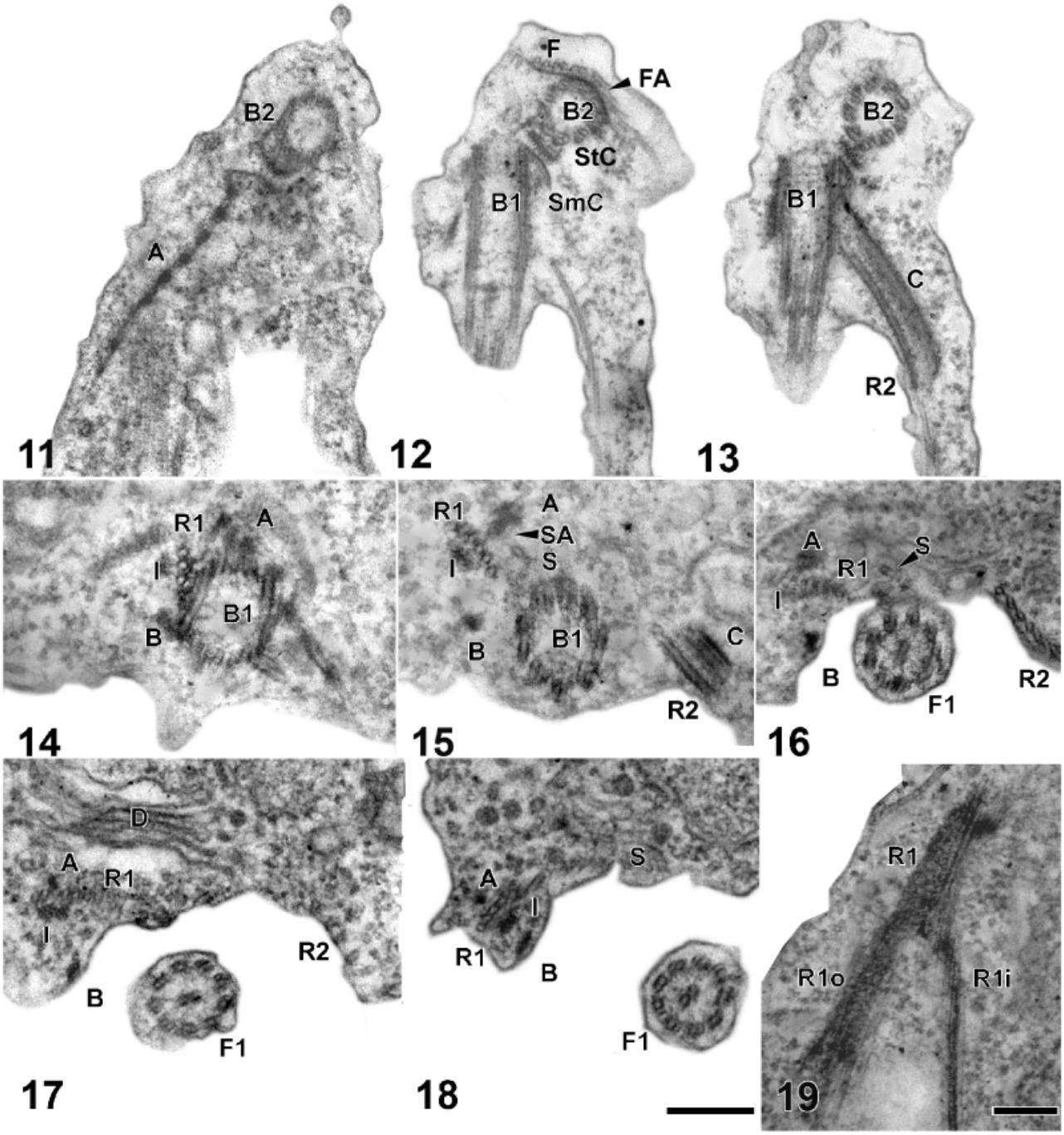

Fig. **11–19.** Transmission electron micrographs of *Andalucia godoyi* n. gen., n. sp. **11.** Transverse section of the anterior basal body (B2). A, A fibre. **12.** Section cutting B2 transversally and B1 (posterior basal body) obliquely. SmC, smooth crescent; StC, striated crescent. **13.** Similar view showing the origin of the R2 root and C fibre (C). **14–18.** Non-consecutive series showing the appearance of B1 and the emergence of F1. B, B fibre; D, dictyosome (Golgi apparatus); I, I fibre; S, singlet root; SA, singlet associated fibre. **19.** Longitudinal section showing the split of R1. All scale bars represent 200 nm (Scale bar in Fig. 18 applies for Fig. 14–18, Scale bar in Fig. 19 applies for Fig. 11, 13 and Fig. 19).

The R2 microtubular root, with about seven microtubules, originates near the left side of basal body 1 and is directed posteriorly (Fig. 14). The non-microtubular C fibre is associated with the dorsal side of R2 (Fig. 13, 15). In cross-section, the C fibre is 100 nm thick and up to 200 nm broad and has a multilayered appearance, where two conspicuous dense sheets appear to be separated by a finer sheet, with adjacent sheets ca 20 nm apart (Fig. 13, 15).

Shortly after the groove opens posterior to the flagellar apparatus, R1 splits into inner and outer portions (R1i, R1o), with the I fibre continuing with R1o only (Fig. 9, 18, 19). The A fibre terminates shortly after the origin of the groove (Fig. 11, 18). The C fibre terminates around the origin of the groove, at which point the microtubules of R2 begin to splay (Fig. 13, 17). The B fibre eventually connects to the R1o/I fibre complex. Thus, in the anterior portion of the groove, its right margin is supported by the B fibre and its right wall by R1o. The floor of the groove is supported by R1i, and to its left, the singlet (S). The left wall and its margin are supported by microtubules originating from R2.

