Acta Protozool. (2006) 45: 265 - 270

ACTA
PROTOZOOLOGICA

Phylogenetic Positions of Two Crytophorid Ciliates, *Dysteria procera* and *Hartmannula derouxi* (Ciliophora: Phyllopharyngea: Dysteriida) Inferred from the Complete Small Subunit Ribosomal RNA Gene Sequences

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Summary. The complete small subunit rRNA (SSrRNA) gene was sequenced for two poorly known marine crytophorid ciliates, *Dysteria procera* Kahl, 1931 and *Hartmannula derouxi* Gong *et* Song, 2004. The phylogenetic positions within the class Phyllopharyngea were deduced using Bayesian, distance matrix and maximum parsimony methods. *Dysteria procera* and *Hartmannula derouxi*, together with other available ciliates of the class Phyllopharyngea, form a monophyletic clade with strong bootstrap support in all the tree construction methods (values of 100% Bay, 100% LS, 100% NJ, 100% MP). The SSrRNA genealogy showed that the class Phyllopharyngea contained two monophyletic subclasses Chonotrichia and Suctoria, while the subclass Phyllopharyngia was paraphyletic. Our results revealed that some taxa of the subclass Chonotrichia (e.g. *Isochona* species) had a close relationship with the subclass Phyllopharyngia, suggesting that heteromeric nuclear apparatus and the dorsal-reduced ciliature are likely phylogenetically informative. Additionally, consisting with traditional morphological descriptions, dysteriids may be a specialized group within the class in our phylogenetic trees.

Key words: *Dysteria procera*, *Hartmannula derouxi*, marine ciliate, Phyllopharyngea, phylogenetic position, SSrRNA.

INTRODUCTION

The Phyllopharyngea, an understudied class in the phylum Ciliophora, is named for the radially arranged mocrotubular structures (phyllae) around the cytopharynx (de Puytorac *et al.* 1974; de Puytorac 1994; Lynn and Corliss 1991; Lynn 1996; Lynn and Small 1997, 2002). Taxonomic and systematic studies on this class are traditionally based morphological characters revealed with silver impregnation methods (Song and Wilbert 2002).

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Molecular analyses have been used recently to refine our knowledge of phylogenetic relationships within the ciliated protozoa (phylum Ciliophora). The application of the polymerase chain reaction (PCR) to phylogenetic studies of ciliates finally provides us with the opportunity to acquire molecular sequence information. The genes used to reconstruct phylogenetic trees have mainly included small subunit rRNA (SSrRNA), large subunit rRNA (LSrRNA), tubulin, histone, Hsp70, and DNA polymerase á sequences (Hoffman and Prescott 1997, Baroin-Touranchean *et al.* 1998, Bernhard and Schlegel 1998, Budin and Philippe 1998, Lynn *et al.* 2000). However, sequence data for phyllopharyngean ciliates remain comparatively rare and incomplete. To date, SSrRNA gene sequences have been determined for only about 23 species within this species-rich class (Leipe *et*

al. 1994, Riley and Katz 2001, Snoeyenbos-West *et al.* 2004).

As a part of a comprehensive analysis of ciliate phylogeny carried out recently in the authors' group, we have sequenced the SSrRNA gene for these two poorly-known marine crytophorid ciliates, *Dysteria procera* Kahl, 1931 and *Hartmannula derouxi* Gong *et* Song, 2004. Results were presented to provide more molecular information on these rare-known organisms.

MATERIALS AND METHODS

Ciliate collection and identification. *Dysteria procera* and *Hartmannula derouxi* were collected from the littoral area off Qingdao (Tsingtao, 36°08'N; 120°43'E), China. Isolated specimens of both species were maintained in the laboratory for about 1 week as raw cultures in Petri dishes for observation and further studies.

Their morphology was described recently by Gong and Song (2003, 2004) (Fig. 1). Systematic and terminology at the order and above level are mainly referred from Lynn and Small (2002).

