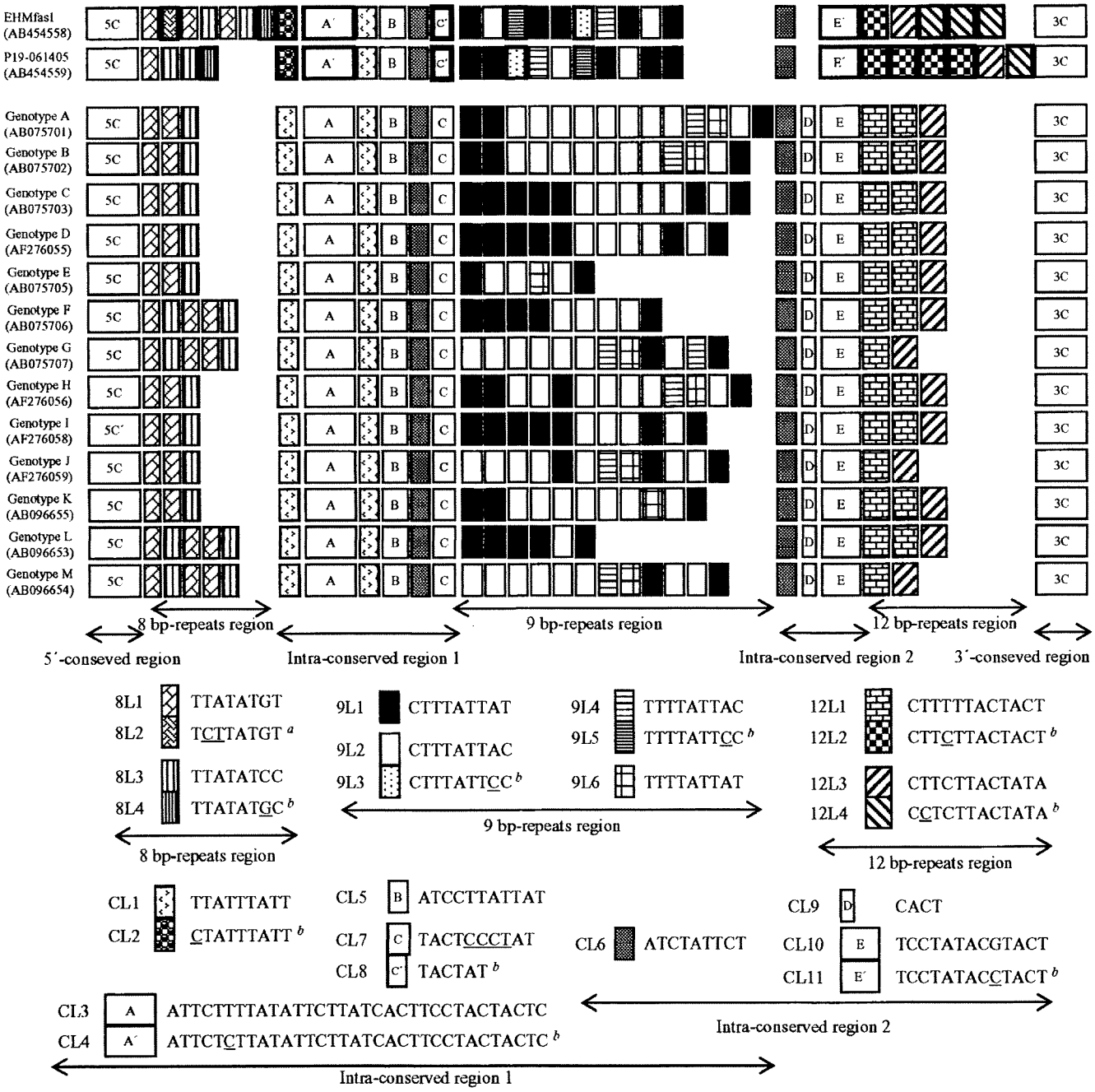

Fig. 4 Schematic representation of polymorphism in the repeat-containing region of the locus 1–2 region among simian *E. histolytica*-like strains and available *E. histolytica*. Nucleotide sequences pattern was shown. Each nucleotide sequence of unit was tentatively given a number. Nucleotide sequences of these units are also shown. *Enclosed units with bold line* were EHMfas1-specific or simian *E. histolytica*-like strain-specific units. EHMfas1-specific or simian *E. histolytica*-like strain-specific mutations in nucleotide sequences are *underlined*. Simian *E. histolytica*-like strain-specific deletion from human isolates is underlined at comparative sequence. *a* EHMfas1-specific unit sequence. *b* Simian *E. histolytica*-like strain-specific unit sequences