Morphological analysis. A parsimony analysis of morphological data from ''excavate taxa only'' recovers 35 trees of length 149 (Fig. 20), while the ''all excavates'' analysis recovers four trees of length 176 (data not shown). In both analyses, jakobids, including *Andalucia*, form a clade with relatively high Bremer support (3 or 4). The synapomorphies that unambiguously support the jakobids clade in all most parsimonious trees in both analyses are (i) an absence of an R4 root, (ii) an origin of cortical microtubules in close association with basal body 2, and (iii) tubular mitochondrial cristae (transforming to flat cristae in *Jakoba libera*). Two other characters, (i) an absence of a ventral flagellar vane (i.e. only having a dorsal flagellar vane), and (ii) a ''three-spaced-sheets'' structure of the C fibre, are unique to jakobids, but are not reconstructed as unambiguous synapomorphies of jakobids in all trees (some other taxa in the analysis lack flagellar vanes or a C fibre, and are coded as uncertainties for characters detailing the organization of the vanes or C fibre). In the ''all excavates'' analysis *Andalucia* is basal within jakobids, although with minimal Bremer support. In the ''excavate taxa only'' analysis, *Andalucia* is basal within jakobids in only a slight majority of the shortest trees. The deep-level relationships amongst excavate groups differ in the two analysis, are always weakly supported, and often not even consistent amongst the shortest trees (see also Simpson 2003).

Jakobids are sister to a weak clade of Heterolobosea, Euglenozoa, and parabasalids in the ''all excavates'' analysis (with Bremer support 1) (data not shown), and are sister to *Malawimonas* in a majority of the shortest trees in the ''excavate taxa only'' analysis (Fig. 20).

