Molecular Phylogenies of *Blastocystis* Isolates from Different Hosts: Implications for Genetic Diversity, Identification of Species, and Zoonosis

Christophe Noël,[1][†][‡] Fabienne Dufernez,[1][†] Delphine Gerbod,[1][§] Virginia P. Edgcomb,[2]
Pilar Delgado-Viscogliosi,[3] Lip-Chuen Ho,[4] Mulkit Singh,[4] René Wintjens,[5]
Mitchell L. Sogin,[6] Monique Capron,[1] Raymond Pierce,[1]
Lionel Zenner,[7] and Eric Viscogliosi[1]*

*Unité Inserm U547[1] and Département Eaux et Environnement, Laboratoire de Recherches Appliquées,[3] Institut Pasteur
de Lille, Lille, and Ecole Nationale Vétérinaire de Lyon, UMR DGER-INRA 958, Marcy l'Etoile,[7] France;
Biology Department, Woods Hole Oceanographic Institution,[2] and Josephine Bay Paul Center for
Molecular Evolution, Marine Biological Laboratory,[6] Woods Hole, Massachusetts;
Department of Microbiology, Faculty of Medicine, National University of
Singapore, Singapore[4]; and Institut de Pharmacie, Chimie Générale,
Université Libre de Bruxelles, Brussels, Belgium[5]*

Small-subunit (SSU) rRNA gene sequences were obtained by PCR from 12 *Blastocystis* isolates from humans, rats, and reptiles for which elongation factor 1α (EF-1α) gene sequences are already available. These new sequences were analyzed by the Bayesian method in a broad phylogeny including, for the first time, all *Blastocystis* sequences available in the databases. Phylogenetic trees identified seven well-resolved groups plus several discrete lineages that could represent newly defined clades. Comparative analysis of SSU rRNA- and EF-1α-based trees obtained by maximum-likelihood methods from a restricted sampling (13 isolates) revealed overall agreement between the two phylogenies. In spite of their morphological similarity, sequence divergence among *Blastocystis* isolates reflected considerable genetic diversity that could be correlated with the existence of potentially ≥12 different species within the genus. Based on this analysis and previous PCR-based genotype classification data, six of these major groups might consist of *Blastocystis* isolates from both humans and other animal hosts, confirming the low host specificity of *Blastocystis*. Our results also strongly suggest the existence of numerous zoonotic isolates with frequent animal-to-human and human-to-animal transmissions and of a large potential reservoir in animals for infections in humans.

Blastocystis hominis is an anaerobic enteric parasite that inhabits the human intestinal tract. Although the role of *B. hominis* as a human pathogen has been criticized mainly because of the impossibility of eliminating all other causes of symptoms, it is commonly considered a causative agent of intestinal disease (for reviews, see references 10, 14, 36, and 39). Moreover, *B. hominis* is probably the most frequent protozoan reported in human fecal samples (45), with a prevalence ranging between 30 and 50% in some developing countries (36). In addition, infection with *B. hominis* appears to be common and more severe in immunocompromised patients (12, 30, 40).

Microorganisms identified as *Blastocystis* have also been isolated from a wide range of animals, such as nonhuman primates, pigs, cattle, birds, amphibians, and, less frequently, rodents, reptiles, and insects (for reviews, see references 2 and 10), but the taxonomy within the genus *Blastocystis* remains controversial. Indeed, most *Blastocystis* isolates have remained indistinguishable from *B. hominis* by light and electron microscopy. However, new species have been differentiated from *B. hominis* in different nonhuman hosts (8, 11, 34, 41), but until further confirmation using molecular data is available, *B. hominis* defines the parasite isolated from humans and *Blastocystis* sp. represents those isolated from other animal hosts.

Although the designations of individual species of *Blastocystis* have not been adequately resolved, extensive genetic diversity among both *B. hominis* and *Blastocystis* sp. isolates has been described mostly by random amplified polymorphic DNA (RAPD) (3–5, 22, 46–50) and restriction fragment length polymorphism (RFLP) analyses of PCR-amplified small-subunit (SSU) rRNA (3–5, 9, 13, 19, 20, 25, 35, 49). By RFLP, at least 10 subspecies, or ribodemes (the term used to describe populations that share the same riboprint pattern), have been characterized (13, 25, 48). Some of these different ribodemes were found in human hosts, which raised the possibility that more than one species of *Blastocystis* infects humans and suggests the existence of zoonotic strains of this parasite. Clark (13) and Yoshikawa et al. (46, 47) showed that two *Blastocystis* isolates, one from guinea pigs and one from chickens, exhibited RFLP profiles or RAPD patterns similar to those observed in some *B.*

* Corresponding author. Mailing address: Unité Inserm U547, Institut Pasteur, 1 Rue du Professeur Calmette, BP 245, 59019 Lille Cedex, France. Phone: 33-3-20-87-79-61. Fax: 33-3-20-87-78-88. E-mail: eric.viscogliosi@pasteur-lille.fr.

† C. Noël and F. Dufernez contributed equally to this work.

‡ Present address: Molecular Biology Unit, Microbiology Group, Department of Zoology, The Natural History Museum, Cromwell Rd., London SW7 5BD, United Kingdom.

§ Present address: Department of Biology, Life Sciences Centre, 1355 Oxford St., Halifax, Nova Scotia, B3H 4J1 Canada.

hominis strains, suggesting animal-to-human or human-to-animal transmission. This is supported by several studies (14, 31, 32) confirming that animal and food handlers are more likely to be infected. Recently, diagnostic PCR using known sequenced-tagged site (STS)-specific primer sets was developed to distinguish and classify *Blastocystis* populations into seven different subtypes based on genomic similarity (47, 48, 50). By this method, Abe et al. (3–5) suggested the existence of numerous zoonotic isolates from various animal hosts. However, molecular data obtained by RAPD-, RFLP-, and PCR-based genotype classification reflected limited sequence divergence among taxa and provided only preliminary information on genetic relatedness between human and nonhuman isolates.

