

Molecular phylogeny of *Einfeldia* (Diptera: Chironomidae) inferred from sequencing of mitochondrial cytochrome oxidase subunit 1 (COX1) gene

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Abstract

By using universal primer 911 and 912, sequencing of mitochondrial cytochrome oxidase subunit 1 (COX1) gene of *Einfeldia* was carried out and got contig nucleotide sequence of 708 base pairs. After getting contig sequence, this sequencing result was run on open access NCBI BLAST software and got hits, taxonomy report, lineage report and phylogeny tree. Data shows the close relation between *Einfeldia* and *Polypedilum*.

Keywords: Chironomidae, Udaipur, *Einfeldia*, NCBI, BLAST

1. Introduction

The family Chironomidae, commonly known as non-biting midges, is one of the most abundant, diverse, and cosmopolitan groups of aquatic insects (Ferrington 2008) [3]. They occur on all continents including Antarctica and have adapted too many aquatic and semi-aquatic habitats. Chironomids are holometabolous aquatic insects, and have four distinct life stages: egg, larva, pupa and adult. The larval stage has four instars, and may last from less than two weeks to several years depending on species and environmental conditions. Most of the species are restricted to freshwater habitats, and the larvae of some have adapted to anoxic conditions. Most Chironomid adults do not feed, living usually only for a few days. Chironomids are in the dipterans sub-order Nematocera, and so are related to other well-known fly groups such as the Ceratopogonidae (biting midges), Simuliidae (blackflies), Culicidae (mosquitoes) and Chaoboridae (phantom midges). The subfamilies Tanypodinae, Orthocladiinae and Chironominae are most common. Of these, Chironominae are often encountered in lentic and warm-water systems. Other subfamilies, such as Telmatogetoninae and Podonominae are relatively restricted in habitats (e.g. warm seashores; lentic littoral), and Diamesinae and Prodiamesinae are relatively uncommon (Armitage *et al.*, 1995) [1].

2. Materials and methods

Larval *Einfeldia* were collected from Udaisagar Lake, Udaipur. Then collected larvae were carried to the laboratory. Collected larvae were examined under a compound microscope for identification up to genera using keys (Epler, 2001) [2]. At last, we did the sequencing of mitochondrial cytochrome oxidase subunit 1 (COX1) gene to get its molecular phylogeny. For this larval sample was send to Xcelris Lab Ahmedabad. The final contig sequence received was analyzed using open access NCBI BLAST software. Methods adopted by them are described below.

2.1 DNA Isolation

DNA was isolated from Larvae sample using XcelGen Plant DNA mini kit (XG2611-01). Extracted DNA was eluted in 20µl of nuclease-free water.

2.2 NanoDrop-8000: Quantitative and Qualitative analysis of total gDNA

S. No.	Sample ID	OD 260/280	Concentration (ng)
1	Larvae	2.07	159.9 ng/µl

2.3 Quality Check of DNA on Agarose

The isolated DNA, products were analyzed on 0.8% of Agarose gel. The methodology is given below:

Protocol of Agarose Gel Electrophoresis: p

- 0.8% Agarose was prepared in 100 ml 1xTAE buffer and was dissolved by heating.
- It was then, cooled down and Ethidium Bromide (10µg/ml) was added.
- Comb and the casting tray was set, into that, the molten agarose containing ethidium bromide was pour and was allowed to solidify.
- The DNA (100 ng) was loaded with 6X loading dye, along with *Hind* III Ladder DNA ladder.
- The resolved DNA was documented through gel Documentation system.

2.4 Polymerase Chain Reaction amplification through Cytochrome Oxidase Specific Primer (911-912)

Isolated DNA of larvae was subjected to amplification through Cytochrome Oxidase Specific Primer (911-912), in 25µl reaction volume, using thermocycler (Applied Biosystems, Veriti®). Composition of reaction mixture for PCR is given in Table 1. The PCR was carried out according to the cycle condition mentioned in Table 2.

Table 1: Composition of reaction mixture for PCR

Components	Quantity
Nuclease free water	To make-up volume for 25 µl
DNA	50ng
Primer (10pmole)	1.0µl
2X PCR Master Mix	12.5µl
Total Volume	25µl

Table 2: Thermal Cycler reaction condition.