DNA extraction, PCR, and cloning. Cells were starved overnight, rinsed with sterile artificial marine water and then centrifuged at low speed. Lysis buffer (Shang *et al.* 2003) was added and the mixture incubated at 56°C for 1~2 h to extract DNA, then 94°C for 15 min to denature the proteinase K. An equal mixture of the regular DNA polymerase (Promega, USA) and the *Pfu* Taq DNA polymerase (High Fidelity, Sangon, Canada) was utilized for PCR reaction (Chen and Song 2002). Oligonucleotide primer sequences used in this work are available from the authors.

The amplified products were purified (UNIQ-5 DNA Cleaning Kit), inserted into a pUCm-T vector (Sangon, Canada), and sequenced on an ABI Prism 377 Automated DNA Sequencer. Subsequent sequencing was performed using primer walking.

The GenBank/EMBL accession numbers are DQ057347 (*Dysteria procera*) and AY378113 (*Hartmannula derouxi*).

Sequence availability. The nucleotide sequences used for analysis are available from the GenBank/EMBL databases under the following accession numbers: *Isochona* sp. OOSW-1 AY242116, *Isochona* sp. OOSW-2 AY242117, *Isochona* sp. OOSW-3 AY242118, *Isochona* sp. OOSW-4 AY242119, *Chilodonella uncinata* AF300281, *Trithigmostoma steini* X71134, *Chlamydodon excocellatus* AY331790, *Chlamydodon triquetrus* AY331794, *Dysteria derouxi* AY378112, *Dysteria* sp. 1 AY331797, *Dysteria* sp. 2 AY331798, *Tokophrya quadripartita* AY102174, *Discophrya collini* L26446, *Heliophrya erhardi* AY007445, *Prodiscophrya* sp. OOS-2003 AY331802, *Ephelota* sp. RJL2001 AF326357, *Ephelota* sp. OOS-2003 AY331804, *Pseudocohnilembus marinus* Z22880, *Mesanophrys carcini* AY103189, *Pseudocohnilembus hargisi* AY212806, *Aspidisca steini* AF305625, *Diophrys appendiculata* AY004773, *Uronychia transfuga* AF260120, and a karyorelictid ciliate, *Loxodes magnus* L31519 was used as the outgroup species.

Phylogenetic analyses. SSrRNA gene sequences were aligned using the Clustal W, ver. 1.80 (Thompson *et al.* 1994). The computer program, MrBayes v3.0b4 (Huelsenbeck and Ronquist 2001) was used for the Markov Chain Monte Carlo (MCMC) algorithm to construct Bayesian tree. PHYLIP package, version 3.57c (Felsenstein 1985) was used to calculate the sequence similarity and evolutionary distances between pairs of nucleotide sequences using the Kimura (1980) two-parameter model. Distance-matrix trees were constructed using the least-squares [LS] method (Fitch and Margoliash 1967) and the neighbor-joining [NJ] method (Saitou and Nei 1987). Maximum parsimony (MP) tree was constructed by a heuristic search in the program PAUP* v4.0b10 (Swofford 2002). For maximum parsimony analysis, nucleotide sequences were reduced to phylogenetically informative sites. Data were bootstrap resampled 1,000 times.

RESULTS

Sequences and comparisons

The complete SSrRNA gene sequences were determined consisting of 1728 nucleotides (for *Dysteria procera*) and 1708 nucleotides (for *Hartmannula derouxi*) in length. The GC content (44.44% in *D. procera* and 44.91% in *H. derouxi* respectively) is in the same range as in other ciliates (Elwood *et al.* 1985, Sogin and Elwood 1986, Schlegel *et al.* 1991).

The sequence of *Dysteria procera* differs in 131 nucleotides from the sequence of *Dysteria derouxi* (structural similarity 92.40%). 284 sites are different from that in *Hartmannula derouxi* (structural similarity 83.34%), while 233 sites differ from that of *Isochona* sp. OOSW-1 (structural similarity 86.27%), 242 sites are different from that in *Chlamydodon excocellatus* clone 1 (structural similarity 85.96%).

Bayesian and distance matrix analysis

Both Bayesian and distance-matrix based analyses provided strong bootstrap support for the monophyly of the class Phyllopharyngea, Oligohymenophorea and Spirotrichea *sensu* Lynn and Small 2002 (see Fig. 2).

