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Equisetum ramosissimum Desf. Subsp. *debile* (Roxb. ex Vaucher) Hauke – is really extinct from Gujarat, India?

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Abstract
A recent study for pteridophytic flora in the westernmost state, Gujarat of India reports the occurrence of extinct *Equisetum ramosissimum* Desf. subsp. *debile* (Roxb. Ex Vauch.) Hauke from the wild with its molecular confirmation and phylogenetic position within other species of *Equisetum* across the world. Phylogenetic analysis confirms that the *E. ramosissimum debile* is a sister taxa of the clade containing *E. hyemale affine* and *E. x ferrissii* (*E. hyemale* female and *E. laevigatum* male). Phylogenetic position of other *Equisetum* species obtained in the present study was also supported by the previous studies.

Keywords: biodiversity, extinct, Gujarat, molecular phylogeny

1. Introduction
Equisetaceae is a well-defined group of extant and extinct plants which dates back to the Devonian period and is unique in Pteridophytes [1]. Their phylogenetics position is controversial as they have been considered as basal to the euphyllophyte lineage [2]; sisters to the ferns [3]; sisters to the angiosperms [4] and more in recent times, in the lineage of eusporangiate ferns [5]. *Equisetum*, commonly known as horsetails and scouring rushes, is the only living genus of the class Equisetopsida. *Equisetum* comprises more than 15 species worldwide except Australasia and Antarctica [6] of which 4 species are reported from India till date [7]. The westernmost state, Gujarat accounts only one species [8-10].
In earlier studies of pteridophytes of Gujarat, *Equisetum ramosissimum* Desf. subsp. *debile* (Roxb. ex Vaucher) Hauke was only reported from the Savli taluka, Vadodara district of the state by Chavan and Padate [8], Padate (1969) [9] and in report of Gujarat Ecological Commission (1996) [10]. In recent years, Patel *et al.*, (2015) [11] and Rajput *et al.*, (2016) [12] were not able to relocate the species from the same and other regions of the state Gujarat. Therefore, *E. ramosissimum debile* was declared as regionally extinct in wild from the state of Gujarat [11, 12]. The present study aims to relocate *E. ramosissimum debile* from Gujarat region with its molecular confirmation and phylogenetic position within other species of *Equisetum* present around the world.

2. Materials and methods
During a botanical expedition in July to September-2017, *E. ramosissimum* subsp. *debile* was collected from the Mayadevi temple at Bhenskatri, Waghai, Gujarat, India (20º55'47.49" N 73º31'59.15" E, elevation-125m). Photographs were taken in its natural habitat and few samples were collected for morphological, molecular analysis and for herbarium preparations. Taxonomic identification of collected taxa was carried out using available literatures [7, 13]. Herbarium sheets were deposited in herbarium of Bapalal Vaidya Botanical Research Centre (BVBRC), Department of Biosciences, Veer Narmad South Gujarat University, Surat, Gujarat.

2.1 Molecular analysis
Genomic DNA was extracted from sterilized plant material following Doyle and Doyle (1987) [14]. *RbcL* choloroplast DNA (cpDNA) region was amplified using 20 µL reaction mixture contained isolated genomic DNA template (1 µL), forward primer (1 µL), reverse primer (1 µL), 1× final concentration of ReadyMix™ Taq PCR reaction mixture (Sigma) (10 µL) and nuclease free water (7 µL). The reaction was carried out in thermal cycler (Applied Biosystems Veriti®). PCR program was adjusted as: 94 °C for 4 minutes, 30 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 1.30 minutes, and a final elongation step at 72 °C for 10 minutes and stored at −4 °C for ∞ time. PCR product was purified using GenElute™ PCR clean-up kit and sequenced at Eurrofins Genomics India Pvt Ltd., Bangalore. Sequences of all *Equisetum* species (17 sequences of *rbcL* gene) were downloaded from GeneBank in FASTA format. Downloaded sequences were supplemented with the newly generated sequences of *E. ramosissimum* Desf. subsp. *debile*. *Psilotum nudum* was used as outgroup taxa. Sequences were aligned with clustalw [15] embedded in MEGA7.0 [16]. Aligned data was analyzed with PartitionFinder [17] for optimal partitioning strategy and evolutionary substitution model. Maximum Likelihood (ML) analyses were employed to infer phylogenetic relationships in RaxML [18]. 1000 bootstrap replicates were run under GTR + I model to assess clade support.