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	4 Minutes	1
Denaturation	94°C	40 Seconds	
Annealing	47°C	45 Seconds	35
Extension	72°C	45 Seconds	
Final Extension	72°C	20 Minutes	1

2.5 Quality Check amplified PCR product on Agarose

To check the quality of the PCR amplicons, PCR amplified products were analyzed on 1.2% Agarose gel.

2.6 Sequencing PCR products

The PCR amplicon was purified enzymatically using Exo-SAP, as per the manufacturer instructions (Applied Biosystem). After the purification the products were subjected to Sanger sequencing using ABI, 3730XL DNA analyzer using BdT v3.1 chemistry. Forward and Reverse DNA sequencing reaction of PCR amplicons (Sample) of PCR product was carried out with 911-912 primers, separately.

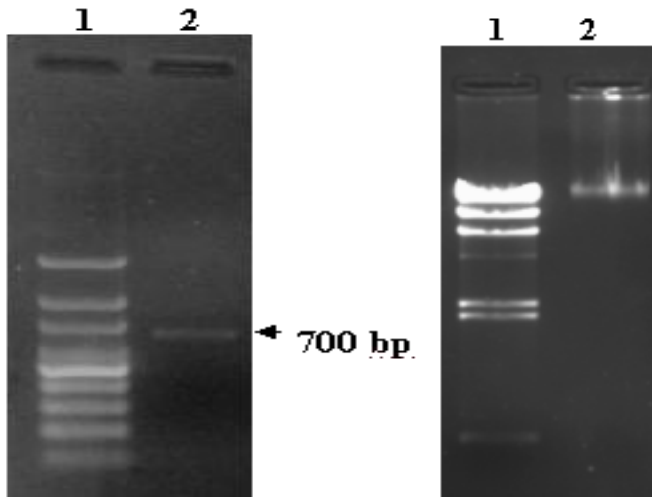


Fig 1: Gel photograph 1: (Lane 1. *Hind* III DNA Ladder; Lane 2. DNA of Larvae); Gel photograph 2: (Lane 1. 100 bp DNA Ladder; Lane 2. Amplicon generated with 911-912 prime).

3. Results and Discussion

Cytological practices on Chironomidae are not new but sequencing is rare for Chironomids. Sequencing of Chironomids gave significant tool for assessment of phylogeny and taxonomy. Montagna *et al* (2016) [5] investigated the integrated taxonomy and DNA barcoding of Alpine Chironomidae. Their study focused principally on larvae of the genus *Diamesa* Meigen. A fragment of the mitochondrial CO1 gene was obtained from 112 larvae, pupae and adults (*Diamesinae*, *Orthoclaudiinae* and *Tanypodinae*) that were collected in different mountain regions of the Alps and Apennines. On the basis of morphological characters 102 specimens were attributed to 16 species and the remaining 10 species were identified to the genus level. Molecular species delimitation was performed using various models. On basis of their study we can conclude that molecular identification represents a promising tool that could be adopted in evaluating biodiversity. Previous study of Gusev *et al* (2014) [4] derived the phylogeny of Chironomids by sequencing mitochondrial cytochrome oxidase subunit 1 gene. Larval *Einfeldia* were send

to Xcelris Genomics, Ahmedabad to get nucleotide sequence of mitochondrial cytochrome oxidase subunit 1 (COX1) gene. By using universal primer 911 and 912, sequencing of mitochondrial cytochrome oxidase subunit 1 (COX1) gene was carried out and got following nucleotide sequence.

Sequence got using primer 911-

TAATAACAATCGATCATACAAACAATGGTATTTCGAT
CTAGAGTGATTCTCTCGATCGTATATTAATTACTGT
TGTAATAAAATTTACTGATCCTAAAATAGAAGAAAC
ACCTGCGAGATGTAGAGAGAAAATGGCAAGATCTA
CTGAAGCTCCTCTATGAGCAATTCTTGCTGATAAAG
GGGGTAAACTGTTTCATCCGGTTCCTGCTCCATTTTC
TACAATAGAAGACTGAAAGAAGAAGAGATGAAGAAAG
GAGGTAAGAGTCAAAGCTTATGTTATTTATTTCGAG
GAAAGGCTATATCTGGGGCTCCTAGTATTAAGGGA
CAAGTCAGTTTCCAAATCCCCCAATTAATAATAGGTA
TAACTATAAAAAAATTATAATAAATGCGTGAGCTG
TAACGATAACATTGTAAATTTGGTCATCTCCAATTA
AGTACCTGGATGCCCTAATTCAGCTCGAATTAATAT
ACTAAGGGAAGTACCTACTATTCCAGATCAAGCTCC
GAAAATAAAATAAAGGGTTCCAATATCTTTATGTTT
GGGT