The SSrRNA genealogy showed that the class Phyllopharyngea contains two monophyletic subclasses Chonotrichia and Suctoria, while the subclass Phyllopharyngia is paraphyletic. The family Hartmannulidae (e.g. *Hartmannula derouxi*), together with Chilodonellidae, Chlamydodontidae and Dysteriidae, was clustered within the subclass Phyllopharyngia, but closely related to Isochonidae. The members of the subclass Suctoria, represented by Endogenida, Evaginogenida and Exogenida, were grouped as a sister group to the traditional crytophorids though the suctorian ciliates exhibit extraordinary differences both in general morphology and other biological feathers, e. g. life style, morphogenetic pattern, reproduction behaviors etc.

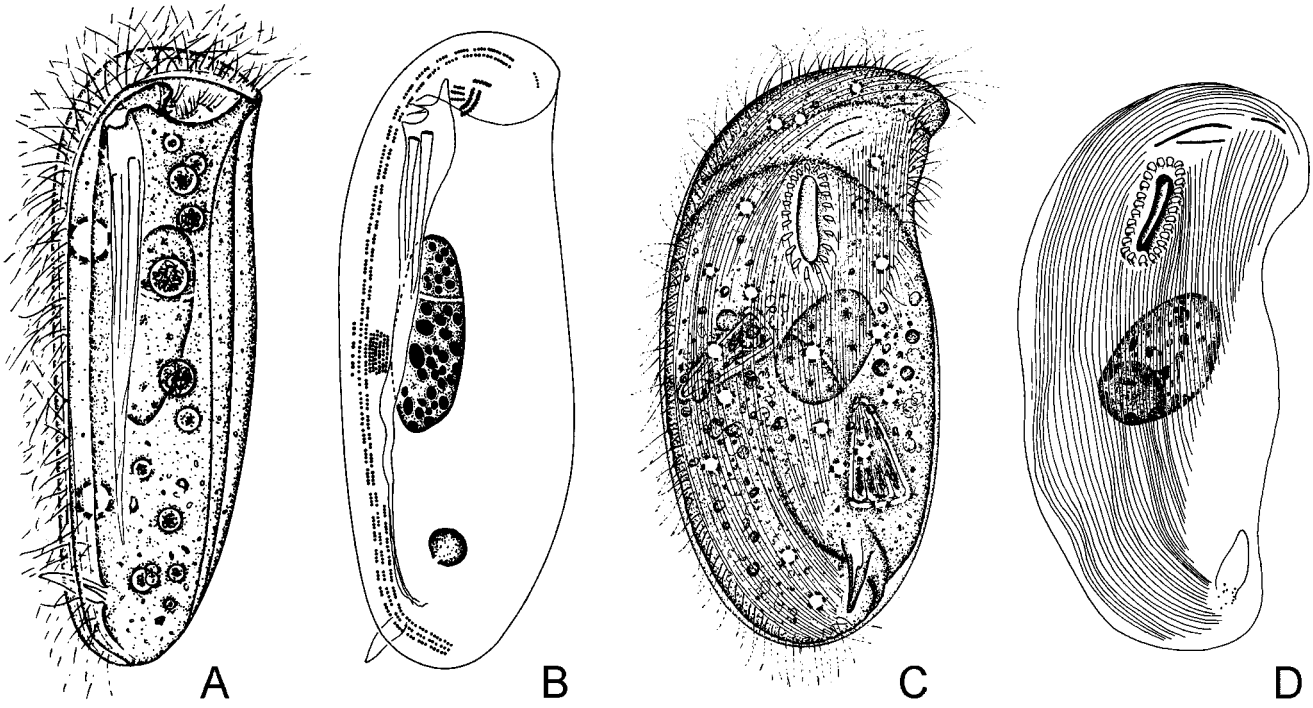

Fig. 1. Morphology and infraciliature of *Dysteria procera* (A, B) and *Hartmannula derouxi* (C, D), from life (A, C) and after protargol impregnated specimens (B, D). Reproduced with permission from Gong and Song (2003, 2004).