Thus, molecular phylogenies were recently constructed from nearly full-length SSU rRNA (1, 7, 29, 43, 49, 50) and elongation factor 1α (EF-1α) gene sequences (18). These analyses suggested low host specificity for *Blastocystis* and the zoonotic potential of the parasite. However, the restricted number of *Blastocystis* sequences obtained or phylogenetically analyzed from human and nonhuman hosts was insufficient to determine the extent to which the parasite can be transmitted among host species and the potential reservoir for infection of humans. In addition, comparative phylogenetic studies have demonstrated that, depending on the group in question, single-gene phylogenies based on either RNA or protein can be very misleading. It was therefore critical to compare the topology of *Blastocystis* SSU rRNA-based analyses with those of protein-coding genes.

In this study, we obtained almost full-length SSU rRNA gene sequences of 12 *Blastocystis* isolates from humans, rats, and reptiles for which EF-1α gene sequences are available (18). These sequences were used in a large phylogenetic analysis including 78 other *Blastocystis* sequences available in databases. For the first time for *Blastocystis*, this allowed the parallel construction of phylogenetic trees from two molecular markers with the same sample of isolates, comparing protein (EF-1α)- and rRNA-based trees. All these data allowed us to clarify genetic diversity, species identities, and host specificities among *Blastocystis* isolates.

MATERIALS AND METHODS

Source of *Blastocystis* and DNA extraction. Axenic cultures of 12 *Blastocystis* isolates (Table 1) from humans, rats, and reptiles were maintained in Iscove's modified Dulbecco's medium supplemented with 10% horse serum, as previously described (17). Genomic-DNA isolation was performed as described by Ho et al. (18). The DNA preparations were treated with RNase A at 37°C for 30 min and kept at −20°C until they were used.

PCR amplification, cloning, and sequencing. SSU ribosomal DNA (rDNA) genes from each *Blastocystis* isolate were amplified using the eukaryote-specific primers A and B (without 5′ restriction site linkers) designed by Medlin et al. (27). PCRs were carried out according to standard conditions for Platinum *Taq* DNA polymerase (Invitrogen, Groningen, The Netherlands). After the denaturation step at 94°C for 5 min, 40 cycles of amplification were performed with a GeneAmp PCR System 9700 apparatus (Applied Biosystems, Courtaboeuf, France) as follows: 1 min at 94°C, 2 min at 48°C, and 3 min at 72°C. The final extension step was continued for 15 min. The products were separated by agarose gel electrophoresis, and the band of the expected size (~1,800 bp) was purified using the QIAEX II Gel Extraction kit (QIAGEN, Hilden, Germany). The purified PCR products were cloned in the T vector pCR 2.1-TOPO (Invitrogen) and amplified in TOP10 competent cells. Minipreparations of plasmid DNA were done using the QIAprep Spin Miniprep kit (QIAGEN). Clones containing an insert were sequenced on both strands by primer walking using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) and an automated PRISM 377 DNA sequencer (Applied Biosystems).

Phylogenetic analyses. SSU rRNA gene sequences from *Blastocystis* isolates obtained in this study were aligned with a set of eukaryotic sequences including 78 other *Blastocystis* isolates retrieved from databases (Table 1) using the ARB package (http://www.arb-home.de/) according to conservation of primary and secondary structures. EF-1α amino acid sequences of *Blastocystis* isolates available in databases were aligned by use of the BioEdit version 5.0.9 package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). In each analysis, we restricted phylogenetic inference to sites that could be unambiguously aligned. Full-length alignments and sites used in analyses are available upon request. Phylogenetic analyses of SSU rRNA and EF-1α sequences were carried out using MrBAYES version 2.01 (21). Bayesian analyses of the SSU rRNA datasets were performed using the GTR (general time reversible) +Γ (gamma distribution of rates with four rate categories) +I (proportion of invariant sites) model of sequence evolution, with base frequencies, the proportion of invariant sites, and the shape parameter alpha of the Γ distribution estimated from the data. The data set of EF-1α amino acid sequences was analyzed as described above using the JTT amino acid replacement model (24). In all Bayesian analyses, starting trees were random, four simultaneous Markov chains were run for 500,000 generations, burn-in values were set at 30,000 generations (based on empirical values of stabilizing likelihoods), and trees were sampled every 100 generations. Bayesian posterior probabilities were calculated using a Markov chain Monte Carlo sampling approach (15) implemented in MrBAYES version 2.01. Phylogenetic analyses were also performed for EF-1α and restricted SSU rRNA data sets by using TREE-PUZZLE version 5.0 (38) and 10,000 quartet puzzling steps. The JTT and HKY (16) models were used for the amino acid and SSU rRNA data sets, respectively. The proportion of invariant sites and the alpha shape parameter were estimated from the data. For the large SSU rRNA data set, analyses were carried out both including and excluding outgroups to determine if this substantially affected relationships within *Blastocystis* isolates.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study have been deposited in GenBank under accession numbers AY590105 to AY590116.

RESULTS AND DISCUSSION

Phylogenetic analysis of *Blastocystis* SSU rRNA gene sequences. The amplification of the SSU rDNA coding regions of the 12 *Blastocystis* isolates from humans, rats, and reptiles analyzed in this study produced a DNA fragment of the expected size (~1.8 kb in length) as determined by gel electrophoresis. This fragment was eluted and cloned into the pCR 2.1-TOPO vector, and two clones were completely sequenced for each isolate, which always proved to be identical. Each of the new SSU rRNA gene sequences showed the highest similarity (from 82.6 to 99.9%) to homologous sequences of the other *Blastocystis* isolates reported so far (Table 1). In the common part of our alignment, the lengths of all *Blastocystis* SSU rRNA gene sequences available in databases and obtained in this work range from 1,666 to 1,777 bp, and the G+C contents range from 53.9 to 64.4%.