Molecular phylogenetic analysis. The ML analysis of 18S rRNA sequences recovers the *A. godoyi* isolates from soil, And28 and And19, as a strongly supported clade (bootstrap support 100%, Fig. 21). *Andalucia godoyi* in turn forms a strongly supported clade with *A. incarcerata* (bootstrap support 96%). All other jakobids (*J. libera*, *Reclinomonas*, ''*Seculamonas*'') form a separate strong clade (bootstrap support 100%). *Jakoba libera* and ''*Seculamonas*'' are related to the exclusion of *Reclinomonas*, with moderate bootstrap support (64%) (Fig. 21). The best trees placing *A. incarcerata*, or *Andalucia* as a whole, specifically with *J. libera* (or with *J. libera* and ''*Seculamonas*'') confer much less likelihood on the data and are strongly rejected by AU tests ($\Delta \ln L > 90$; $P < 0.002$, Table 1). The *Andalucia* clade is not specifically related to the ''other jakobids'' clade in the ML tree. Instead, the two clades form successive branches attached to the base of a moderately strong Heterolobosea-Euglenozoa clade (Fig. 21). In the ML tree, the ''other jakobids'' clade is closest to Heterolobosea and Euglenozoa, but bootstrap support for this position is weak (47%). In fact, trees in which jakobids are monophyletic confer only slightly less likelihood on the data than the ML tree and are not rejected by AU tests (Table 1). The entire jakobids–Euglenozoa–Heterolobosea clade receives moderate bootstrap support (63%) (Fig. 21).

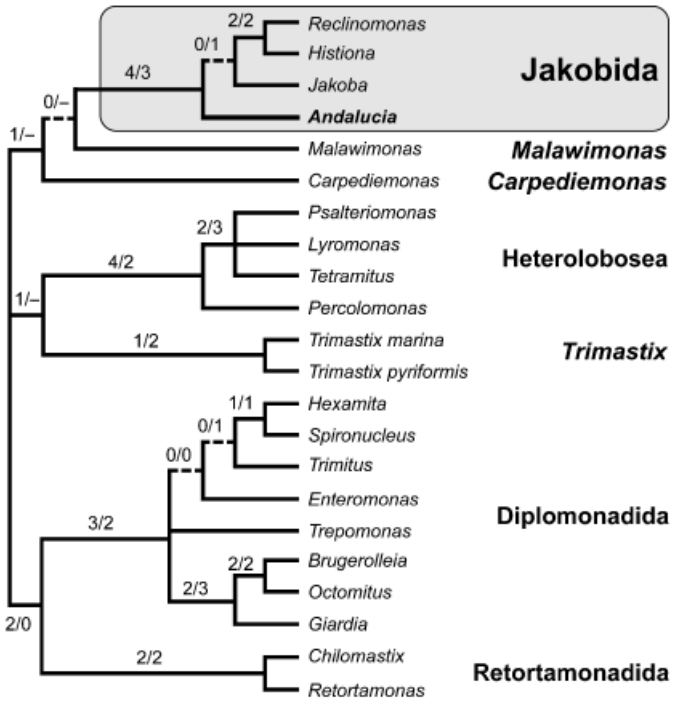

Fig. **20.** Maximum parsimony tree for morphological data on excavates (''excavate taxa only'' analysis; Majority-rule consensus tree of 35 trees of length 149 shown). Branch lengths are arbitrary. Dashed lines represent branches present in $<100\%$ of the best trees. Numbers at internal branches are Bremer support values (decay indices). Numbers to the left of the divider are for the ''excavate taxa only'' analysis, numbers to the right are for the ''all excavates'' analysis. Hyphens indicate that a bipartition is not present in the best trees for the ''all excavates'' analysis.

DISCUSSION

Phylogenetic placement of *Andalucia godoyi* n. gen., n. sp. Our 18S rRNA analysis confirms that *A. godoyi* is strongly and specifically related to *J. incarcerata*. By contrast, *J. libera* groups strongly with *Reclinomonas* (and ''*Seculamonas*''), not its congener *J. incarcerata*, and statistical tests strongly reject trees where *Jakoba* and *Andalucia* are forced to be a clade. In other words, 18S rRNA shows clearly that the genus *Jakoba* is currently not monophyletic, and it would be inappropriate to assign *A. godoyi* to *Jakoba* solely because of its close relationship to *J. incarcerata* (*J. libera* is the type of *Jakoba*). There is no existing genus that can house *A. godoyi*, and therefore, we have created a new genus, *Andalucia* n. gen.