Maximum parsimony analysis

The major aspects of the topology of the maximum parsimony tree (Fig. 3) were similar to those of the Bayesian and distance-matrix trees (Fig. 2). *Dysteria procera* and *Hartmannula derouxi* were placed within the subclass Phyllopharyngia. The family Dysteriidae and Hartmannulidae are in variably close with the Chlamydodontidae, where the "well-known" Chilodonellidae forms a clearly outlined group. A remarkable degree of correlation was the relative positions of *Isochona*, *Hartmannula* and *Dysteria*, which means that the subclass Chonotrichia (e.g. *Isochona* species) showed a very close relationship with the subclass Phyllopharyngia (Fig. 3).

DISCUSSION

As demonstrated in the present work, the class Phyllopharyngea was strongly confirmed as a monophyletic clade containing three major subclasses so far represented by SSrRNA gene sequences (100% Bay, 100% LS, 100%NJ, 100% MP, Figs 2, 3). It is consistent with the traditional taxonomy based on the shared morphological characters.

Within the class Phyllopharyngea, the subclass Suctoria branched basally with a strong bootstrap support (100% Bay, 100% LS, 100% NJ, 100% MP), whereas the clades for the Phyllopharyngia and Chonotrichia branched later with moderate support (see Figs 2, 3).

As shown in Figs 2, 3, Chilodonellidae was separated from other Phyllopharyngia groups, while the families Chlamydodontidae, Dysteriidae (represent by *Dysteria*) and Hartmannulidae branched above it and formed a monophyletic clade severally with high bootstrap support in all trees.

Both Dysteriidae and Hartmannulidae were characterized by laterally compressed body; ventral cilia not thigmotactic, juxtaposed heteromerous macronucleus, etc, but differed in the pattern of the left ventral somatic kineties. According to the molecular characters inferred from SSrRNA gene sequences, two families were closely related, which was consistent with the morphological