3. Results
3.1 *Equisetum ramosissimum* Desf. Subsp. *debile* (Roxb. *ex* Vauch.) Hauke Amer. Fern J. 52: 33. 1962. (Figure 1A-E).
Equisetum debile Roxburgh ex Vaucher, Mém. Soc. Phys. Genève 1:387. 1822; *Hippochaete debilis* (Roxburgh ex Vaucher) Holub; *H. ramosissima* subsp. *debilis* (Roxburgh ex

Vaucher) Á. Löve & D. Löve. Terrestrial, growing on wet ground along streams in open areas at low altitude and scrambles on grass, bushes and shrubs. Plants medium to large in size; rhizome blackish brown, erect or creeping, deep-seated, nodes and roots with numerous long brown coloured trichomes. Fronds monomorphic; sterile and fertile branches alike, green, 200 × 1-1.5 cm, lax, simple or irregularly branched with few branches; branches 1-3 in a whorl, long, slender; ribs 8-20, less prominent. Internodes 3-8 cm long; leaf sheaths tight, 0.5 cm long, lower portion green, upper portion slightly blackish brown including 10-22 lanceolate-acuminate dark brown margined deciduous teeth. Cones solitary, terminal on the stems or their branches, oblong, apiculate, shortly stalked or sessile, 0.5-2 cm long. Sporangiophores orbicular or oblong bearing several sporangia. Sporangia oblong, yellowish.

3.1.1 Habitat
Growing near river banks and torrential waters.

3.1.2 Fertile
September-December

3.1.3 Distribution
World
Bangladesh, Indonesia, Japan, Laos, Malaysia, Myanmar, Nepal, New Guinea, Philippines, Singapore, Thailand, Vietnam and India.

India
Rajasthan, Haryana, Uttar Pradesh, Jarkhand, Madhya Pradesh, Tamil Nadu, Arunachal Pradesh, Assam, Meghalaya, Sikkim, West Bengal and Gujarat.

3.2 Phylogenetic analyses
Taxonomically, the genus *Equisetum* is divided in two subgenera *Equisetum* & *Hippochaete* on the basis of stomatal position as superficial in subgenus *Equisetum* (*E. arvense*, *E. bogotense*, *E. diffusum*, *E. fluviatile*, *E. palustre*, *E. pratense*, *E. sylvaticum*, and *E. telmateia*) and sunken below the epidermal surface in subgenus *Hippochaete* (*E. giganteum*, *E. hyemale* subsp. *affine*, *E. laevigatum*, *E. myriochaetum*, *E. ramosissimum* subsp. *debile*, *E. scirpoides*, *E. variegatum*, *E.*× *ferrissii* (*E. hyemale* (female and *laevigatum* male), *E.*× *ferrissii* (*E. laevigatum* (female and *hyemale* male). Our ML phylogenetics analysis of *rbcl* gene was also support the taxonomic classification and in which two monophyletic clades belongs to subg. *Hippochaete* and subg. *Equisetum* (minus *E. bobotense*). Moreover, *E. ramosissimum debile* showed 100 % match with the samples collected from Taiwan by Des Marais *et al.*, (2003) [19] and found as a sister to the clade containing *Equisetum hyemale affine* and *Equisetum x ferrissii* (*E. hyemale* female and *laevigatum* male) (Figure 2).

4. Discussion
Till date, except from Savli taluka, no other localities were recorded from Gujarat in wild [8-10]. According to Patel *et al.* (2015) [11] natural habitat of *Equisetum ramosissimum* subsp. *debile* from Savli was lost due to human activities like deepening of lakes and increasing of residential boundaries. So far considered as extinct in wild, we relocated it from a new habitat at Mayadevi temple in South Gujarat. Moreover, a new locality is a religious and picnic place which also calls for an imperative need for conservation from extinction from its new locality and further surveys are needed to document occurrence of this species from south Gujarat and in other parts of the state as well.

Description of a new species as world's smallest terrestrial pteridophyte with two new record of genus *Ophioglossum*, a new record of fern belongs to genus *Hypodematium*, a new record of fern belongs to genus *Cheilanthes* [20-22] and the present communication on relocation of an extinct species of *Equisetum* in merely a year, highlights the poor nature of pteridophyte documentation in the state Gujarat of India warranting dedicated pteridophytic surveys across the state.