SSU rRNA gene sequences obtained in this study were added to an existing database of 77 other *Blastocystis* sequences (Table 1). Tree construction was performed using the stramenopile *Proteromonas lacertae*, a commensal flagellate of reptiles and amphibians, as the outgroup in view of its position closely related to *Blastocystis* in previous phylogenetic studies (26, 28, 29, 33). This data set included 1,563 unambiguously aligned positions, which we analyzed by the maximum-likelihood method using MrBAYES. The rooted maximum-likelihood tree (Fig. 1) identified seven clades called groups I to VII, each highly supported by Bayesian posterior probabilities (BP) of 100% plus several discrete lineages. In a subsequent analysis from which the outgroup was excluded, permitting the inclusion of more characters (1,606 positions), no significant deviation from this topology was noted (data not shown). We

TABLE 1. Origins of *Blastocystis* isolates analyzed in our study and accession numbers of their SSU rRNA gene sequences

Isolate	Species	Host	Country of origin	Reference	Clones[a]	Accession no.
B	*B. hominis*	Human	Singapore	7; Present study	a, b	AF408427, AY590105
C	*B. hominis*	Human	Singapore	Present study		AY590106
E	*B. hominis*	Human	Singapore	Present study		AY590107
G	*B. hominis*	Human	Singapore	Present study		AY590108
H	*B. hominis*	Human	Singapore	Present study		AY590109
S	*B. hominis*	Human	Pakistan	Present study		AY590110
HE87-1	*B. hominis*	Human	Japan	6	a, b	AB023499, AB023578
HG00-10	*B. hominis*	Human	Japan	50		AY244619
HG00-12	*B. hominis*	Human	Japan	50		AY244620
HJ00-4	*B. hominis*	Human	Japan	50		AF408425
HJ00-5	*B. hominis*	Human	Japan	50		AF408426
HJ01-7	*B. hominis*	Human	Japan	50		AY244621
HJ96-1	*B. hominis*	Human	Japan	7		AB070987
HJ96A-26	*B. hominis*	Human	Japan	7	a, b, c	AB070988, AB091234 and -5
HJ96A-29	*B. hominis*	Human	Japan	7		AB070989
HJ96AS-1	*B. hominis*	Human	Japan	7	a, b, c, d	AB070990, AB091236 to -8
HJ97-2	*B. hominis*	Human	Japan	7	a, b	AB070991, AB091239
HT98-1	*B. hominis*	Human	Thailand	7		AB070992
HV93-13	*B. hominis*	Human	Japan	7	a,b	AB070986, AB091233
Nand II	*B. hominis*	Human	United States	33		U51151
	B. hominis	Human	France	29		AY135402
	B. hominis	Human	Thailand	43		AF439782
JM92-2	*Blastocystis* sp.	Monkey	Japan	7		AB070997
MJ99-116	*Blastocystis* sp.	Monkey	Japan	1		AB107969
MJ99-123	*Blastocystis* sp.	Monkey	Japan	1		Identical to AB107968
MJ99-130	*Blastocystis* sp.	Monkey	Japan	1		Identical to AB107969
MJ99-132	*Blastocystis* sp.	Monkey	Japan	1		AB107970
MJ99-424	*Blastocystis* sp.	Monkey	Japan	1		AB107967
MJ99-568	*Blastocystis* sp.	Monkey	Japan	1		AB107968
PJ99-148	*Blastocystis* sp.	Pig	Japan	1		Identical to AB107964
PJ99-154	*Blastocystis* sp.	Pig	Japan	1		AB107961
PJ99-162	*Blastocystis* sp.	Pig	Japan	1		AB107963
PJ99-172	*Blastocystis* sp.	Pig	Japan	1		AB107962
PJ99-188	*Blastocystis* sp.	Pig	Japan	1		AB107964
SY94-3	*Blastocystis* sp.	Pig	Japan	7	a, b	AB070998, AB091248
SY94-7	*Blastocystis* sp.	Pig	Japan	7	a, b, c	AB070999, AB091249 and -50
	Blastocystis sp.	Pig	Thailand	43		AF538348
	Blastocystis sp.	Pig	Czech Republic	29	1, 2, 3, 4	AY135403-6
CJ99-284	*Blastocystis* sp.	Cattle	Japan	1		AB107966
CJ99-344	*Blastocystis* sp.	Cattle	Japan	1		Identical to AB107961
CJ99-350	*Blastocystis* sp.	Cattle	Japan	1		Identical to AB107964
CJ99-353	*Blastocystis* sp.	Cattle	Japan	1		Identical to AB107963
CJ99-363	*Blastocystis* sp.	Cattle	Japan	1		AB107965
	Blastocystis sp.	Horse	Thailand	43		AF538349[b]
CK86-1	*Blastocystis* sp.	Chicken	Japan	7	a, b, c	AB070993, AB091240 and -1
CK92-4	*Blastocystis* sp.	Chicken	Japan	7	a, b	AB070994, AB091242
	Blastocystis sp.	Chicken	France	29	1, 2	AY135409 and -10
	Blastocystis sp.	Duck	France	29		AY135412
	Blastocystis sp.	Turkey	France	29		AY135411
QQ93-3	*Blastocystis* sp.	Quail	Japan	7	a, b	AB070995, AB091243
QQ98-4	*Blastocystis* sp.	Quail	Japan	7	a, b, c, d, e	AB070996, AB091244 to -7
BJ99-310	*Blastocystis* sp.	Partridge	Japan	1		AB107972
BJ99-319	*Blastocystis* sp.	Pheasant	Japan	1		AB107971
BJ99-569	*Blastocystis* sp.	Goose	Japan	1		AB107973
RN94-9	*Blastocystis* sp.	Rat	Japan	7	a, b	AB071000, AB091251
	Blastocystis sp.	Rat	France	29	1, 2	AY135407 and -8
S1	*B. ratti*	Rat	Singapore	Present study		AY590111
WR1	*B. ratti*	Rat	Singapore	Present study		AY590113
WR2	*B. ratti*	Rat	Singapore	Present study		AY590114
NIH:1295:1	*Blastocystis* sp.	Guinea pig	United States	33		U51152
	Blastocystis sp.	Guinea pig	United States	26		U26177
	B. cycluri	Lizard	Singapore	Present study		AY590116
	B. lapemi	Sea snake	Singapore	Present study		AY590115
	B. pythoni	Python	Singapore	Present study		AY590112