Sequence got using primer 912-

GTTATCGTTACAGCTCACGCATTTATTATAATTTTTT
TTATAGTTATACCTATTTTAATTGGGGGATTTGAAA
CTGACTTGTCCTTTAATACTAGGAGCCCAGATATA
GCCTTTCCTCGAATAAATAACATAAGCTTTTGACTTT
TACCTCCTTCTTACTCTTCTTCTTTCAAGTTCATT
GTAGAAAATGGAGCAGGAACCGGATGAACAGTTTA
CCCCCTTTATCAGCAAGAATTGCTCATAGAGGAGC
TTCAGTAGATCTTGCCATTTTCTCTTACATCTCGCA
GGTGTTCCTTCTATTTTAGGATCAGTAAATTTTATTA
CAACAGTAATTAATATACGATCGAGAGGAATCACTC
TAGATCGAATACCATTGTTTGTATGATCGATTGTTAT
TACTACAGTTCTACTTCTCCTTTCATTACCAGTATTA
GCTGGAGCTATACAATACTACTAAGTACCAGGAAAT
TTAAATACTTCTTTTTCGACCCAGCTGGAGGAGGTG
ATCCTATCTTTACCAACATTTATTTTGATTTTTTGGT
CACCTGGAAAATTTAAA

Contig. Sequence-

TTAAATTTTCCAGGTGACCAAAAAATCAAATAAA
TGTTGGTAAAGAATAGGATCACCTCCTCAGCTGGG
TCGAAAAGGAAGTATTTAAATTTCCGGTCAGTTAGT
AGTATTGTGATAGCTCCAGCTAATACTGGTAATGAA
AGGAGAAGTAGAACTGTAGTAATAACAATCGATCAT
ACAAACAATGGTATTCGATCTAGAGTGATTCTCTC
GATCGTATATTAATTACTGTTGTAATAAAATTTACTG
ATCCTAAAATAGAAGAAACACCTGCGAGATGTAGA
GAGAAAATGGCAAGATCTACTGAAGCTCCTCTATGA
GCAATTCCTTGCTGATAAAGGGGGTAAACTGTTTCAT
CCGGTTCCTGCTCCATTTTCTACAATAGAAGTGGAAA
GAAGAAGAGTAAGAGAAGGAGGTAAAAGTCAAAG
CTTATGTTATTTATTCGAGGAAAGGCTATATCTGGGG
CTCCTAGTATTAAGGGACAAGTCAAGTTTCCAAATC
CCCCAATTAATAATAGGTATACTATAAAAAAATTA
TAATAAATGCGTGAGCTGTAACGATAACATTGTAAA
TTTGGTCATCTCCAATTAAGTACCTGGATGCCCTAA
TTCAGCTCGAATTAATATACTAAGGGAAGTACCTAC
TATTCCAGATCAAGCTCCGAAAATAAAATAAAGGGT

TCCAATATCTTTATGTTTGGGT

Contig Sequence size- 708bp

After getting contig sequence, this sequencing was run on open

access NCBI BLAST software and got hits, taxonomy report, lineage report and phylogeny tree (Figure 2, 3, 4 and 5). Data shows the close relation between *Einfeldia* and *Polypedilum*.