Figures

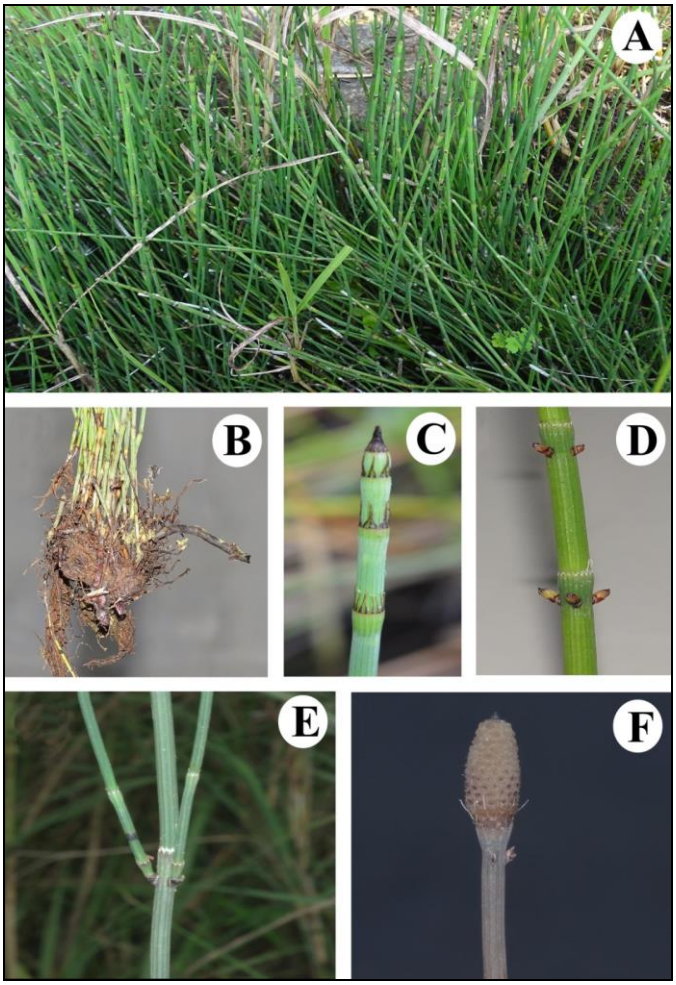

Fig 1: *Equisetum ramosissimum* Desf. Subsp. *debile* (Roxb. ex Vaucher) Hauke A - Plants in wild. B - Rhizome. C - Sheath. D - Aerial stem showing ridges and furrows. E - Branching pattern. F - Cone.

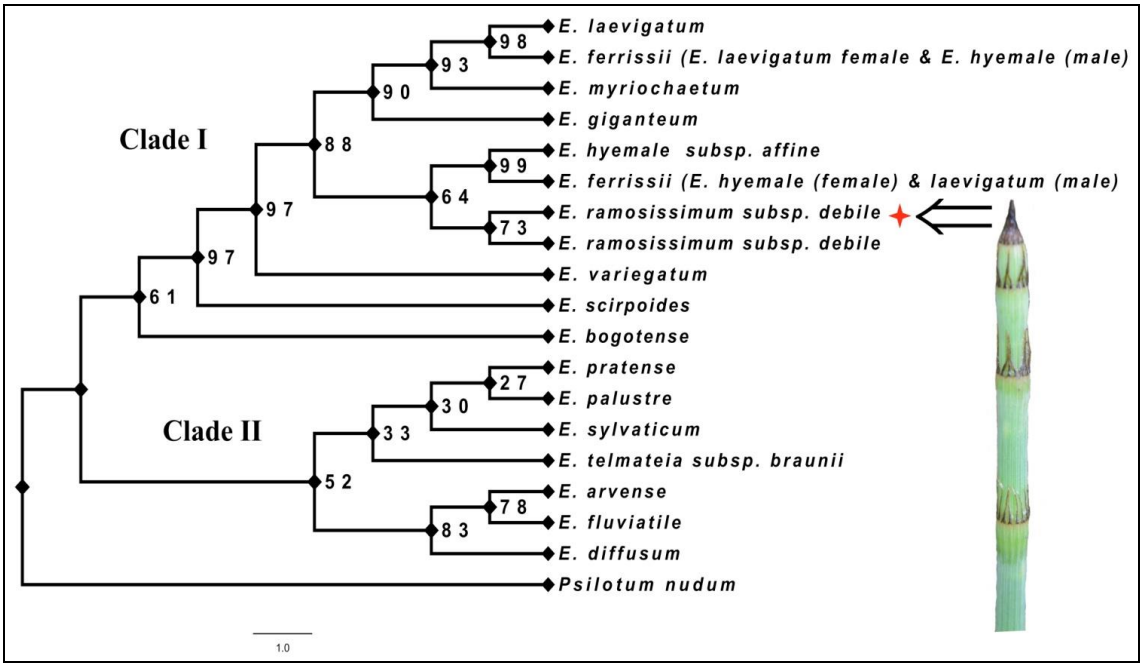

Fig 2: Maximum-likelihood phylogeny of *Equisetum* based on choloroplast ribulose bisphosphate carboxylase/oxygenase gene (*rbcL*). Nodes are supported by ML bootstrap percentages (BP).

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6. Data availability

Rbcl generated sequence have been submitted in GenBank with accession number MH730060.

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