[a] Clones with different sequences obtained from the same isolate.
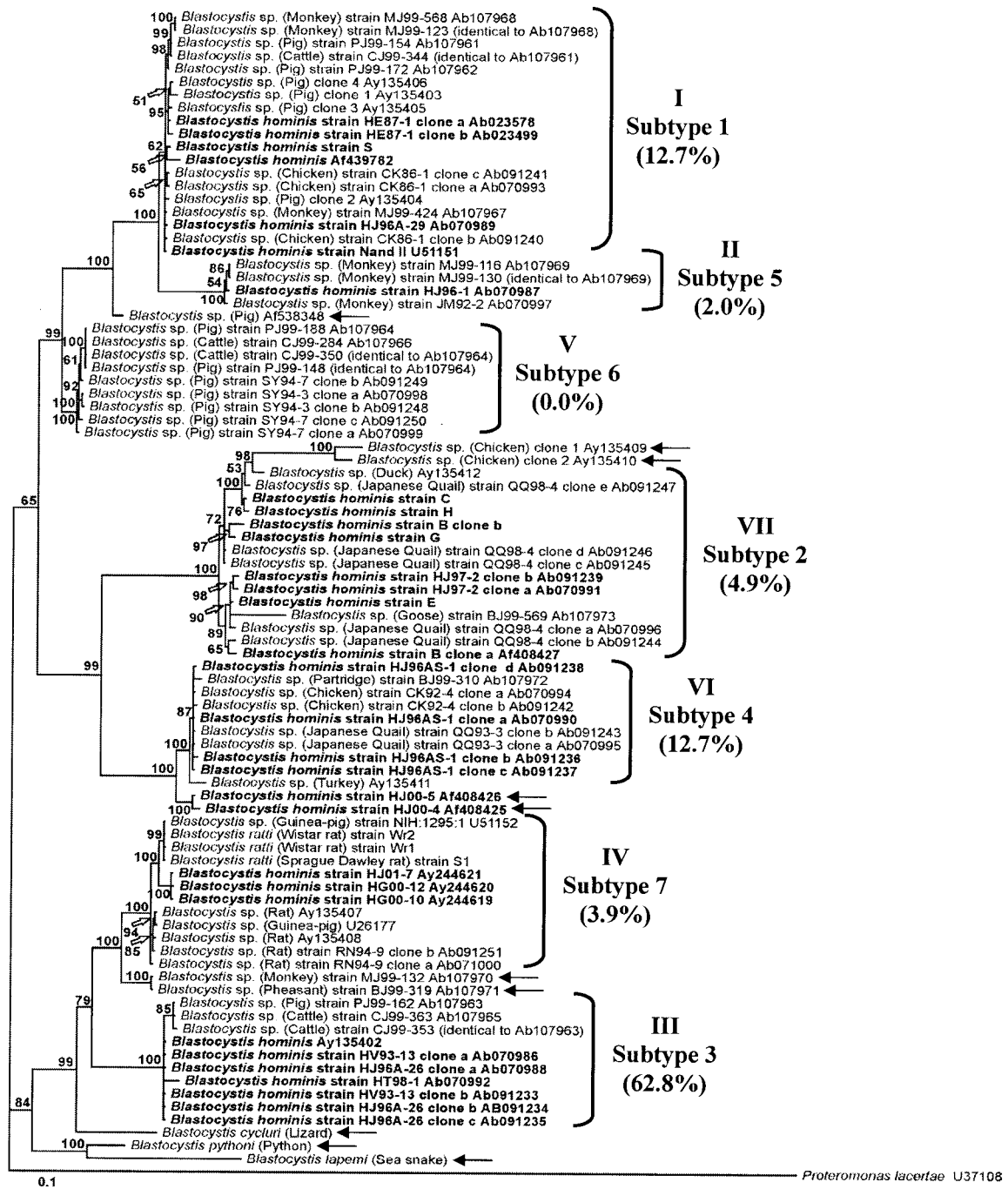[b] Partial sequence.

FIG. 1. Maximum-likelihood phylogeny of *Blastocystis* isolates inferred from SSU rRNA gene sequences. *P. lacertae* served as the outgroup. Bayesian posterior probabilities are given as percentages near the individual nodes. Nodes with values of <50% are not shown. *Blastocystis* isolates from humans are indicated in boldface. Groups I to VII are those previously described by Arisue et al. (7), and the arrows indicate isolates for which classification is uncertain. Scale bar, 0.1 substitutions (corrected) per base pair.

also noted that there was only limited or moderate support for the relative branching order within most of the groups.

The major groups identified in our analysis were in agreement with those defined in recent SSU rRNA-based studies that included a more restricted sample of *Blastocystis* isolates (1, 7). For this reason, we have conserved the same numbering of the groups used by these authors in order to facilitate the

comparison of both SSU rRNA-based trees. In addition, as stated by Abe (1), Arisue et al. (7), and Yoshikawa et al. (49, 50), there is a correlation between these phylogenetically different groups and the subtypes identified by PCR-based genotype classification. Indeed, groups I to VII correspond to subtypes 1, 5, 3, 7, 6, 4, and 2, respectively, as indicated in Fig. 1.

Group I (subtype 1) mostly unites *Blastocystis* isolates from

pigs, cattle, monkeys, and humans. In a subsequent phylogenetic analysis restricted to 986 positions, we observed that the partial SSU rDNA sequence of a horse isolate was also included in this group (data not shown). We also note that group I is closely related to group II (subtype 5), which includes only primate and human isolates. In addition, the higher-order clade of groups I, II, and V (subtype 6, which is composed of pig and cattle isolates) cluster together with strong BP (99%). Within this large group I-II-V, we also describe the emergence of a discrete lineage composed only of a single pig isolate (accession number AF538348) that could be the only known representative of a new clade. Interestingly, the large group I-II-V is mostly restricted to mammalian isolates (humans, monkeys, bovids, equids, and pigs) with rodents excluded. However, it also includes the isolate CK86-1 from chickens, which was already identified as a zoonotic strain (12, 45, 46). This isolate occupies a position distant from the other bird isolates analyzed in this study and included in groups VI and VII.