Sequences producing significant alignments:

Select: [All](#) [None](#) Selected:0

[Alignments](#) [Download](#) [GenBank](#) [Graphics](#) [Distance tree of results](#)

Description	Max score	Total score	Query cover	E value	Ident	Accession
<input type="checkbox"/> Polypedilum vanderplanki mitochondrion, complete genome	702	702	97%	0.0	85%	KT251040.1
<input type="checkbox"/> Phortica nudiarista isolate 1931 cytochrome oxidase subunit I gene, partial cds, mitochondrial	706	706	97%	0.0	85%	KU243347.1
<input type="checkbox"/> Phortica foliiseta isolate 1915 cytochrome oxidase subunit I gene, partial cds, mitochondrial	706	706	97%	0.0	85%	KU243341.1
<input type="checkbox"/> Chironominae sp. BOLD:ACG9457 voucher MDFRC_DI0062 cytochrome oxidase subunit 1 (COI) gene, partial cds, mitochondrial	701	701	97%	0.0	85%	KP697476.1
<input type="checkbox"/> Phortica nudiarista isolate 1919 cytochrome oxidase subunit I gene, partial cds, mitochondrial	695	695	97%	0.0	85%	KU243346.1
<input type="checkbox"/> Phortica speculum voucher DIP124111 cytochrome oxidase subunit 1 (COI) gene, partial cds, mitochondrial	695	695	97%	0.0	85%	KJ083036.1
<input type="checkbox"/> Phortica speculum voucher DIP124110 cytochrome oxidase subunit 1 (COI) gene, partial cds, mitochondrial	695	695	97%	0.0	85%	KJ083035.1
<input type="checkbox"/> Chironomidae sp. GES-2010 isolate C5 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	736	736	97%	0.0	86%	GU565707.1
<input type="checkbox"/> Chironomidae sp. GES-2010 isolate C4 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	732	732	97%	0.0	86%	GU565708.1
<input type="checkbox"/> Phortica nudiarista isolate 1953 cytochrome oxidase subunit I gene, partial cds, mitochondrial	699	699	97%	0.0	85%	KU243348.1
<input type="checkbox"/> Phortica pavriarista voucher DIP124098 cytochrome oxidase subunit 1 (COI) gene, partial cds, mitochondrial	713	713	96%	0.0	85%	KJ083023.1
<input type="checkbox"/> Chironomidae sp. GES-2010 isolate D5 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	713	713	96%	0.0	85%	GU565715.1
<input type="checkbox"/> Chironomidae sp. GES-2010 isolate B9 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	713	713	96%	0.0	85%	GU565712.1
<input type="checkbox"/> Phortica nudiarista cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	708	708	96%	0.0	85%	HQ011972.1
<input type="checkbox"/> Phortica subradiata voucher DIP123287 cytochrome oxidase subunit 1 (COI) gene, partial cds, mitochondrial	699	699	96%	0.0	85%	KJ130864.1

Fig 2: NCBI hits obtained.

Taxonomy Report

Taxonomy	Number of hits	Number of Organisms	Description
<input type="checkbox"/> Diptera	145	29	
<input type="checkbox"/> Chironomidae	134	23	
<input type="checkbox"/> Chironominae	114	15	
<input type="checkbox"/> Chironomini	93	11	
<input type="checkbox"/> Polypedilum	92	10	
<input type="checkbox"/> Polypedilum sp. BOLD:ACC5662	1	1	Polypedilum sp. BOLD:ACC5662 hits
<input type="checkbox"/> Polypedilum nubifer	74	1	Polypedilum nubifer hits
<input type="checkbox"/> Polypedilum scalaenum	3	1	Polypedilum scalaenum hits
<input type="checkbox"/> Polypedilum unifascium	2	1	Polypedilum unifascium hits
<input type="checkbox"/> Polypedilum sp. TE02	2	1	Polypedilum sp. TE02 hits
<input type="checkbox"/> Polypedilum sp. BOLD:AAO2005	1	1	Polypedilum sp. BOLD:AAO2005 hits
<input type="checkbox"/> Polypedilum convictum	3	1	Polypedilum convictum hits
<input type="checkbox"/> Polypedilum vanderplanki	1	1	Polypedilum vanderplanki hits
<input type="checkbox"/> Polypedilum sp. BOLD:AAO2164	4	1	Polypedilum sp. BOLD:AAO2164 hits
<input type="checkbox"/> Polypedilum sp. BOLD:AAN8908	1	1	Polypedilum sp. BOLD:AAN8908 hits
<input type="checkbox"/> Phaenopsectra sp. BOLD:AAN8990	1	1	Phaenopsectra sp. BOLD:AAN8990 hits