Andalucia godoyi and *J. incarcerata* are very similar in morphology at the light- and electron microscopical level. The most conspicuous differences are that the anterior flagellum is always long in *Andalucia godoyi* ($\sim 2\times$ the length of the cell body in live cells) and has not been observed to attach to the substrate during feeding, while *J. incarcerata* lacks mitochondrial cristae (see below). We have not found totally clear-cut synapomorphies uniting *A. godoyi* and *J. incarcerata* to the exclusion of other eukaryotes, because most of the similarities they share are also found in other jakobids. The singlet-associated fibre is more pronounced in these

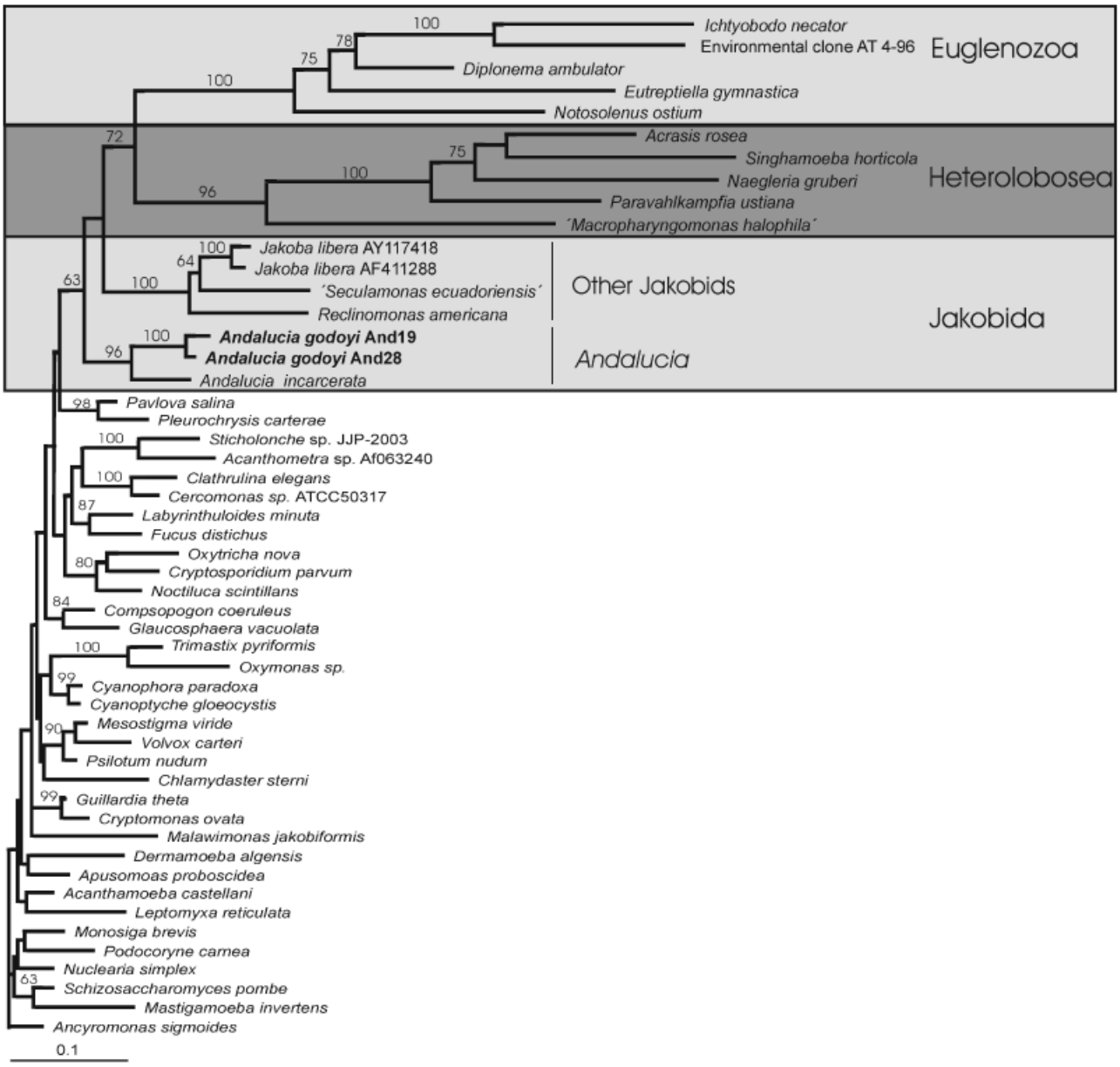

Fig. **21.** Maximum likelihood tree of 18S rRNA sequences from selected eukaryotes, indicating the position of *Andalucia godoyi* (GTR+Γ+*I* model with $\alpha = 0.57$ and $I = 0.21$; $\ln L = -14645.1$). The numbers at internal branches are maximum likelihood bootstrap support values under the same model, based on 375 replicates (values indicated only when $>60\%$).

Table **1.** Comparison of alternative tree topologies by likelihood difference and ''approximately unbiased'' (AU) tests

Tested topology	$\Delta\ln L$	AU test (*P*)
ML tree [Fig. 21: $\ln L - 14645.1$]	n.a.	n.a.
Jakobids monophyletic	5.9	0.33–0.40
(*Jakoba, Andalucia*)	110.0	<0.001*
(*Jakoba, Andalucia*, ''*Seculamonas*'')	90.8	<0.001*
(*Jakoba, A. incarcerata*)	140.5	<0.001*
(*Jakoba, A. incarcerata*, ''*Seculamonas*'')	119.9	<0.001–0.002*

*Best trees corresponding to alternative topology significantly worse ($\alpha = 0.05$).

n.a., not applicable.

two taxa than in other jakobids, but this feature is also found in some other excavates, such as *Trimastix* (Simpson, Bernard, and Patterson 2000). Due partly to the probable basal position of *Andalucia* within jakobids, it is difficult to determine ancestral and derived states for this character in