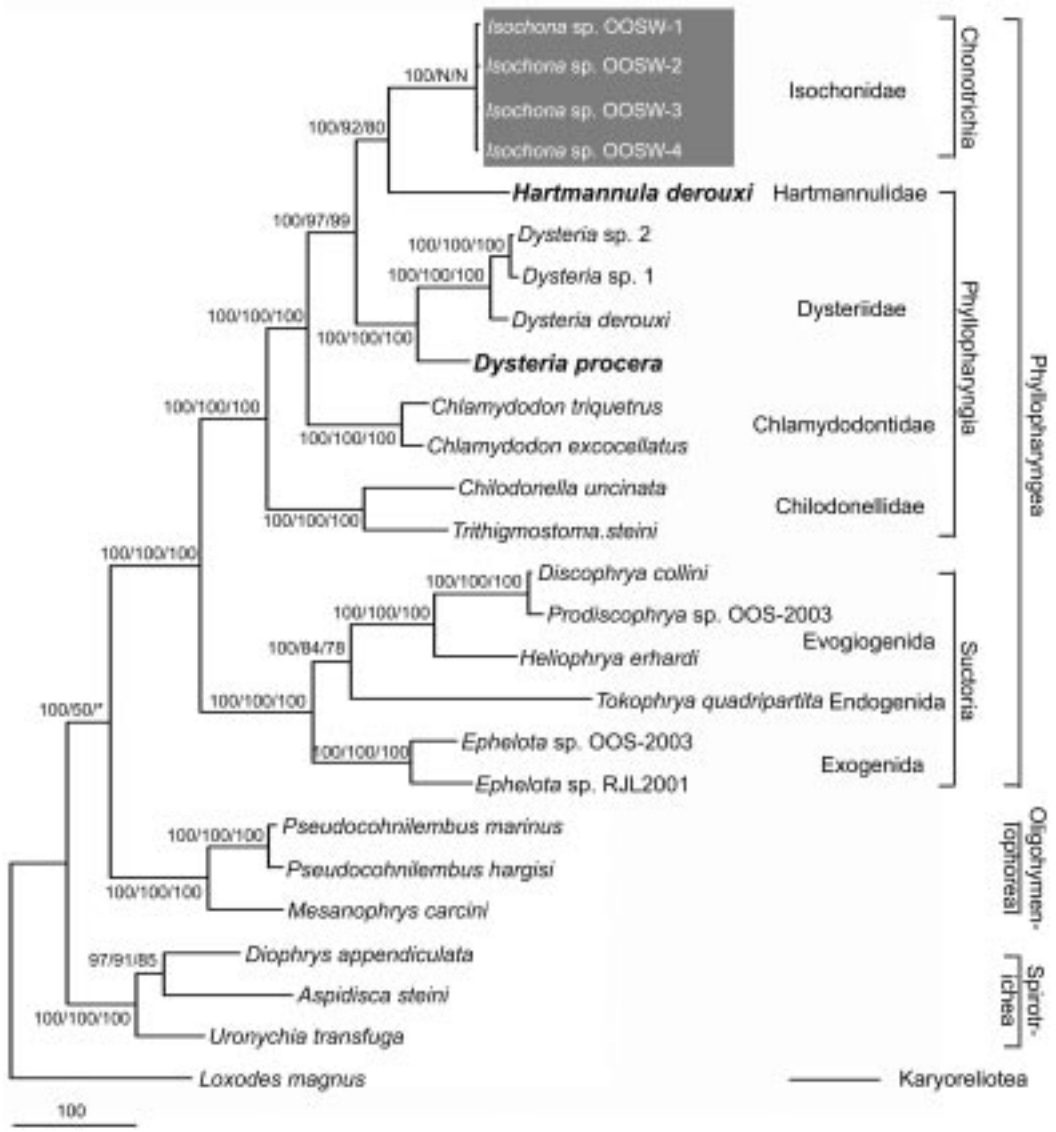

Fig. 2. Bayesian tree inferred from the nucleotide sequences of complete small subunit rRNA (SSrRNA) of phyllopharyngean ciliated protozoa. Numbers on branches indicate support indices from 1000 bootstrap estimations using each of the algorithms in the following order: Bayesian credibility value using the MrBayes, distance-matrix based least-squares (LS) and neighbor joining (NJ) bootstrap percentage using the Phylip package. Asterisks indicate bootstrap values less than 50%. "N" reflects disagreement between a method and the reference Bayesian tree at a given node. Evolutionary distance is represented by the branch length to separate the species in the figure. The scale bar corresponds to five substitutions per 100 nucleotide positions. Numbers at nodes represent bootstrap values (%) out of 1,000 replicates. The new sequences are represented in boldface.

comparison (Lynn and Small 2002, Gong and Song 2003).

The SSrRNA genealogy showed that the class Phyllopharyngea contained two monophyletic subclasses Chonotrichia and Suctoria, while the subclass Phyllopharyngia was paraphyletic. Our results revealed that some taxa of subclass the Chonotrichia (e.g. *Isochona* species) had a close relationship with the subclass Phyllopharyngia, suggesting that heteromeric nuclear apparatus and the dorsal-reduced ciliature are phylogenetically informative (Corliss 1979, Grell and Meister 1982, Puytorac *et al.* 1994, Foissner 1996, Snoeyenbos-West *et al.* 2004). Migratory "larval" form of Chonotrichs resembles adult Phyllopharyngia (Kent