. . . Riethia stictoptera	10	1	Riethia stictoptera hits
. . . Tanytarsus	10	2	
. . . . Tanytarsus inextentus	6	1	Tanytarsus inextentus hits
. . . . Tanytarsus bispinosus	4	1	Tanytarsus bispinosus hits
. . . Chironominae sp. BOLD:ACG9457	1	1	Chironominae sp. BOLD:ACG9457 hits
. . Unclassified Chironomidae	20	8	
. . . Chironomidae sp. GES-2010	4	1	Chironomidae sp. GES-2010 hits
. . . Chironomidae sp. BOLD:ACC8266	6	1	Chironomidae sp. BOLD:ACC8266 hits
. . . Chironomidae sp. BOLD:ACG2764	5	1	Chironomidae sp. BOLD:ACG2764 hits
. . . Chironomidae sp. BE-2012	1	1	Chironomidae sp. BE-2012 hits
. . . Chironomidae sp. BOLD:ACL4023	1	1	Chironomidae sp. BOLD:ACL4023 hits
. . . Chironomidae sp. BOLD:ABA0772	1	1	Chironomidae sp. BOLD:ABA0772 hits
. . . Chironomidae sp. BOLD:ACB0738	1	1	Chironomidae sp. BOLD:ACB0738 hits
. . . Chironomidae sp. BOLD:ACB0996	1	1	Chironomidae sp. BOLD:ACB0996 hits
. Diptera sp. KMGHap_100	1	1	Diptera sp. KMGHap_100 hits
. Phortica	10	5	
. . Phortica	9	4	
. . . Phortica pavriarista	1	1	Phortica pavriarista hits
. . . Phortica nudiarista	5	1	Phortica nudiarista hits
. . . Phortica foliiseta	1	1	Phortica foliiseta hits
. . . Phortica speculum	2	1	Phortica speculum hits
. . Phortica subradiata	1	1	Phortica subradiata hits

Fig 3: Taxonomy report.

Lineage Report				
Organism	Blast Name	Score	Number of Hits	Description
Diptera	flies		145	
. Chironomidae	flies		134	
. . Chironominae	flies		114	
. . . Chironomini	flies		93	
. . . . Polypedilum	flies		92	
. Polypedilum sp. BOLD:ACC5662	flies	1179	1	Polypedilum sp. BOLD:ACC5662 hits
. Polypedilum nubifer	flies	1013	74	Polypedilum nubifer hits
. Polypedilum scalaenum	flies	780	3	Polypedilum scalaenum hits
. Polypedilum unifascium	flies	760	2	Polypedilum unifascium hits
. Polypedilum sp. TE02	flies	726	2	Polypedilum sp. TE02 hits
. Polypedilum sp. BOLD:AAO2005	flies	726	1	Polypedilum sp. BOLD:AAO2005 hits
. Polypedilum convictum	flies	721	3	Polypedilum convictum hits
. Polypedilum vanderplanki	flies	702	1	Polypedilum vanderplanki hits
. Polypedilum sp. BOLD:AAO2164	flies	699	4	Polypedilum sp. BOLD:AAO2164 hits
. Polypedilum sp. BOLD:AAN8908	flies	695	1	Polypedilum sp. BOLD:AAN8908 hits
. Phaenopsectra sp. BOLD:AAN8990	flies	732	1	Phaenopsectra sp. BOLD:AAN8990 hits
. . . Riethia stictoptera	flies	750	10	Riethia stictoptera hits
. . . Tanytarsus inextentus	flies	732	6	Tanytarsus inextentus hits
. . . Chironominae sp. BOLD:ACG9457	flies	701	1	Chironominae sp. BOLD:ACG9457 hits
. . . Tanytarsus bispinosus	flies	693	4	Tanytarsus bispinosus hits
. . Chironomidae sp. GES-2010	flies	736	4	Chironomidae sp. GES-2010 hits