jakobids. It is also possible that such subtle features might be fixation-sensitive. However, *A. godoyi* and *J. incarcerata* do share a C:G base pair within the basal stem of ''helix 27'' of 18S rRNA (positions 1050 and 1085 in the *J. incarcerata* 18S rRNA gene sequence AY117419). This nucleotide pair is not present in other jakobids and is present in only in a very few other eukaryote species. Further, phylogenetic analyses of at least two nuclear-encoded protein genes strongly support an *A. godoyi-J. incarcerata* clade, albeit with a weaker sampling of other jakobid species (Perley, T. A., EL. and AGBS., unpubl. data).

Jakoba incarcerata was originally described as a member of *Jakoba* because it shared distinctive jakobid cytoskeletal features, but was aloricate and often attached to the substrate by its anterior flagellum (F2) during feeding (Bernard, Simpson, and Patterson 2000). This genus assignment was made in the absence of an explicit phylogenetic analysis, and essentially by default. Until our current work, *Jakoba* was the only legitimate generic vehicle for aloricate jakobids, and the flagellar attachment behaviour was held to be distinctive for the genus. In fact, the aloricate condition is almost certainly plesiomorphic for jakobids. Bearing in mind that 18 rRNA trees strongly reject the monophyly of *J. libera* and *J. incarcerata*, the flagellar attachment behaviour may also be plesiomorphic. It is unclear that *J.incarcerata* has any more significant phenotypic similarity to *J. libera* than it does to *A. godoyi*. Given our molecular phylogenetic results, it is untenable to retain *J. incarcerata* within *Jakoba*, and given the phenotypic similarity between *J. incarcerata* and *A. godoyi*, we believe that the least disruptive and most convenient solution is to consider *J. incarcerata* as part of the new genus *Andalucia*. We therefore rename *Jakoba incarcerata* as *Andalucia incarcerata* n. comb.

