Description and phylogenetic characterization of hydra from Naukuchiatal (Uttarakhand, India) and comparison with other *Hydra* strains

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Hydra, a fresh water Cnidarian, has been used as a model system to study regeneration and pattern formation. Here we report a newly identified hydra from Naukuchiatal, India. Comparison of *Hydra vulgaris* Naukuchiatal with *Hydra magnipapillata*, *Hydra vulgaris* AEP and *Hydra vulgaris* Ind-Pune showed variations in morphology. Nema arrangement in holotrichous isorhiza nematocytes showed transverse coiling pattern, a characteristic feature of the '*vulgaris*' group. Phylogenetic analysis using conserved mitochondrial genes confirmed that Naukuchiatal hydra belongs to the '*vulgaris*' group though it is a different strain particular to the type locality. Its morphological peculiarities could be the result of pristine environment.

Keywords: Hydra, mitochondrial genes, morphology, nematocysts, taxonomy.

ONE of the major challenges in hydra biology is correct taxonomic identification of specimens from different localities. Hydra belonging to the phylum Cnidaria has been previously included in the genus *Hydra* by Carl Linne[1] which was followed by the three genera classification by Schulze[2] in 1914. Later, the genus *Hydra* was divided into four different groups: oligactis (stalked hydra), *vulgaris* (common hydra), braueri (gracile hydra) and viridissima (green hydra), based on both morphological differences[3] and molecular phylogenetic analysis[4]. However, the field of hydra taxonomy is believed to be an actively evolving field and much remains to be done[5]. This needs continued inputs from all over the globe. In India, the study on different hydra types was initiated by Annandale[6,7]. Later, 16 types of hydra from 15 distantly located ecosystems were collected and compared at morphological and physiological levels[8].

Our laboratory uses hydra extensively as a model to understand evolution of cell signalling and pattern-forming mechanisms. The taxonomic status of hydra strain, *H. vulgaris* Ind-Pune used in our studies was revised on the basis morphological and molecular characteristics[9]. We initiated studies to collect and characterize hydra polyps from different localities in India. A morphologically distinct hydra was collected from Naukuchiatal Lake in Uttarakhand in India (30°15′N and 79°15′E) in June 2012 and its culture is since being maintained under laboratory conditions. Using morphological and molecular taxonomic analysis we have identified it to be *Hydra vulgaris*. Interestingly, in spite of being the same species, the Naukuchiatal strain differs from the Pune strain significantly.

Materials and methods

Strains used and culture conditions

A single hydra polyp was collected from Naukuchiatal Lake in Uttarakhand. Clonal culture from this polyp is being maintained in the laboratory for the past four years in hydra medium: 1 mM NaCl, 1 mM $CaC1_2$, 0.1 mM KCl, 0.1 mM $MgSO_4$ and 1 mM Tris-chloride, pH 8.0 (ref. 10). Other strains used for comparison included *Hydra magnipapillata* (which is the same as *H. vulgaris* reg 16)[5], *Hydra vulgaris* AEP and *Hydra vugaris* Ind-Pune. Polyps were fed with freshly hatched *Artemia salina* nauplii and the medium was changed six hours post-feeding. The cultures were maintained at a constant temperature of 18 ± 1°C with 12 h of day and night cycle.

Body column length measurements

Adult polyps with a single bud were transferred to a glass petri plate under which a graph paper was placed. The length of their body column was measured after allowing them to relax naturally. Body column length of fixed hydra was measured by exposing adult polyps to 2% urethane for 2 min for relaxing them and they were fixed overnight in 4% paraformaldehyde at 4°C (ref. 11). The polyps were then washed twice with 1X PBS. Images were taken using Olympus SZX16 microscope to measure the length of the body column.

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Comparison of budding rate and doubling time among four different strains of hydra

Budding rates of all the four strains were compared. Polyps (n = 24) of each strain with a single bud were maintained separately till the buds detached from the parent hydra. Uniform age of all hydra polyps was ensured by transferring each detached bud to an individual well in a 24 well plate. These were fed and cleaned thrice a week. The detached buds from the separated polyps of all strains were counted for one year and the total number of buds was recorded.