and 5.8S rDNA with ITS 1 and ITS 2 and Cpn60 and PNT genes of EHMfas1 and P19-061405 have been used for previous phylogenetic studies of the genus *Entamoeba* (Clark and Diamond 1997; Silberman et al. 1999; Som et al. 2000; Bakatselou et al. 2003; Clark et al. 2006). All phylogenetic studies indicated that EHMfas1 and P19-061405 generated an original cluster beside or under *E. histolytica*, including the PNT gene, which is known to contribute to an anomalous-shaped phylogenetic tree (Bakatselou et al. 2003).

Comparisons of polymorphic loci used for genotyping revealed notable differences from *E. histolytica* and

similarities between simian *E. histolytica*-like strains in sequence variation of the constructing units (Figs. 2, 3, and 4). The chitinase genes, the SREHP genes, and the locus 1–2 sequences of *E. histolytica* have been categorized into seven, 37, and 13 genotypes, respectively, from 79 strains, based on the combination pattern of constructing units in the polymorphic regions, respectively (Haghighi et al. 2002, 2003). The most kinds of mutation units were observed in locus 1–2, although the most kinds of genotype have been reported in the SREHP gene. In all polymorphic loci, most mutated units that were observed in EHMfas1 and P19-061405 were shared as simian *E. histolytica*-like strain-specific units, including deletions, among EHMfas1 and P19-061405. These mutated units had single-nucleotide substitutions relative to the corresponding units in *E. histolytica*. On the other hand, unshared mutated units were also observed. In the SREHP gene, SN16 was not observed in P19-061405, and the second insertion unit (GATGAA-GAA; DEE) was only observed in EHMfas1. In locus 1–2, 8L2 was specific for EHMfas1. The EHMfas1-specific insertion unit was likely derived from the non-repeating SN3 (GATGAAGAT; DED), with a single-nucleotide substitution. These slight disparities may reflect the differences between the host monkey species or the habitats of EHMfas1 and P19-061405; however, not enough strains have been studied to date. Analyses of these polymorphic loci also indicated that simian *E. histolytica*-like strains can also be classified by genotyping with original mutated units as *E. histolytica*.

It is interesting that *E. dispar* exhibit the same repetitive units as *E. histolytica* in the chitinase (CN2, CN4, and CN6) and the SREHP (SN8 and SN16) genes (Ghosh et al. 2000). Although *E. dispar* exhibits *E. dispar*-specific units, most of those mutated units were not repetitive. On the other hand, EHMfas1 and P19-061405 exhibited many repetitive mutated units, CN3, CN5, CN7, SN9, and SN17. EHMfas1 and P19-061405 exhibited not only mutated CN3 and SN17 but also the corresponding CN2 and SN16. However, all CN4, CN6, and SN8 were changed to CN5, CN7, and SN9. These findings indicate that *E. histolytica* is more closely related to *E. dispar* than the simian *E. histolytica*-like strains, in contrast to the results of other sequence analyses. It is considered that these contradictory findings are probably caused by specific effects, other than general evolution, because SREHP, in particular, is known to be a trophozoite surface antigen (Stanley et al. 1995). In other words, these contradictory findings may indicate differences in the manner of immune escape between different host species. On the other hand, locus 1–2 is a non-coding region and has the most kinds of mutation units. These polymorphic loci must be exposed to other evolutionary effects. This specific contradictory finding is likely a consequence of "concerted evolution" (Dover 1982, 1993; Dover and Tautz 1986; Wilkinson and Chapman 1991; Jinks-Robertson and Petes 1993; Liao 1999).

Concerted evolution is a universal biological phenomenon for repetition of DNA elements and has been observed in many repetitive DNA sequences and multi-gene families. Concerted evolution is thought to result from various mechanisms of DNA turnover, including unequal crossing-over, DNA amplification, gene conversion, and replication slippage. In *Entamoeba* species, concerted evolution has been discussed as it relates to the 5.8S rRNA gene and rRNA-linked *E. histolytica* short tandem repeats (Som et al. 2000; Tawari et al. 2008). It is suggested that diversification of the chitinase gene, the SREHP gene, and locus 1–2 can be caused by replication slippage (Ghosh et al. 2000; Bhattacharya et al. 2005). The repetitive units developing by concerted evolution are homogeneous within species, but differ somewhat between species. The similarities of each kind of unit in the chitinase and SREHP genes, locus 1-2 within simian *E. histolytica*-like strains or *E. histolytica*, and the differences of those between the both are accountable by concerted evolution. Concerted evolution of these polymorphic loci indicates that the repetitive simian *E. histolytica*-like strain-specific units had been amplified and conserved according to speciation as opposed to general DNA evolution, whereas some of these units had been eliminated or were not apparent in *E. histolytica* and *E. dispar*.