To give the taxon *Andalucia* an explicit and stable phylogenetic definition, we introduce it as a node-based taxon (de Querioz and Gauthier 1992), with qualifying clauses to reasonably ensure that it does not subsume established genera and higher taxa (see ''taxonomic summary'' below). We note the recent creation of suprageneric node-based taxa within Kinetoplastea (Moreira, Lopez-Garcia, and Vickerman 2004). There is no particular reason why a taxon that fulfills the nomenclatural role of a genus cannot also be defined this way. The ''description'' supplied in the taxonomic summary below is akin to a conventional diagnosis, but explicitly subordinate to the definition (i.e. to be emended if in conflict with the definition, rather than vice versa). While it has been considered desirable for heterotrophic flagellate genera to be distinguished diagnostically at the light microscopical level, this ideal is arguably already violated in small free-living excavates (e.g. *Malawimonas* vs. *Carpediemonas*), and is destined to be become still more impractical with the formal description of other organisms currently in culture.

On the monophyly of jakobids. Our analysis is arguably the first study of 18S rRNA specifically designed to examine jakobid phylogeny—more full-length jakobid sequences are available than in previous analyses, and we have excluded most ''long-branch'' eukaryotes. Nonetheless, our ML tree agrees with most recent analyses in placing jakobids as two separate groups joined successively to the base of a Heterolobosea-Euglenozoa clade (Cavalier-Smith 2003, 2004; Berney, Fahrni, and Pawlowski 2004; EL. et al., unpubl. data; Nikolaev et al. 2004a, b). At first reading, this repeated result might suggest that jakobids are not monophyletic. In fact, partly because of such results it has been proposed that jakobids are paraphyletic, and gave rise to a Heterolobosea-Euglenozoa clade (Cavalier-Smith 2003). However, in both our current work, and previous analyses, the bipartition separating the jakobids is very weakly supported (note that in one analysis, the bipartition is supported by >0.95 posterior probability, but by $<50\%$ bootstrap support under a near-identical evolutionary model, Nikolaev et al. 2004a). Furthermore, different analyses recover different jakobid clades as the immediate sister group to Heterolobosea and Euglenozoa. ''Other jakobids'' are closest to Heterolobosea and Euglenozoa in our analysis, and two previous studies (Cavalier-Smith 2004; EL. et al., unpubl. data), while *Andalucia* is closest in several works (Berney et al. 2004; Cavalier-Smith 2003; Nikolaev et al. 2004a, b). Finally, jakobid monophyly is not rejected by our statistical examination (Table 1). Thus, 18S rRNA analyses do not support jakobid monophyly, but provide only weak evidence for jakobid paraphyly or polyphyly.

In contrast to molecular analyses, morphological data positively support jakobid monophyly and the identity of *A. godoyi* as a jakobid. Three features have been identified as distinctive for jakobids amongst excavates: (i) a single flagellar vane on the dorsal side of the ventral flagellum; (ii) the organization of the C fibre appearing as three spaced sheets; and (iii) the dorsal fan of microtubules originating in very close association with basal body 2 (Simpson and Patterson 2001). *Andalucia godoyi* shows all of these features. Our formal morphological analysis recovers the monophyly of jakobids within excavates. The jakobid clade is actually the most strongly supported group in our analysis, in contrast to a previous morphological study where jakobid monophyly was moderately supported (Simpson 2003). We tentatively favour the hypothesis that jakobids, including *Andalucia*, are monophyletic to the exclusion of all other excavates, and all other eukaryotes. This proposal requires further scrutiny using different molecular markers (Perley, T. A., EL. and AGBS., unpubl. data).