In order to compare the doubling time among all the strains, an equal number of hydra polyps (n = 10) was fed daily with freshly hatched *A. salina* nauplii and transferred to a fresh hydra medium after 6 h. The number of polyps was recorded daily until the population of all strains doubled. This experiment was repeated four times and cumulative data for each strain were pooled to obtain the average number of days required for doubling the population. Statistical significance was calculated by confirming the normal distribution of samples for budding rate by Shapiro–Wilks and Anderson–Darling tests. Data were presented as mean ± SD. Significance was measured by one way ANOVA and unpaired t-test with Welch's unequal variance correction using PAST 3.0 and GraphPad prism 6.01 software.

Pattern of tentacle emergence

Hydra polyps at various stages of budding were relaxed with 2% urethane for 2 min and fixed in 4% paraformaldehyde overnight at 4°C (ref. 11). The polyps were washed with 1X PBS and the emergence pattern of tentacles was observed and recorded under an Olympus SZX16 stereo-microscope.

Preparation of nematocysts

Nematocysts for microscopic study were prepared as described previously[12]. Adult non-budding polyps (n = 20) were starved for 36 h and their hypostomal (head) tissue collected by cutting off the remaining part of the polyp. The tissue was dissociated in 200 µl of maceration medium (glycerin : glacialacetic acid : 1X hydra medium in the proportion 1 : 1 : 13) and incubated for 10 min at room temperature. Single cell suspension was prepared using a 2 ml syringe with 20 gauge needle followed by fixation with 20% formaldehyde and mounted on a microscopic slide in 50% glycerol. Images of nematocysts were captured using a Zeiss Axio Imager Z1 Apotome microscope.

Molecular taxonomy

Genomic DNA was isolated from 50 polyps using phenol/chloroform extraction[13]. To amplify the partial coding sequences of mitochondrial genes, reported primers[4] were used for 16S rRNA gene, while universal invertebrate primers[14] (Fw: GGTCAACAAATCATAAAGATATTG and Rev: TAAACTTCAGGGTGACCAAAAAAT) were used to amplify cytochrome oxidase subunit-I (COI) gene. PCR conditions for amplifying partial coding sequences of the two mitochondrial genes were as follows: Initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 16S rRNA gene and 47°C for COI gene for 1 min and extension at 72°C for 1 min 30 sec and final extension for 10 min. The amplified PCR products were cloned into pGEMT-Easy vector system (Promega) and confirmed by sequencing.

Phylogenetic trees were constructed by neighbour joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods using MEGA 6.0 software[15]. Sequence alignments for the two genes from four hydra groups and members from Aplanulata (*Euphysa intermedia*, *Euphysa aurata* and *Ectopleura larynx*) and *Obelia geniculate* (Supplementary Table 1) were carried out using ClustalW and Muscle from MEGA. In all methods bootstrap test with 1000 replicates was conducted and random addition of sequences for 10 replicates was carried out for MP method to represent the evolutionary history of the taxa analysed. Topology of the tree was depicted by NJ method and the tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances measured. P-distance method was used to compute the evolutionary distances. Bootstrap values indicated at the branch points were inferred from NJ, MP and ML methods respectively. *Obelia geneculata* was used as outgroup for both datasets (Supplementary Table 1 for accession numbers of genes used).

Results and discussion

Description

General morphology: The progeny of the original polyp retained their pinkish red colour (Figure 1 *A*) for a long period of 18 months (up to December 2013) after which the polyps became lighter in colour. Body column length of live adult polyps without bud (Figure 1 *A*(*a*)) can extend up to 15 mm with an average length of 8.88 ± 1.23 mm (n = 50). Adult polyps when fixed, measure 3.0–6.0 mm with an average body column length of 3.7 ± 0.60 mm (n = 50). Polyps are without a distinct stalk and the number of tentacles observed in majority of the polyps (n = 100) was 7 (80%). Polyps with 6 (3%), 8 (13%) and 9 (4%) tentacles were also noticed. Usual mode of reproduction in hydra is asexual (by budding), though sexual reproduction was also observed due to environmental stress[16]. In the current strain, asexual mode of reproduction was generally seen. Polyps with female gonads were also observed indicating dioecious nature of the