Tachibana et al. (2007) proposed that P19-061405 should be distinguished from *E. histolytica* by revival of the named *E. nuttalli* (Castellani 1908). Because EHMfas1 exhibited slight differences but had the same genetic characteristics as P19-061405, EHMfas1 also should be identified as *E. nuttalli*. This study indicates that *E. nuttalli* is endemic to monkey but we cannot exclude the possibility of zoonotic infection from monkey to human. We believe that further comparative study of *E. nuttalli* will contribute to more insights into the phylogeny and pathogenicity of *Entamoeba* species because *E. nuttalli* is very closely related to *E. histolytica* and *E. nuttalli* is the only pathogenic species other than *E. histolytica* among the *Entamoeba* species.

Acknowledgment This work was supported by a Grant-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science.

References

Ali IKM, Zaki M, Clark CG (2005) Use of PCR amplification of tRNA gene-linked short tandem repeats for genotyping *Entamoeba histolytica*. J Clin Microbiol 43:5842–5847

Ayeh-Kumi PF, Ali IM, Lockhart LA, Gilchrist CA, Petri WA Jr, Haque R (2001) *Entamoeba histolytica*: genetic diversity of

clinical isolates from Bangladesh as demonstrated by polymorphisms in the serine-rich gene. Exp Parasitol 99:80–88

Bakatselou C, Beste D, Kadri AO, Somanath S, Clarc CG (2003) Analysis of genes of mitochondrial origin in the genus *Entamoeba*. J Eukaryot Microbiol 50:210–214

Bhattacharya D, Haque R, Singh U (2005) Coding and noncoding genomic regions of *Entamoeba histolytica* have significantly different rates of sequence polymorphisms: implications for epidemiological studies. J Clin Microbiol 43:4815–4819

Castellani A (1908) Note on a liver abscess of amoebic origin in a monkey. Parasitology 1:101–102

Clark CG, Diamond LS (1993) *Entamoeba histolytica*: a method for isolate identification. Exp Parasitol 77:450–455

Clark CG, Diamond LS (1997) Inraspecific variation and phylogenetic relationships in the genus *Entamoeba* as revealed by riboprinting. J Euk Microbiol 44:142–154

Clark CG, Kaffashian F, Tawari B, Windsor JJ, Twigg-Flesner A, Davies-Morel MC, Blessmann J, Ebert F, Peschel B, Le Van A, Jackson CJ, Macfarlane L, Tannich E (2006) New insights into the phylogeny of *Entamoeba* species provided by analysis of four new small-subunit rRNA genes. Int J Syst Evol Microbiol 56:2235–2239

Diamond LS, Harlow DR, Cunnick CC (1978) A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans R Soc Trop Med Hyg 72:431–432

de la Vega H, Specht CA, Semio CE, Robbins PW, Eichinger D, Caplivski D, Ghosh S, Samuelson J (1997) Cloning and expression of chitinases of *Entamoebae*. Mol Biochem Parasitol 85:139–147

Dover G (1982) Molecular drive: a cohesive mode of species evolution. Nature 299:111–117

Dover GA (1993) Evolution of genetic redundancy for advanced players. Curr Opin Genet Dev 3:902–910

Dover GA, Tautz D (1986) Conservation and divergence in multigene families: alternatives to selection and drift. Philos Trans R Soc Lond B Biol Sci 312:275–289

Ghosh S, Frisardi M, Ramirez-Avila DS, Sturm-Ramirez K, Newton Sanchez OA, Sntos-Preciado JI, Ganguly C, Lohia A, Reed S, Samuelson J (2000) Molecular epidemiology of *Entamoeba* spp.: evidence of a bottleneck (Demographic Sweep) and transcontinental spread of diploid parasites. J Clin Microbiol 38:3815–3821

Haghighi A, Kobayashi S, Takeuchi T, Masuda G, Nozaki T (2002) Remarkable genetic polymorphism among *Entamoeba histolytica* isolates from a limited geographic area. J Clin Microbiol 40:4081–4090