Indeed, groups VI (subtype 4) and VII (subtype 2) strongly unite some isolates from humans and most of the isolates from birds, with the exception of isolates CK86-1 from chickens (group I) and BJ99-319 from pheasants. We also note that distinct isolates from both Japanese quails and chickens are represented in each of these two different groups. Moreover, as previously stated by Yoshikawa et al. (50), the isolates HJ00-4 and HJ00-5 from humans are clustered in an additional monophyletic clade at the base of group VI. However, both isolates HJ00-4 and HJ00-5 composing this new lineage were negative with all STS primers used in PCR-based genotype classification, suggesting that the isolates HJ00-4 and HJ00-5 do not belong to subtype 4 and could represent a new genotype (50). This hypothesis could be also tested with STS primers for two isolates from chickens (accession numbers AY135409 and AY135410) that could form another new clade.

Additional clades are represented by groups III (subtype 3) and IV (subtype 7). The two groups cluster together with a moderate BP of 79%. Group IV is mostly composed of isolates from rodents but also includes three human isolates, whereas group III unites four isolates from humans and three isolates from cattle and pigs. The isolates MJ99-132 and BJ99-319, from monkeys and pheasants, respectively, are clustered in an additional monophyletic clade, which is strongly united with group IV (BP value, 100%). Although both isolates MJ99-132 and BJ99-319 exhibit the same subtype 7 as isolates composing group IV (1), this clade could represent a new well-defined phylogenetic group.

At the base of the higher-order clade III-IV, we note the emergence of two additional discrete lineages, composed of the three isolates from reptiles, that could represent new phylogenetic groups. These isolates form a polyphyletic group. Indeed, *Blastocystis cycluri*, isolated from lizards, clusters together with isolates of groups III and IV with high support (BP value, 99%), whereas *Blastocystis lapemi* and *Blastocystis pythoni*, isolated from snakes, exhibit a derived position and group together with BP of 100%. This dichotomy observed between *Blastocystis* isolates from reptiles could naturally reflect the evolutionary divergence between their respective hosts.

Comparative phylogeny of *Blastocystis* isolates inferred from EF-1α and SSU rRNA gene sequences. Comparison of SSU rRNA gene sequences confirms that *Blastocystis* organisms are genetically highly divergent in spite of their morphological identity and defines a large number of potential phylogenetic groups among *Blastocystis* isolates. However, congruence between independent data sets is the strongest argument in favor of a given topology, so the SSU rRNA tree of *Blastocystis* isolates needs to be compared with phylogenies based on other molecules. To this end, we obtained SSU rRNA gene sequences from 12 *Blastocystis* isolates for which a major part of the coding region of the EF-1α gene had already been sequenced (18). SSU rRNA and EF-1α sequences were also available for the human isolate HE87-1 and were added to our database. This allowed us to compare SSU rRNA- and EF-1α-based trees with the same sampling of 13 *Blastocystis* isolates.

As shown in Fig. 1 and 2, 10 of the 13 isolates studied in this comparative analysis represented three groups (I, IV, and VII), whereas the three other isolates from reptiles composed two newly described clades. For EF-1α and restricted SSU rRNA data sets, phylogenetic analyses were performed using 289 amino acid and 1,644 nucleotide aligned positions, respectively, and unrooted maximum-likelihood trees were constructed using both MrBAYES and TREE-PUZZLE. Identity matrices calculated from both alignments revealed that sequence similarity among all pairs ranged from 83.0 to 99.9% and 88.5 to 100% for the SSU rRNA and EF-1α data sets, respectively (data not shown), and indicated that EF-1α sequences are more highly conserved than SSU rRNA sequences among *Blastocystis* isolates.

The topologies of SSU rRNA- (Fig. 2A) and EF-1α-based (Fig. 2B) trees are identical for the taxa selected. We recovered with high support the well-defined groups I, IV, and VII described above with the larger SSU rRNA data set and confirmed the large genetic diversity between groups I and VII, both of which include human isolates. Among these clades, better resolution is observed within group VII in the SSU rRNA- than in the EF-1α-based tree. This is in agreement with the percentage sequence identity calculated for each marker. Both trees also emphasize the polyphyly of isolates from reptiles, which emerge as two independent lineages. In the SSU rRNA-based tree, *B. cycluri* clusters with rodent isolates with low support values, whereas the same grouping is strongly supported (100% by both reconstruction methods) in the EF-1α-based tree. Overall, phylogenetic trees of *Blastocystis* isolates based on SSU rRNA and EF-1α sequences are well supported and entirely congruent with each other. Thus, our data suggest that EF-1α should be a valuable complement to SSU rRNA to elucidate phylogenetic relationships among *Blastocystis* isolates.

Identification of species and zoonotic potential of *Blastocystis* organisms. New species designations in the *Blastocystis* genus were proposed for isolates from different nonhuman hosts (8, 11, 34, 41) on the basis of questionable criteria, such as differences in the hosts of origin, growth characteristics, and electrophoretic karyotypes. However, it is clear that these differences alone are not sufficient for the proposal of new species (23, 36, 39, 44). On the other hand, the genetic diversity observed in this work, as in previous molecular studies (1, 7, 29,
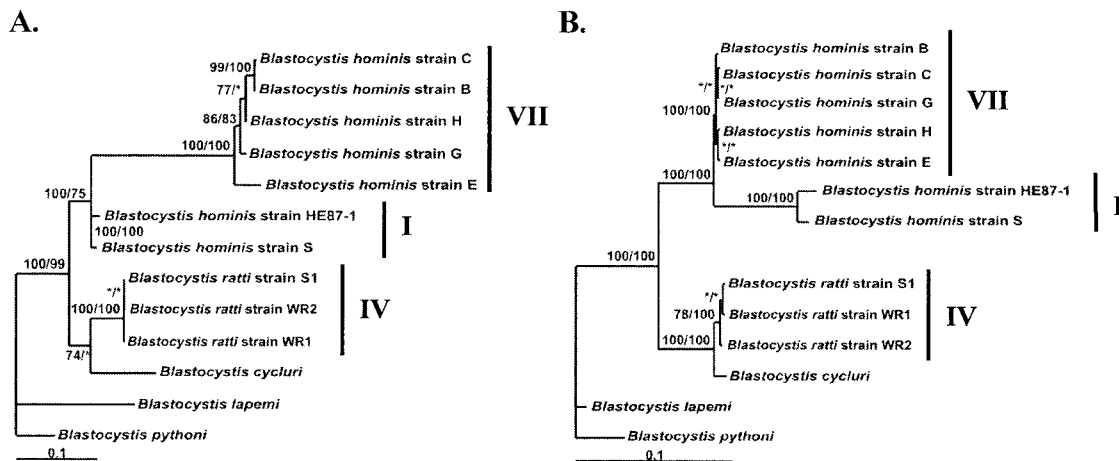
FIG. 2. Comparison of unrooted maximum-likelihood trees inferred from SSU rRNA (A) and EF-1α (B) sequences for the same sample of *Blastocystis* isolates. EF-1α nucleotide sequences are available in the GenBank database (accession numbers AF090737, AF091356 to AF091365, AF223379, and D64080). The numbers at the nodes correspond to Bayesian posterior probabilities given as percentages (left of the slash) and percentages of occurrence in 10,000 quartet puzzling steps (right of the slash). Asterisks designate nodes with values of <50%. Scale bars, 0.1 substitutions (corrected) per site.

