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European Species of Subaerial Green Alga *Trentepohlia annulata* (*Trentepohliales, Ulvophyceae*) Caused Blood Rain in Kerala, India

Felix Bast[1*], Satej Bhushan[1], Aijaz Ahmad John[1], Jackson Achankunju[2], Nadaraja Panikkar MV[3], Christina Hametner[4] and Elfriede Stocker-Wörgötter[4]

[1]*Centre for Biosciences, Central University of Punjab, Bathinda, Punjab, 151001, India*

[2]*Department of Botany, SB College, Changanassery, Kerala, 686 101, India*

[3]*Department of Botany, SN College, Kollam, Kerala, 691001, India*

[4]*Ökologie und Diversität der Pflanzen, Universität Salzburg, Hellbrunnerstraße 34, 5020 Salzburg, Austria*

[*]**Corresponding author:** Felix Bast, Centre for Biosciences, Central University of Punjab, Bathinda, Punjab, 151001, India, Tel: +91 98721 52694; Fax: +91 164 2430586; E-mail: felix.bast@cup.ac.in

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Abstract

In 2011, isolated parts in south Indian state of Kerala as well as neighboring Sri Lanka experienced sporadic spell of red colored "blood rain", cause of which was later attributed to terrestrial subaerial microalgae of the genus *Trentepohlia*. Green algae of this genus is commonly found living in symbiosis as phycobionts of lichens in the free-living form in adjoining tropical rain forests, however, specific identity of which have never been determined. It is known that lichens disperse small algal-hyphal packages, so-called soredia, for vegetative reproduction, which can explain the content of *Trentepohlia*-spores in the water. Given its extraordinary spore-dispersal mechanism via rain, we were specifically interested to know species-level identity of a randomly collected *Trentepohlialean* algae of "blood rain" region, and to investigate the possibility that this might had been introduced from elsewhere through areal route. Results of our comparative morphology and molecular phylogenetic analyses using the nuclear ribosomal DNA Internal Transcribed Spacers region concluded that this alga is *Trentepohlia annulata* – a species of which no previous reports exist from India. These two geographical isolates were separated by Kimura-2-Parameter pair-wise distance of 0.06-which in turn indicate a low rate of evolution at these loci that are renowned for rapid molecular evolution, suggestive of a recent introduction. Phylogeny reconstruction using Bayesian Inference and Maximum Likelihood methods resulted in well-resolved phylograms with a robust clade composed of these isolates. This finding indicates the existence of areal dispersal of algal spores on continental and global scales through "clouds over oceans"- a phenomenon earlier reported for bacteria and fungi, but for the first time in algae.

Keywords: Green alga; Trentepohlia annulata; Trentepohlialean

Introduction

Sporadic spells of red colored rains have been reported from South Indian state of Kerala since 1896, with most recent episode in June 2012, which was later attributed to the aerial spores of locally abundant terrestrial green alga *Trentepohlia* [1]. Similar red rains were also reported from neighboring Sri Lanka for several times, with the latest episode in December 2012. Probably this is the first incident in modern times that an alga became center of national media coverage in India and Sri Lanka. It was only very recently that DNA molecules were discovered in the spores collected from colored raindrops [2], however, specific identity of this specimen have not yet been resolved. Although algae of the genus *Trentepohlia* have been first reported in India more than 3 decades ago [3] no reports exist that have attempted to identify the Indian isolates beyond the genus level. This is partly owing to the challenging task of differentiating simple morpho-forms of *Trentepohliales*, with a number of recent molecular systematic studies suggesting inherent problems associated with morphometry and its incongruence with phylogenetic systematics in this order [4-6].