Whether jakobids are monophyletic or paraphyletic, 18S rRNA phylogenies indicate that *Andalucia* is the jakobid group most distantly related to *Reclinomonas*. Thus, *Andalucia* may prove particularly important for understanding the evolution of excavate eukaryotes and mitochondrial genomes.

The ultrastructure of jakobids. The study of *A. godoyi* highlights additional morphological similarities between *Andalucia* (including *A. incarcerata* n. comb) and other jakobids. Traditionally, mitochondrial cristae morphology has been a principal diagnostic feature in protist systematics (Patterson 1994; Taylor 1976). Jakobids are one of the few eukaryote groups that are widely known to have multiple forms of mitochondrial cristae—*J. libera* has flattened cristae while *Reclinomonas* and *Histiona* have tubular cristae (Flavin and Nerad 1993; Mylnikov 1989; O'Kelly 1993; Patterson 1990). No mitochondrial cristae have been seen in *A. incarcerata*, which is cultured under oxygen-poor conditions (Simpson and Patterson 2001). The presence of tubular cristae in *A. godoyi* indicates that this form is likely to be ancestral in jakobids. Tentatively, we now consider jakobids as a tubulocristate lineage. This is similar to Euglenozoa being considered a discicristate lineage despite diplonemids and some kinetoplastids having non-discoidal cristae (Brugerolle et al. 1979; Simpson 1997; Triemer and Ott 1990). The conservatism of crista morphology within established groups is often overestimated and should not be overvalued relative to other data. Nonetheless, it is interesting that jakobids are the only excavate group in which tubular cristae predominate.

The original account of the cytoskeleton of *A. incarcerata* included a two-membered microtubular root (R4) associated with basal body 2 (Simpson and Patterson 2001). A similar root is present in most excavates, but is absent from other jakobids (O'Kelly and Nerad 1999; Simpson 2003). Consequently, there has been doubt as to whether the structure in *A. incarcerata* is really R4, or whether it is a different structure, such as part of the dorsal fan or a prodigious protoroot that transforms into R2 during division (Simpson 2003; Simpson and Patterson 2001) (O'Kelly, C. J., pers. commun.). We did not observe any microtubular root associated with basal body 2 in *A. godoyi*. This increases suspicion that the R4 root of *A. incarcerata* was misidentified, and that jakobids ancestrally lack the R4 root.

The B fibre is reported as originating in several different places in excavates: either against root R1, or against R2, or directly against basal body 1 (Simpson 2003; Simpson and Patterson 2001; Simpson et al. 2002a). In *A. incarcerata*, the B fibre reportedly originates against R1, as in Preaxostyla (i.e. *Trimastix* and oxymonads). In *A. godoyi*, the B fibre clearly originates against basal body 1, as in other studied jakobids (and *Malawimonas*). This observation eliminates another morphological distinction between the two groups of jakobids.

Novel observations for jakobids. The dense sheet that underlies the origin of the dorsal fan near basal body 2 is, to date, a unique feature of *A. godoyi* among jakobids. Other jakobids, especially *A. incarcerata*, have some dense material between the dorsal fan and basal body 2, but it does not appear as a distinct sheet (Simpson and Patterson 2001). A dense sheet, the ''lapel'', underlies the origin of the dorsal fan in retortamonads (Bernard, Simpson, and Patterson 1997; Brugerolle 1973). A sheet is also present in the heteroloboseid *Percolomonas* (Brugerolle and Simpson 2004; Fenchel and Patterson 1986). In these other cases, the originating microtubules are separated by some distance from basal body 2, although the dense sheet may originate in close association with basal body 2 in *Percolomonas* (Fig. 1i in Brugerolle and Simpson 2004).