43, 49, 50), among *Blastocystis* isolates strongly suggests that the genus consists of several species.

Indeed, the evolutionary distances observed among the different groups defined in our phylogenetic analysis are comparable to or greater than those observed among other stramenopile species (data not shown [obtained from an alignment including 1,458 unambiguous positions]). In addition, it is likely that each of the three reptile isolates analyzed here could represent a new species, as could one isolate from chickens (accession numbers AY135409 and AY135410) and one isolate from pigs (accession number AF538348). In contrast, it seems unlikely from our data that the newly described lineage composed of the isolates MJ99-132 and BJ99-319, from monkeys and pheasants, respectively, could be elevated to the species rank. Moreover, both isolates have been shown to exhibit subtype 7 and have been included in group IV (1). In addition, although the human isolates HJ00-4 and HJ00-5 could represent a new genotype (50), the evolutionary distances calculated between the closely related sequences of the two isolates and those composing group VI are too short to consider isolates HJ00-4 and HJ00-5 representatives of a new *Blastocystis* species. Overall, the genus *Blastocystis* could include at least 12 different species, reflective of the great genetic diversity observed among isolates. However, such taxonomic statements should be regarded cautiously until further confirmation is obtained, since an accelerated evolutionary rate for *Blastocystis* SSU rRNA genes cannot yet be ruled out. Additional studies involving more isolates and other conserved genes, such as that for EF-1α, should help to clarify taxonomic relationships among these organisms.

As demonstrated in our study, groups I to VII are not host specific, and most of them comprise isolates from humans and other animals, suggesting that *Blastocystis* may be cross-infective among various animal hosts. Indeed, as previously stated by Arisue et al. (7) based on a restricted sample of isolates, the relationships among hosts are not in agreement with the genuine phylogeny of the animals in any part of our *Blastocystis*

tree, and this could be explained by the existence of numerous zoonotic isolates. Thus, our study raises questions about the transmission of *Blastocystis*. The fecal-oral route is considered to be the main mode of transmission, but food-borne and waterborne transmission of *Blastocystis* via untreated water or poor sanitary conditions have also been suggested (reviewed in references 10, 36, and 39), although not confirmed experimentally. Human-to-human transmission of *B. hominis* infection between two small communities has been proposed (48), and experimental cross-transmission has been achieved in rodents with *Blastocystis* isolated from humans (for reviews, see references 10 and 36). Moreover, people working closely with animals in zoos and abattoirs and food handlers (14, 31, 32) are at higher risk of acquiring *Blastocystis* infection. In addition, transmission of *Blastocystis* organisms could also be frequent between animals, especially in confined areas, such as zoological gardens and circuses, where fecal contamination of grass and fodder is common (37, 42). Thus, in light of our data and those from the literature, it is reasonable to speculate that contamination by *Blastocystis* organisms can occur via animal-to-animal, human-to-animal, and animal-to-human routes. However, given the large number of potential zoonotic isolates found in our study, it remains difficult to identify the host origins and transmission routes of some isolates.

As stated above, groups VI (genotype 4) and VII (genotype 2) included only bird and human isolates. In a recent study, Yoshikawa et al. (50) studied the prevalence of each *Blastocystis* genotype in five different countries. These values are indicated in Fig. 1 and were based on a total of 99 human isolates. These authors showed that genotypes 4 and 2 represented 17.6% of the human isolates tested. Therefore, these results suggest that human isolates of both groups are zoonotic and could represent examples of animal-to-human transmission. In addition, since the same *Blastocystis* species is able to infect several bird species, animal-to-animal transmission could also be proposed. Group I (genotype 1) is composed only of mammalian isolates (pig, cattle, monkey, and human), with

the exception of the chicken isolate CK86-1. The prevalence of human isolates in this group is 12.7%. According to the diversity of hosts found in this group, it is likely composed mostly of zoonotic isolates. Although the host origins of the *Blastocystis* species representative of this group remain extremely difficult to define, they are probably mammalian. Therefore, transmissions both among bovines, pigs, monkeys, and humans and from mammals to birds for the chicken isolate CK86-1 could be suggested. Group IV (genotype 7) is composed mostly of rodent isolates. Thus, the same *Blastocystis* species (probably *Blastocystis ratti*) could colonize both rats and guinea pigs. Moreover, few human isolates have been shown to possess the same genotype 7 (3.9%). Therefore, this grouping of rodent and human isolates could reflect animal-to-human transmission. Finally, in accordance with its prevalence among human isolates (62.8%), group III (genotype 3) could represent a specific genotype of human origin. Isolates from pigs and cattle sharing genotype 3 are likely to reflect contamination by humans. These preliminary hypotheses concerning the zoonotic potential of these organisms could be reinforced by the analysis of additional isolates from different hosts.

In conclusion, our data confirm that extensive genetic diversity exists among *Blastocystis* isolates from humans and other animals and suggest that more than one species could infect humans. Moreover, our findings emphasize the low host specificity of this organism and indicate that numerous human *Blastocystis* infections may be of zoonotic origin. Thus, the number and range of animals found to be infected by *Blastocystis* may represent a huge potential reservoir for infection of humans.