Methods

With an objective for its specific identification and molecular assessment, we sought out for sampling a random natural population of *Trentepohlia* from Anandapally, Kerala, India (9.17769 N, 76.73473 E)-a place known for its sporadic episodes of red rain. No special permission was required for sampling at this location as the site is not part of any protected areas designated by government of India. Algae were found to be growing on the barks of rubber tree *Hevea brasiliensis* (Figure 1A). On external morphology, the algae were in reddish-green powdery form. Wet slides of the specimen were observed under an upright microscope (BX53, Olympus, Japan) and microphotographs were taken using a digital camera (EOS 60D, Canon, Japan) attached to the microscope. The algal filaments consisted of linear arranged spherical cells of around 12 µm - resembling streptococcal bacteria albeit the size is being much larger, and color being green (Figure 1B and 1D). Thalli were irregularly branched, and a few gametangial cells-that can be distinguished from the rest by its large cell size of 21-28 µm-were observed either from intercalary or apical regions. Developing gametes were clearly discernible inside the globular gametangia, although ostiole was not observed.

Unabridged protocols used for DNA extraction, amplification and sequencing used in the present study are available at Lab Archives Protocols (http://dx.doi.org/10.6070/H4R20Z9). In summary, total genomic DNA was extracted from dried specimens using HiPurATM Algal Genomic Extraction Kit (HiMedia Laboratories, India). A region consisting of ITS1-5.8S-ITS2 was amplified from the extracted rDNA using ITS1-forward primer and ITS4-reverse primer as per [7] and

subsequently subjected to bidirectional Sanger sequencing (Applied Biosystems 3730xl Genetic Analyzer, Foster City, CA, USA). Sequences were then assembled and deposited in GenBank under the accession KF318886.

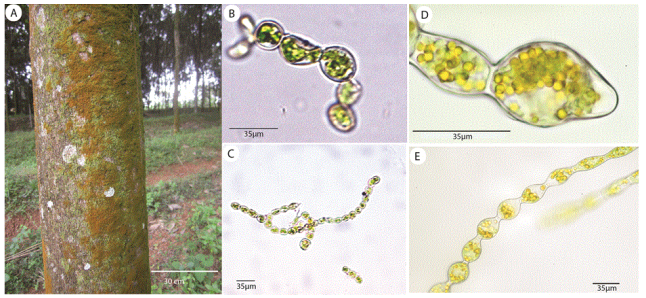

Figure 1: Morphology of *Trentepohlia annulata* isolates. A-C: Isolate from India. D-E: Isolate from former Czechoslovakia. A: Algae growing on the rubber tree bark. B and D: intercalary and apical gametangia. C and E: Axial growth of globular/barrel shaped cells.

Phylogenetic analyses using Maximum Likelihood (ML) and Bayesian Inference (BI) methods were subsequently conducted. Step-by-step protocols for sequence analysis used in the present study are as per [8]. All of our alignments, trees and matrices are accessible from TREEBase under Accession # TB2:S14455 (available at http://purl.org/phylo/treebase/phylows/study/TB2:S14455?x-access-code=2a6d1cbcada6c70ac556cd071bd91e54&format=html).

Results and Discussion

BLASTn sequence similarity search indicated that the accession most related to our alga is *Trentepohlia annulata* (JQ687378 E-value =1.62e-138). In the Kimura 2 Parameter (K2P) distance matrix, this accession and our specimen had a distance of 5.75 × 10-2 that is within the conspecific limit considered in *Trentepohliales* [5,6]. In BI as well as ML phylograms, our isolate formed a strong-supported clade along with *T. annulata* (Clade 1 in Figure 2). This clade was monophyletic and distinct from other currently recognized and available species accessions of this genus and other *Trentepohlialean* genera, affirming evolutionary affinity of these two analyzed algal isolates to the species level.