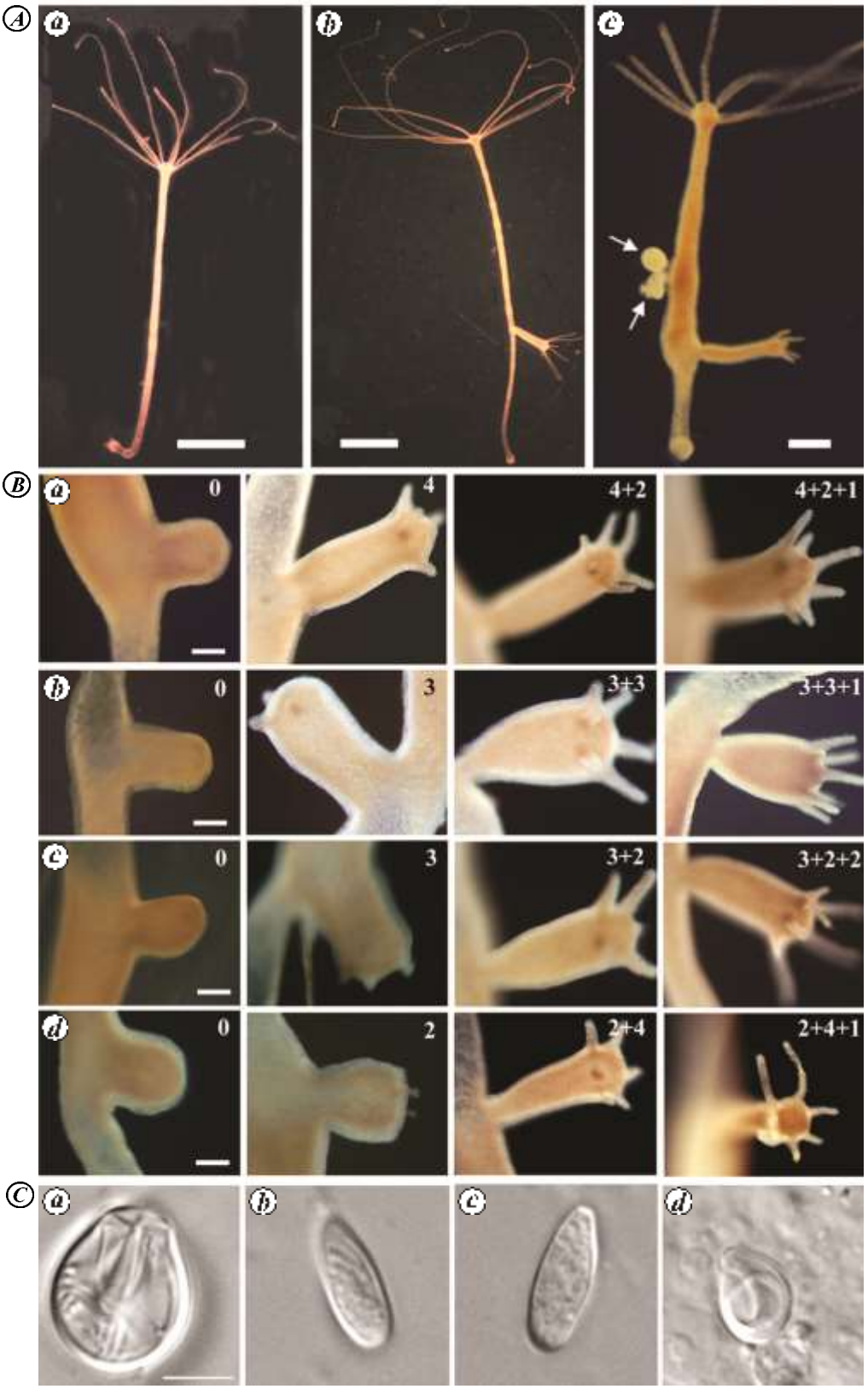

Figure 1. General morphology of *H. vulgaris* Naukuchiatal (***A***): Live polyps (***a***–***c***). (***a***) A polyp without bud (***b***) A polyp with bud and (***c***) represents polyp with two female gonads (shown by arrow). Scale bar: 1 mm. Asynchronous emergence of tentacles on developing bud of *H. vulgaris* Naukuchiatal. (***B***): (***a***) represents 4 + 2 + 1 pattern (***b***) represents 3 + 3 + 1 pattern (***c***) represents 3 + 2 + 2 pattern (***d***) represents 2 + 4 + 1 pattern. Scale bar: 1 mm. Types of nematocysts in *H. vulgaris* Naukuchiatal (***C***): (***a***) Stenotele (***b***) Holotrichous isorhiza (***c***) Atrichous isorhiza (***d***) Desmoneme. Scale bar: 5 μm.

species (Figure 1 *A*(*c*)). Polyps with male gonads have not been found in the culture since the clonal culture was established. This could be because the original polyp used to establish the culture may have been a female polyp.