REFERENCES

1. **Abe, N.** 2004. Molecular and phylogenetic analysis of *Blastocystis* isolates from various hosts. Vet. Parasitol. **120:**235–242.
2. **Abe, N., M. Nagoshi, K. Takami, Y. Sawano, and H. Yoshikawa.** 2002. A survey of *Blastocystis* sp. in livestock, pets, and zoo animals in Japan. Vet. Parasitol. **106:**203–212.
3. **Abe, N., Z. Wu, and H. Yoshikawa.** 2003. Molecular characterization of *Blastocystis* isolates from birds by PCR with diagnosis primers and restriction fragment length polymorphism analysis of the small subunit ribosomal RNA gene. Parasitol. Res. **89:**393–396.
4. **Abe, N., Z. Wu, and H. Yoshikawa.** 2003. Molecular characterization of *Blastocystis* isolates from primates. Vet. Parasitol. **113:**321–325.
5. **Abe, N., Z. Wu, and H. Yoshikawa.** 2003. Zoonotic genotypes of *Blastocystis hominis* detected in cattle and pigs by PCR with diagnostic primers and restriction fragment length polymorphism analysis of the small subunit ribosomal RNA gene. Parasitol. Res. **90:**124–128.
6. **Arisue, N., T. Hashimoto, H. Yoshikawa, Y. Nakamura, G. Nakamura, F. Nakamura, T. A. Yano, and M. Hasegawa.** 2002. Phylogenetic position of *Blastocystis hominis* and of stramenopiles inferred from multiple molecular sequence data. J. Eukaryot. Microbiol. **49:**42–53.
7. **Arisue, N., T. Hashimoto, and H. Yoshikawa.** 2003. Sequence heterogeneity of the small subunit ribosomal RNA genes among *Blastocystis* isolates. Parasitology **126:**1–9.
8. **Belova, L. M., and L. A. Kostenko.** 1990. *Blastocystis galli* sp. n. (Protista, Rhizopoda) from the intestine of domestic hens. Parazitologia **24:**164–168.
9. **Böhm-Gloning, B., J. Knobloch, and B. Walderich.** 1997. Five subgroups of *Blastocystis hominis* isolates from symptomatic and asymptomatic patients revealed by restriction site analysis of PCR-amplified 16S-like rDNA. Trop. Med. Int. Health **2:**771–778.
10. **Boreham, P. F. L., and D. J. Stenzel.** 1993. *Blastocystis* in humans and animals: morphology, biology, and epizootiology. Adv. Parasitol. **32:**1–70.
11. **Chen, X. Q., M. Singh, L. C. Ho, S. W. Tan, G. C. Ng, K. T. Moe, and E. H. Yap.** 1997. Description of a *Blastocystis* species from *Rattus norvegicus*. Parasitol. Res. **83:**313–318.
12. **Cirioni, O., A. Giacometti, D. Drenaggi, F. Ancarani, and G. Scalise.** 1999. Prevalence and clinical relevance of *Blastocystis hominis* in diverse patient cohorts. Eur. J. Epidemiol. **15:**389–393.
13. **Clark, C. G.** 1997. Extensive genetic diversity in *Blastocystis hominis*. Mol. Biochem. Parasitol. **87:**79–83.
14. **Doyle, P. W., M. M. Helgason, R. G. Mathias, and E. M. Proctor.** 1990. Epidemiology and pathogenicity of *Blastocystis hominis*. J. Clin. Microbiol. **28:**116–121.
15. **Green, P. J.** 1995. Reversible jump Markov Chain Monte Carlo computation and Bayesian model determination. Biometrika **82:**711–732.
16. **Hasegawa, M., H. Kishino, and T. Yano.** 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. **22:**160–174.
17. **Ho, L. C., M. Singh, K. Suresh, G. C. Ng, and E. H. Yap.** 1993. Axenic culture of *Blastocystis hominis* in Iscove's modified Dulbecco's medium. Parasitol. Res. **79:**614–616.
18. **Ho, L. C., A. Armugam, K. Jeyaseelan, E. H. Yap, and M. Singh.** 2000. *Blastocystis* elongation factor-1α: genomic organization, taxonomy and phylogenetic relationships. Parasitology **121:**135–144.
19. **Ho, L. C., K. Jeyaseelan, and M. Singh.** 2001. Use of the elongation factor-1α gene in a polymerase chain reaction-based restriction-fragment-length polymorphism analysis of genetic heterogeneity among *Blastocystis* species. Mol. Biochem. Parasitol. **112:**287–291.
20. **Hoevers, J., P. Holman, K. Logan, M. Hommel, R. Ashford, and K. Snowden.** 2000. Restriction-fragment-length polymorphism analysis of small-subunit rRNA genes of *Blastocystis hominis* isolates from geographically diverse human hosts. Parasitol. Res. **86:**57–61.
21. **Huelsenbeck, J. P., and F. Ronquist.** 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics **17:**754–755.
22. **Init, I., J. W. Mak, H. S. Lokman, and H. S. Yong.** 1999. Strain differences in *Blastocystis* isolates as detected by a single set of polymerase chain reaction primers. Parasitol. Res. **85:**131–134.
23. **Isaac-Renton, J. L., C. Cordeiro, K. Sarafis, and H. Shahriari.** 1993. Characterization of *Giardia duodenalis* isolates from a waterborne outbreak. J. Infect. Dis. **167:**431–440.
24. **Jones, D. T., W. R. Taylor, and J. M. Thornton.** 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biol. Sci. **8:**275–282.
25. **Kaneda, Y., N. Horiki, X. J. Cheng, Y. Fujita, M. Maruyama, and H. Tachibana.** 2001. Ribodemes of *Blastocystis hominis* isolated in Japan. Am. J. Trop. Med. Hyg. **65:**393–396.
26. **Leipe, D. D., S. M. Tong, C. L. Goggin, S. B. Slemenda, N. J. Pieniazek, and M. L. Sogin.** 1996. 16S-like rDNA sequences from *Developayella elegans*, *Labyrinthuloides haliotidis*, and *Proteromonas lacertae* confirm that the stramenopiles are a primarily heterotrophic group. Eur. J. Protistol. **32:**449–458.
27. **Medlin, L., H. J. Elwood, S. Stickel, and M. L. Sogin.** 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene **71:**491–499.
28. **Moriya, M., T. Nakayama, and I. Inouye.** 2000. Ultrastructure and 18S rDNA sequence analysis of *Wobblia lunata* gen. et sp. nov., a new heterotrophic flagellate (stramenopiles, incertae sedis). Protist **151:**41–55.
29. **Noël, C., C. Peyronnet, D. Gerbod, V. P. Edgcomb, P. Delgado-Viscogliosi, M. L. Sogin, M. Capron, E. Viscogliosi, and L. Zenner.** 2003. Phylogenetic analysis of *Blastocystis* isolates from different hosts based on the comparison of small-subunit rRNA gene sequences. Mol. Biochem. Parasitol. **126:**119–123.
30. **Prasad, K. N., V. L. Nag, T. N. Dhole, and A. Ayyagari.** 2000. Identification of enteric pathogens in HIV-positive patients with diarrhoea in northern India. J. Health Popul. Nutr. **18:**23–26.
31. **Rajah Salim, H., G. S. Kumar, S. Vellayan, J. W. Mak, A. K. Anuar, I. Init, G. D. Vennila, R. Saminathan, and K. Ramakrishnan.** 1999. *Blastocystis* in animal handlers. Parasitol. Res. **81:**703–705.
32. **Requena, I., Y. Hernandez, M. Ramsay, C. Salazar, and R. Devera.** 2003. Prevalence of *Blastocystis hominis* among food handlers from Caroni municipality, Bolivar state, Venezuela. Cad. Saude Publica **19:**1721–1727.
33. **Silberman, J. D., M. L. Sogin, D. D. Leipe, and C. G. Clark.** 1996. Human parasite finds taxonomic home. Nature **380:**398.
34. **Singh, M., L. C. Ho, A. L. L. Yap, G. C. Ng, S. W. Tan, K. T. Moe, and E. H. Yap.** 1996. Axenic culture of reptilian *Blastocystis* isolates in monophasic medium and speciation by karyotypic typing. Parasitol. Res. **82:**165–169.
35. **Snowden, K., K. Logan, C. Blozinski, J. Hoevers, and P. Holman.** 2000. Restriction-fragment-length polymorphism analysis of small-subunit rRNA genes of *Blastocystis* isolates from animal hosts. Parasitol. Res. **86:**62–66.
36. **Stenzel, D. J., and P. F. L. Boreham.** 1996. *Blastocystis hominis* revisited. Clin. Microbiol. Rev. **9:**563–584.
37. **Stenzel, D. J., M. F. Cassidy, and P. F. L. Boreham.** 1993. Morphology of *Blastocystis* sp. isolated from circus animals. Int. J. Parasitol. **23:**685–687.