To further affirm conspecificity of the two isolates of *T. annulata*, we compared its morphological features. A European isolate was obtained from the culture collection of algae at Goettingen University, Germany (international acronym: SAG) under the accession SAG 20.94, that had earlier been collected from Damasská cesta pobl. Svratky, Ceskomoravská vrchovina, former Czechoslovakia (49.707608 N, 16.021385 E) and maintained as unialgal culture in Bold's basal medium [9] with soil extract. Photographs of 547 days old European isolates were taken using EOS 30D Digital camera (Canon, Japan) attached to a Polyvar light microscope (Reichert-Jung, Germany). Some algal filaments consisted of long elongated, cylindrical cells (41-45 µm × 6-11 µm in size); others had barrel-shaped or globular cells (32-38 × 12-16 µm or 35 × 26 µm in size). Sizes of vegetative, as well as gametangial, cells were comparable with our two isolates. The gametangia of the European culture possessed a globular shape (25-34 µm in diameter) with an ostiole located at the top of a well pronounced neck, while that of Indian isolates were more or less spherical in shape. Differences in morphology could be attributed to the effects of long sub-culturing of European isolate in artificial media.

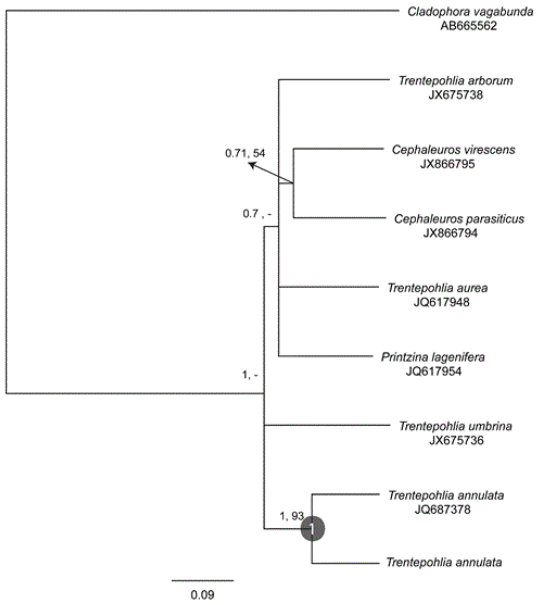

Figure 2: Phylogenetic position of *Trentepohlia annulata* isolates from India among other accessions in ITS- dataset using Bayesian Inference phylogenetic reconstruction (*LnL*=-3800.625) with Kimura 2 Parameter model of molecular evolution with gamma distribution (K2P+G). Numbers near nodes represent Bayesian Posterior Probabilities, followed by Maximum Likelihood bootstrap proportions with 1000 replicates. Dash (-) indicates absence of bootstrap support. This phylogram is rooted with *Cladophora vagabunda* as outgroup. Scale bar given on bottom is in the units of average nucleotide substitutions per site.

Our morphological observations further corroborated molecular phylogenetic identification of Indian isolate as *Trentepohlia annulata*, of which no previous reports exist from India. Low K2P distance of 5.75 × 10-2 between these two geographical isolates is most probably suggestive of the low rate of evolution despite substantial geographic distance of more than 7000 km. As this locus is considered to be rapidly evolving, a recent introduction is suspected. Given the reported areal route of *Trentepohlialean* spore dispersal through rain and our serendipitous discovery of a species also distributed in Europe in the locality where episodes of "Blood Rain" were common, an aerial dispersal of a European species to the Indian subcontinent via "clouds over oceans" seems to be emerging as the most plausible explanation. Moderately long-distance (~1000 km) spore dispersal via aerial route have earlier been documented for basidiomycetes [10] and plant pathogens [11] but to best of our knowledge this is the first report for spore dispersal at intercontinental or global scale for any known biological systems, and definitely for the algae.

With this discovery, an avenue for the characterization of long-distance areal spore dispersal has been opened up. It might be of extreme value to search for pathogens of infectious diseases dispersed in this manner. In our perception rain-drops can serve as a good, cost-efficient proxy for the future barcoding attempts targeted towards

areal spores, especially when attempted using metagenomic approaches. On a related note, this is the first report of the molecular assessment of terrestrial algae from India, thereby highlighting dire necessity to catalogue the biodiversity of this important plant lineage.

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