Emergence of tentacles

Tentacle emergence pattern was considered as one of the important characteristic features for classification of hydra[17,18]. Depending on the order of emergence of tentacles on the developing bud, two types of patterns have been observed. The first represents simultaneous emergence of tentacles in which the tentacle rudiments appear at the same time. In the second type, asynchronous/uneven emergence of tentacles occurs. Asynchronous tentacle emergence pattern has been observed in *H. vulgaris* Naukuchiatal. Emergence pattern was studied in the polyps with seven tentacles as they were most predominant. Depending on the number of tentacles emanating from the developing bud, four types of patterns were observed (Figure 1 *B*) which includes 4 + 2 + 1 showing bud with 4, 4 + 2 (4 big and 2 small) and 4 + 2 + 1 (4 big, 2 small and 1 very small) tentacle rudiments (*a*), 3 + 3 + 1 pattern showing bud with 3, 3 + 3 (three big and three small) and 3 + 3 + 1 (three big, three small and one very small) tentacle rudiments (*b*), 3 + 2 + 2 pattern with (three big, two small and two very small) tentacle rudiments (*c*) and 2 + 4 + 1 pattern with (two big, four small and one very small) tentacle rudiments (*d*). Amongst these, the first (4 + 2 + 1) was the most commonly observed pattern. In case of *H. vulgaris* Ind-Pune the tentacles emerge asynchronously on buds with 2 + 2 + 1 pattern. Two tentacles emerge first opposite to each other followed by two more perpendicular to the first pair and the fifth one appears randomly. *H. magnipapillata*, *H. vulgaris* AEP both belonging to the '*vulgaris* group' shows asynchronous emergence of tentacles on buds.

Nema arrangement and size and shape of nematocysts

One of the most reliable features to classify hydra species, in addition to tentacle emergence pattern, is the comparison of size, shape and arrangement of nema (coiled thread in the nematocyte) in holotrichous isorhiza[18,19]. The nema coiling of an asymmetrical, slender shaped holotrichous isorhiza (Figure 1 *C*(*b*)) in *H. vulgaris* Naukuchiatal showed transverse coiling pattern which is the characteristic feature of the '*vulgaris*' group. Further, stenotele is broad and pyriform and less than 1.5 times as long as its breadth. Thus, the presence of slender shaped holotrichous isorhiza with transverse coiling and average length of stenotele of 1.23 times that of its breadth in the current strain suggests that the Naukuchiatal strain belongs to '*vulgaris*' group of species.

The nematocysts ($n = 25$) were microscopically observed and the length × breadth ($l \times b$) measured to examine the size of nematocysts in *H. vulgaris* Naukuchiatal. Stenoteles are broad, pyriform and each measures about 11.74 ± 1.09 μm × 9.51 ± 0.87 μm ($l \times b$). Most of the holotrichous isorhizae appear paramecium-like, while some are slender and measured 10.23 ± 0.47 μm × 4.29 ± 0.39 μm ($l \times b$). Atrichous isorhizae are slender and are of the dimension 7.96 ± 0.49 μm × 3.77 ± 0.29 μm ($l \times b$). Desmonemes are also pyriform with the smallest measuring about 6.28 ± 0.37 μm × 4.63 ± 0.44 μm. Nematocysts in *H. magnipapillata*[4] (asymmetrical holotrichous isorhizae: 10–13 μm × 4.0–6.0 μm), *H. vulgaris* AEP (ref. 4) and *H. vulgaris* Ind-Pune (Stenoteles: 9.72 ± 1.37 μm × 7.73 ± 1.13 μm, holotrichous isorhizae: 10 ± 0.43 μm × 4.18 ± 0.28 μm, atrichous isorhizae: 8 ± 0.69 μm × 3.53 ± 0.29 μm and desmonemes: 4.28 ± 0.28 μm × 3.16 ± 0.28 μm) show same shapes as that of *H. vulgaris* Naukuchiatal with transverse coiling of nema in holotrichous isorhizae.