. . Chironomidae sp. BOLD:ACC8266	flies	726	6	Chironomidae sp. BOLD:ACC8266 hits
. . Chironomidae sp. BOLD:ACG2764	flies	704	5	Chironomidae sp. BOLD:ACG2764 hits
. . Chironomidae sp. BE-2012	flies	704	1	Chironomidae sp. BE-2012 hits
. . Chironomidae sp. BOLD:ACL4023	flies	699	1	Chironomidae sp. BOLD:ACL4023 hits
. . Chironomidae sp. BOLD:ABA0772	flies	695	1	Chironomidae sp. BOLD:ABA0772 hits
. . Chironomidae sp. BOLD:ACB0738	flies	693	1	Chironomidae sp. BOLD:ACB0738 hits
. . Chironomidae sp. BOLD:ACB0996	flies	693	1	Chironomidae sp. BOLD:ACB0996 hits
. Diptera sp. KMGHap_100	flies	915	1	Diptera sp. KMGHap_100 hits
. Phortica pavriarista	flies	713	1	Phortica pavriarista hits
. Phortica nudiarista	flies	708	5	Phortica nudiarista hits
. Phortica foliiseta	flies	706	1	Phortica foliiseta hits
. Phortica subradiata	flies	699	1	Phortica subradiata hits
. Phortica speculum	flies	695	2	Phortica speculum hits

Fig 4: Lineage report.

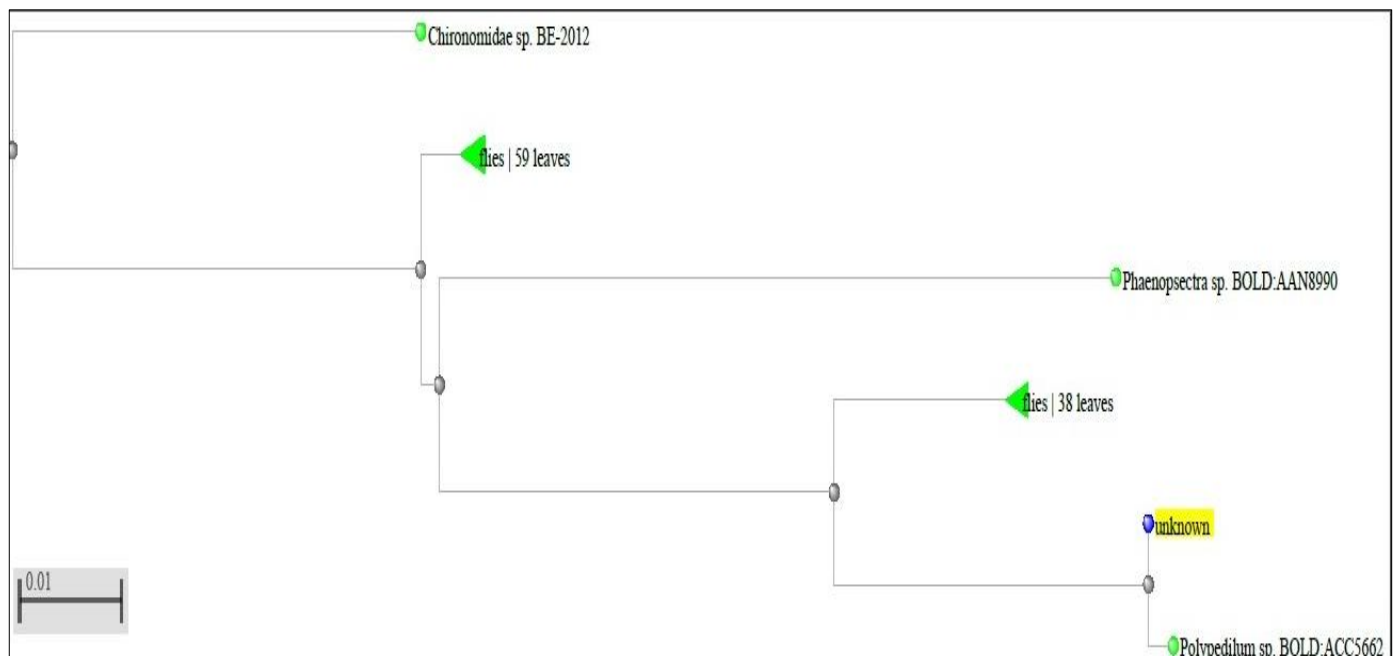


Fig 5: Phylogeny tree obtained.

4. References

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