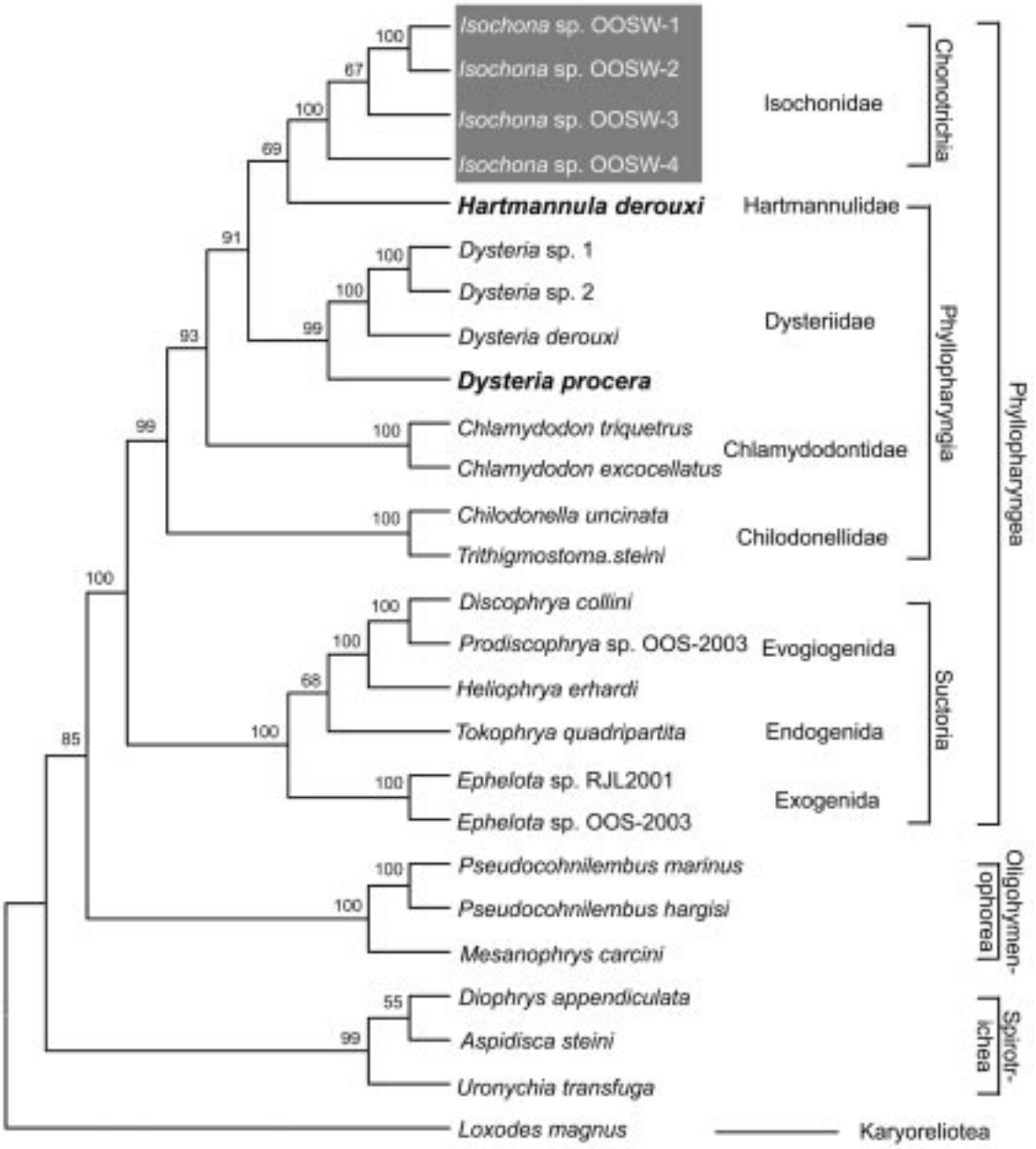

Fig. 3. Maximum Parsimony (MP) tree of the phyllopharyngean ciliates. The numbers at the forks exhibit the percentage of times the group occurred out of the 1,000 trees. No significance is placed on branch lengths connecting the species. The new sequence is highlighted in boldface.

1880-1882, Dobrzańska-Kaczanowska 1963), which implies that morphological differences between adult chonotrichs and Phyllopharyngia may be misleading taxonomically (Snoeyenbos-West *et al.* 2004).

In addition, our results clearly indicated that dysteriids were outlined from other related taxa and may be a specialized group within Phyllopharyngea, which corresponds well with the traditional morphological descriptions (Gong and Song 2003, Li and Song 2006).

Nevertheless, the present study did not provide an unambiguous branching order for all orders within the class Phyllopharyngea, since until now, only 23 species

with this species-rich group have been sequenced and represented on SSrRNA phylogenetic trees. To determine details of their relationships in these highly specialized organisms, more data are definitely needed.

Acknowledgements. This work was supported by the "Nature Science Foundation of China" (Project number 30430090, 40376045). We are grateful to Dr. Jun Gong, ex-graduate student of our laboratory, for his kindly help in identifying the material.

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Received on 11th October, 2005; revised version on 24th March, 2006; accepted on 4th May, 2006