Budding rate and rate of doubling

Rate of budding and doubling time were compared among the four strains. The total number of detached buds from 24 polyps was counted collectively for one year and the average taken for comparison. It was observed that budding rate was highest in Naukuchiatal strain, followed by *H. magnipapillata*, *H. vulgaris* AEP and *H. vulgaris* Ind-Pune with the total number of detached buds per polyp in one year being 79, 68, 62 and 61 respectively (Figure 2 *a*). Comparative rates of budding in the four strains were further confirmed by comparing the doubling time among these strains. Naukuchiatal strain, as expected, exhibited the shortest doubling time of ~9–10 days while doubling time for *H. magnipapillata*, *H. vulgaris* AEP and *H. vulgaris* Ind-Pune strains was 10, 11 and ~11–12 days respectively (Figure 2 *b*). Confidence intervals were calculated to find the accuracy of the mean doubling time, which showed 95% confidence for each condition ranging from ±0.42 to ±1.75.

Isolation and phylogenetic analysis of mitochondrial genes

Mitochondrial genes, viz. 16S rRNA and COI were isolated from *H. vulgaris* Naukuchiatal and used as markers for phylogenetic analyses. COI gene sequence of *H. vulgaris* Naukuchiatal shows 99% similarity with *H. vulgaris* Ind-Pune and *H. magnipapillata*, while 97% with *H. vulgaris* AEP. Also 16S rRNA gene sequence of *H. vulgaris* Naukuchiatal shows 98% similarity with *H. vulgaris* Ind-Pune and *H. magnipapillata*, and 91% with *H. vulgaris* AEP. Sequences of both these genes are available for multiple strains of hydra as well as other species and were used for constructing NJ, MP