Haghighi A, Kobayashi S, Takeuchi T, Thammapalerd N, Nozaki T (2003) Geographic diversity among genotypes of *Entamoeba histolytica* field isolates. J Clin Microbiol 41:3748–3756

Jinks-Robertson S, Petes TD (1993) Experimental determination of rates of concerted evolution. Methods Enzymol 224:631–646

Kohler S, Tannich E (1993) A family of transcripts (K2) of *Entamoeba histolytica* contains polymorphic repetitive regions with highly conserved elements. Mol Biochem Parasitol 59:49–58

Li E, Kunz-Jenkins C, Stanley SL Jr (1992) Isolation and characterization of genomic clones encoding a serine-rich *Entamoeba histolytica* protein. Mol Biochem Parasitol 50:355–358

Liao D (1999) Concerted evolution: molecular mechanism and biological implications. Am J Hum Genet 64:24–30

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Silberman JD, Clark CG, Diamond LS, Sogin ML (1999) Phylogeny of the genera *Entamoeba* and *Endolimax* as deduced from small-subunit ribosomal RNA sequence. Mol Biol Evol 16:1740–1751

Som I, Azam A, Bhattacharya A, Bhattacharya S (2000) Inter- and intra-strain variation in the 5.8S ribosomal RNA and internal transcribed spacer sequences of *Entamoeba histolytica* and comparison with *Entamoeba dispar*, *Entamoeba moshkovskii* and *Entamoeba invadens*. Int J Parasitol 30:723–728

Stanley SL Jr, Becker A, Kunz-Jenkins C, Foster L, Li E (1990) Cloning and expression of a membrane antigen of *Entamoeba histolytica* possessing multiple tandem repeats. Proc Natl Acad Sci U S A 87:4976–4980

Stanley SL Jr, Tian K, Koester JP, Li E (1995) The serine-rich *Entamoeba histolytica* protein is a phosphorylated membrane protein containing O-linked terminal N-acetylglucosamine residues. J Biol Chem 270:4121–4126

Suzuki J, Kobayashi S, Murata R, Yanagawa Y, Takeuchi T (2007) Profiles of a pathogenic *Entamoeba histolytica*-like variant with variations in the nucleotide sequence of the small subunit ribosomal RNA isolated from a primate (De Brazza's guenon). J Zoo Wildl Med 38:471–474

Tachibana H, Kobayashi S, Kato Y, Nagakura K, Kaneda Y, Takeuchi T (1990) Identification of a pathogenic isolate-specific 30, 000-Mr antigen of Entamoeba histolytica by using a monoclonal antibody. Infect Immun 58:955–960

Tachibana H, Yanagi T, Pandey K, Cheng XJ, Kobayashi S, Sherchand JB, Kanbara H (2007) An *Entamoeba* sp. strain isolated from rhesus monkey is virulent but genetically different from *Entamoeba histolytica*. Mol Biochem Parasitol 153:107–114

Takano J, Narita T, Tachibana H, Shimizu T, Komatsubara H, Terao K, Fujimoto K (2005) *Entamoeba histolytica* and *Entamoeba dispar* infections in cynomolgus monkeys imported into Japan for research. Parasitol Res 97:255–257

Takano J, Narita T, Tachibana H, Terao K, Fujimoto K (2007) Comparison of *Entamoeba histolytica* DNA isolated from a cynomolgus monkey with human isolates. Parasitol Res 101:539–546

Tawari B, Ali IK, Scott C, Quail MA, Berriman M, Hall N, Clark CG (2008) Patterns of evolution in the unique tRNA gene arrays of the genus *Entamoeba*. Mol Biol Evol 25:187–198

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25:4876–4882

Verweij JJ, Vermeer J, Brienen EAT, Blotkamp C, Laeijendecker D, Lieshout LV, Polderman AM (2003) *Entamoeba histolytica* infections in captive primates. Parasitol Res 90:100–103

Wilkinson GS, Chapman AM (1991) Length and sequence variation in evening bat D-loop mtDNA. Genetics 128:607–617

Zaki M, Clark CG (2001) Isolation and characterization of polymorphic DNA from *Entamoeba histolytica*. J Clin Microbiol 39:897–905

Zaki M, Meelu P, Sun W, Clark CG (2002) Simultaneous differentiation and typing of *Entamoeba histolytica* and *Entamoeba dispar*. J Clin Microbiol 40:1271–1276