38. **Strimmer, K., and A. Von Haeseler.** 1996. Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. Mol. Biol. Evol. **13:**964–969.

39. **Tan, K. S. W., M. Singh, and E. H. Yap.** 2002. Recent advances in *Blastocystis hominis* research: hot spots in terra incognita. Int. J. Parasitol. **32:**789–804.

40. **Tasova, Y., B. Sahin, S. Koltas, and S. Paydas.** 2000. Clinical significance and frequency of *Blastocystis hominis* in Turkish patients with haematological malignancy. Acta Med. Okayama **54:**133–136.

41. **Teow, W. L., V. Zaman, G. C. Ng, Y. C. Chan, E. H. Yap, J. Howe, P. Gopalakrishnakone, and M. Singh.** 1991. A *Blastocystis* species from the sea-snake, *Lapemis hardwickii* (Serpentes; Hydrophiidae). Int. J. Parasitol. **21:**723–726.

42. **Teow, W. L., G. C. Ng, P. P. Chan, Y. C. Chan, E. H. Yap, V. Zaman, and M. Singh.** 1992. A survey of *Blastocystis* in reptiles. Parasitol. Res. **78:**453–455.

43. **Thathaisong, U., J. Worapong, M. Mungthin, P. Tan-Ariya, K. Viputtigul, A. Sudatis, A. Noonai, and S. Leelayoova.** 2003. *Blastocystis* isolates from a pig and a horse are closely related to *Blastocystis hominis*. J. Clin. Microbiol. **41:**967–975.

44. **Valkiunas, G., and R. W. Ashford.** 2002. Natural host range is not a valid taxonomic character. Trends Parasitol. **18:**528–529.

45. **Windsor, J. J., L. MacFarlane, G. Hughes-Thapa, S. K. A. Jones, and T. M. Whiteside.** 2002. Incidence of *Blastocystis hominis* in faecal samples submitted for routine microbiological analysis. Br. J. Biomed. Sci. **59:**154–157.

46. **Yoshikawa, H., I. Nagono, E. H. Yap, M. Singh, and Y. Takahashi.** 1996. DNA polymorphism revealed by arbitrary primers polymerase chain reaction among *Blastocystis* strains isolated from humans, a chicken, and a reptile. J. Eukaryot. Microbiol. **43:**127–130.

47. **Yoshikawa, H., I. Nagano, Z. Wu, E. H. Yap, M. Singh, and Y. Takahashi.** 1998. Genomic polymorphism among *Blastocystis hominis* strains and development of subtype-specific diagnostic primers. Mol. Cell Probes. **12:**153–159.

48. **Yoshikawa, H., N. Abe, M. Iwasawa, S. Kitano, I. Nagano, Z. Wu, and Y. Takahashi.** 2000. Genomic analysis of *Blastocystis hominis* strains isolated from two long-term health care facilities. J. Clin. Microbiol. **38:**1324–1330.

49. **Yoshikawa, H., Z. Wu, I. Nagano, and Y. Takahashi.** 2003. Molecular comparative studies among *Blastocystis* isolates obtained from humans and animals. J. Parasitol. **89:**585–594.

50. **Yoshikawa, H., Z. Wu, I. Kimata, M. Iseki, I. K. M. D. Ali, M. B. Hossain, V. Zaman, R. Haque, and Y. Takahashi.** 2004. Polymerase chain reaction-based genotype classification among human *Blastocystis hominis* populations isolated from different countries. Parasitol. Res. **92:**22–29.