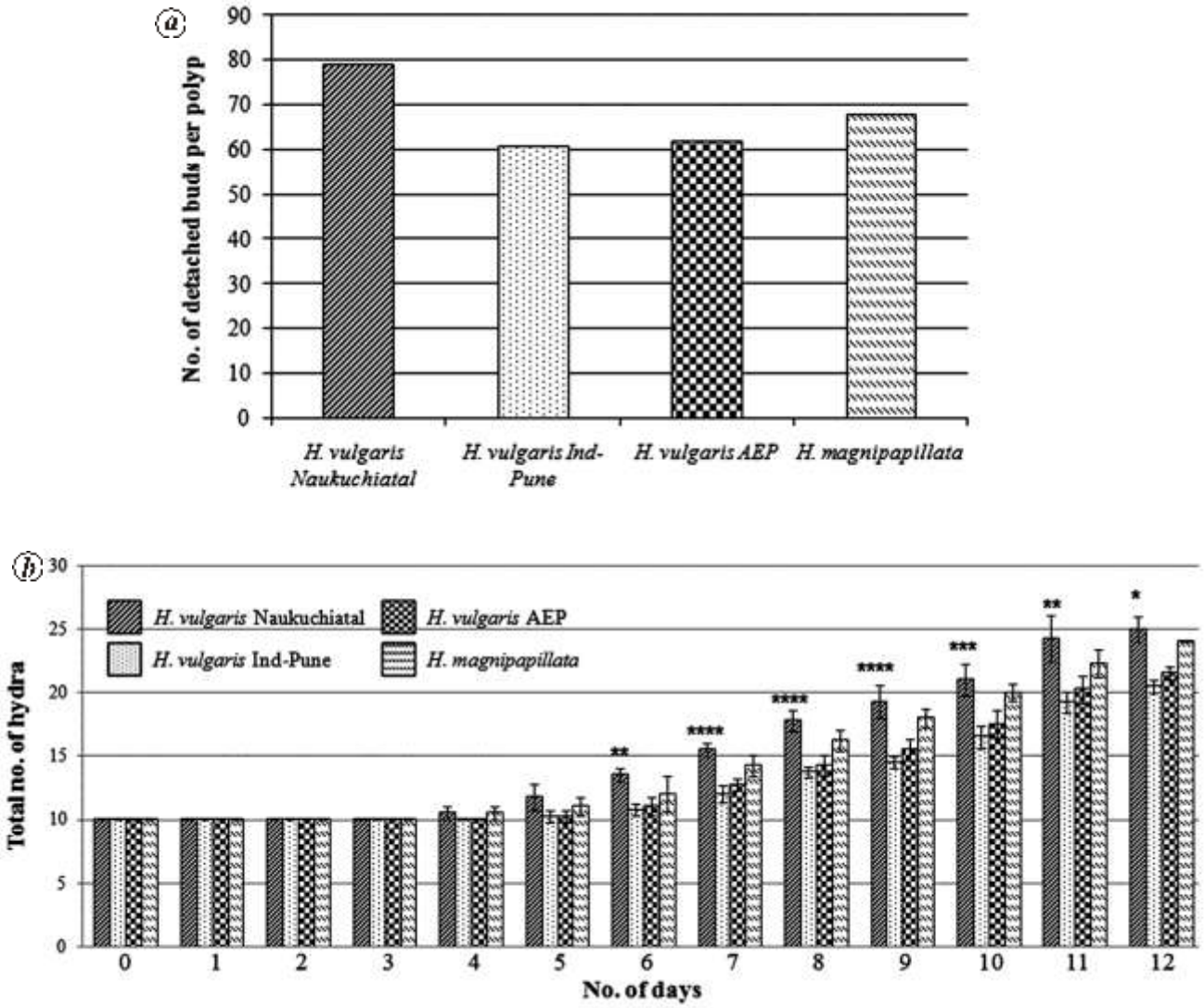

Figure 2. Comparison of rate of budding per polyp (***a***) and rate of doubling (***b***) in *H. vulgaris* Naukuchiatal with other strains. Comparison of average number of detached buds from 24 polyps in 4 different strains after one year (***a***). The average number of detached buds is more in *H. vulgaris* Naukuchiatal (79) as compared to *H. magnipapillata* (68), *H. vulgaris* AEP (62) and *H. vulgaris* Ind-Pune (61). As compared to *H. vulgaris* Ind-Pune, 23.16% higher rate of budding has been observed in *H. vulgaris* Naukuchiatal, while 21.89% and 14.01% more have been observed when compared to *H. vulgaris* AEP and H. magnipapillata respectively. (***b***) Histogram plotted by counting the number of days required for each strain to double. Total number of days required for doubling is 9–10 days for *H. vulgaris* Naukuchiatal and 10 for *H. magnipapillata*, 11 for *H. vulgaris* AEP and 11–12 days for *H. vulgaris* Ind-Pune.

and ML trees. Comparison of both genes in different hydra species showed that *H. vulgaris* Naukuchiatal strain groups with *H. vulgaris* Ind-Pune strain (Figure 3 *a* and *b*).

Presence of four hydra groups (*vulgaris*, *oligactis*, *braueri* and *viridissima*) has been confirmed by phylogenetic analyses using COI gene dataset which supports the previously reported data[5]. *Vulgaris* group in COI dataset showed divergence in the main lineage due to geographic distribution of many individuals[5]. Also, *H. polymorphus*, a Eurasian member from China, showed genetic differentiation from the main '*vulgaris*' lineage in both datasets (Figure 3)[20]. North American hydra members, *H. carnea* and *H. vulgaris* AEP (a laboratory generated strain) were grouped together in both datasets. *H. vulgaris* Naukuchiatal was grouped with *H. vulgaris* Ind-Pune and forms a separate clade either from Japanese *H. magnipapillata* or *H. vulgaris* Basel which is distributed across South Africa and Eurasia. This further confirms that the Naukuchiatal hydra belongs to the '*vulgaris* group' and forms a separate clade from *H. vulgaris* Basel or *H. magnipapillata*, which colonized in Asia due to intercontinental drift[5]. Analysis of 16S rRNA gene dataset also showed grouping of *H. vulgaris* Naukuchiatal and *H. vulgaris* Ind-Pune with *H. vulgaris* Basel and *H. magnipapillata*, but did not fully recover the four hydra groups as was