The A fibre of *A. godoyi* displays a striated appearance with a long period, which has not been observed previously in other excavates, including *A. incarcerata*. It has been suggested that the striated rhizoplast of Heterolobosea is homologous to the A fibre of typical excavates, such as jakobids (Brugerolle and Simpson 2004; Simpson 2003). However the periodicity and appearance of the striations of rhizoplasts are very different to those observed here.

This is the first account of a paranuclear body in jakobids. Similar single-membrane-bounded paranuclear bodies have been found in a sparse, but diverse array of eukaryotes, such as the heterotrophic stramenopile *Placidia cafeteriopsis* (Moriya, Nakayama, and Inouye 2002) and several Cercozoa, including *Cercomonas*/*Neocercomonas* spp. and *Massisteria marina* (Patterson and Fenchel 1990). The version found in Cercozoa is used by Cavalier-Smith and Chao (2003) in the diagnosis of a large Cercozoan subtaxon ''Sarcomonadea.'' The functions and phylogenetic relevance of these structures are not clear. The favoured hypothesis is that some or all of them are peroxisomes (e.g. Cavalier-Smith and Chao 2003).

Taxonomic Summary

Eukaryota
Excavata
Jakobida
Andalucia gen. nov.

Definition. Node: All descendents of the last common ancestor of *Andalucia godoyi* (isolate And28) and *Andalucia incarcerata* (basionym. *Jakoba incarcerata* Bernard et al. 2000). Qualifying clause: Taxon is not to include *Jakoba libera* (Ruinen 1938) Patterson 1990, *Reclinomonas americana* Flavin and Nerad, 1993 or *Naegleria gruberi* Schardinger 1899.

Description. Small, aloricate, heterotrophic flagellates with two flagella inserting apically and subapically at the top of a conspicuous suspension-feeding groove. With a single dorsally located vane on the posterior flagellum (F1) and a multi-layered ''C'' fibre visible by transmission electron microscopy. Lacking the strongly pointed cell apex and flattened mitochondrial cristae of the type of *Jakoba* (*J. libera*), otherwise not readily distinguishable from *Jakoba* by morphology, yet not specifically related in 18S rRNA phylogenies. With a G:C base pair within the base of the stem of ''helix 27'' of the 18S rRNA molecule.

Etymology. Alluding to the region of Spain.

Type species. *Andalucia godoyi* n. sp.

Andalucia godoyi sp. nov.

Diagnosis. Both flagella approximately 2 times the length of the trophic cell. With tubular mitochondrial cristae and a paranuclear body.

Type material. A culture of isolate ''And28'', isolated by EL from soil from Southern Spain (38°16′N, 46°16′W) is deposited with the American Type Culture Collection (ATCC), under the accession number ATCC PRA-185, and is nominated as the name-bearing hapantotype (see article 73.3 of the International Code of Zoological Nomenclature, 4th ed.). Note that Fig. 1–4 of the current paper are light micrographs from this culture.

Etymology. Named after José Godoy, a prominent person in Andújar, Spain, who has helped the poorest people in this region, and who sent us the samples from which *A. godoyi* was isolated.

Andalucia incarcerata nov. comb.

Basionym. *Jakoba incarcerata* Bernard, Simpson, and Patterson 2000.

ACKNOWLEDGMENTS

Thanks to Flemming Ekelund (University of Copenhagen) for the help with isolating *Andalucia godoyi*, Jong Soo Park (Seoul) for the fruitful discussion and practical help, Thomas Perley for practical help, and Charles O'Kelly (Bigelow Laboratory for Ocean Sciences, USA) for sharing prescient concerns about the supposed R4 root in *Andalucia incarcerata*. Cedric Berney (University of Geneva) and Jeffrey Silberman (University of Arkansas) provided seed 18S rRNA alignments. The research was funded by NSERC grant 298366-04 to AGBS, and Swiss National Fund FN 3100-065-272 to EL. Computational resources were funded by Genome Atlantic. AGBS thanks the Canadian Institute for Advanced Research (CIAR) for support as a ''scholar.''

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