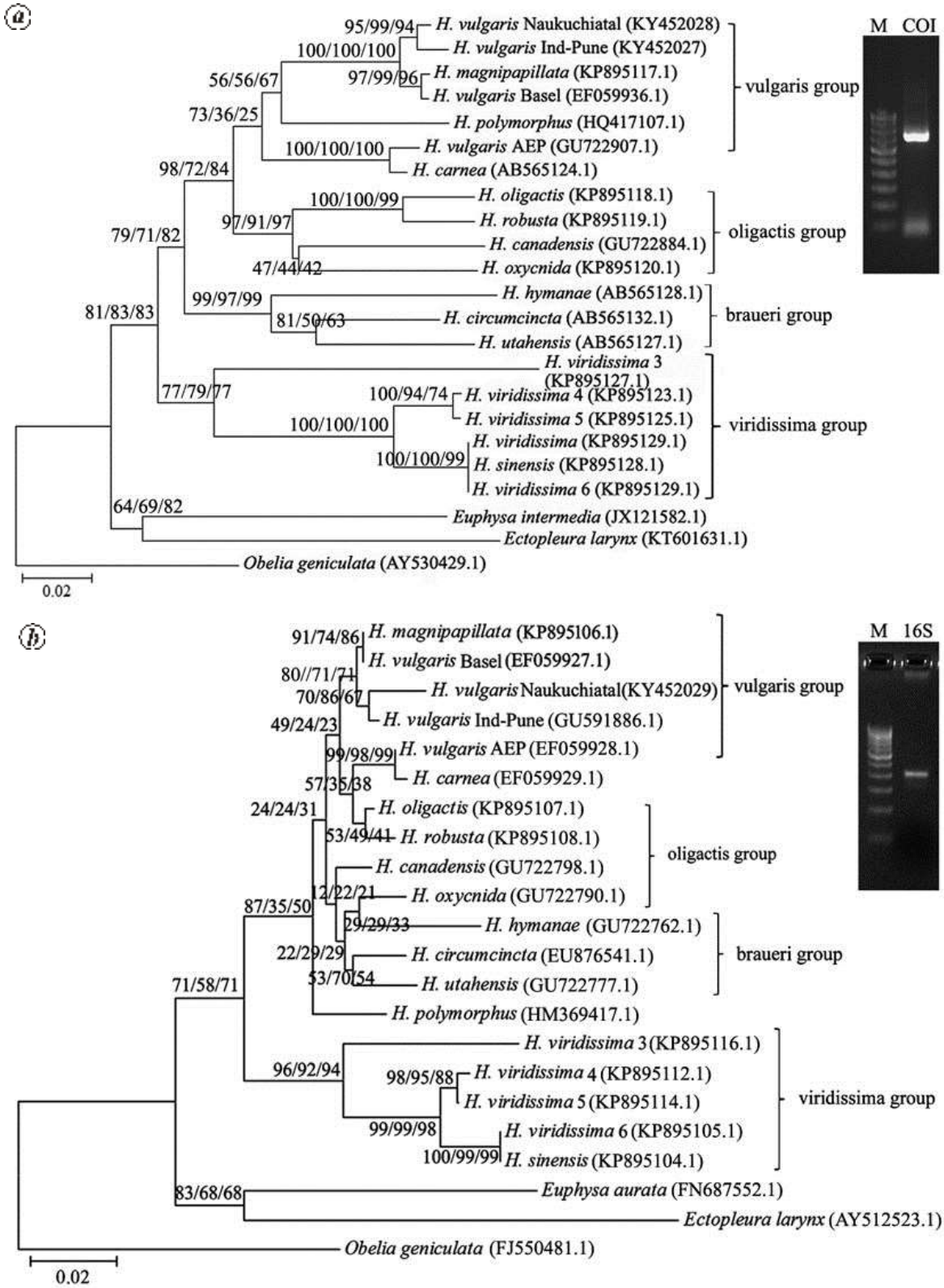

Figure 3. Isolation of mitochondrial genes and their phylogenetic analysis: PCR amplification of partial coding sequences of COI (~700 bp) (***a***) and 16S rRNA (~400 bp) (***b***) genes. M represents 100 bp DNA ladder. Phylogenetic trees based on COI (***a***) and 16S rRNA (***b***) gene sequences. Both trees were inferred by NJ method using MEGA-6. Bootstrap values at the branch points above/below each node are inferred from NJ, MP and ML methods respectively (left to right). Scale bar represents amino acid substitutions per site and are proportional to the branch lengths. Analysis for both genes shows close grouping of *H. vulgaris* Naukuchiatal with *H. vulgaris* Ind-Pune and fall into the *vulgaris* group.

also found previously[5]. Thus, both phylogenetic trees confirm that the Naukuchiatal strain belongs to the '*vulgaris*' group, but is a different strain particular to the type locality. Analysis of water quality at Naukuchiatal collected 11 months before our sample by Govenrment of Uttarakhand[21], shows that the water is non-polluted as judged from standard values made available by the Pennsylvania Lake Management Society (http://www.palakes.org)[22]. Hence it is possible that the morphological peculiarities of this strain are the result of pristine and relatively non-polluted environment with lower ambient temperatures.

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