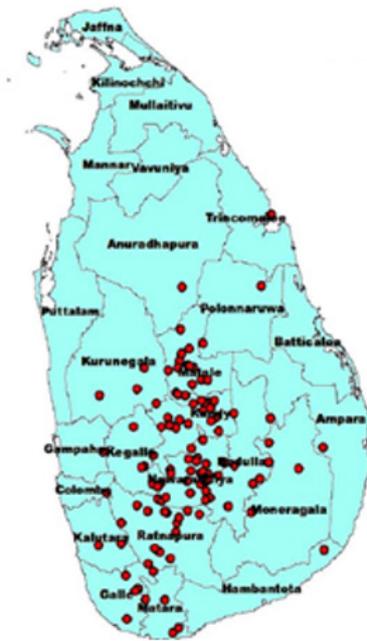


Piper species (Piperaceae) of Sri Lanka and the diversity of the economically important *Piper nigrum* (black pepper): An overview of what has been done and what is yet to be achieved

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Distribution of *Piper* species in Sri Lanka



Wild and cultivated species of *Piper*

Highlights

- *Piper* L. (Piperaceae) is one of the most diverse genera among the basal clades of angiosperms.
- Available chromosome counts indicate that most wild species are diploids and cultivated species are tetraploids.
- Next generation based restriction-site-associated DNA sequencing (RADseq) is a promising genomic tool that could be used to investigate species delimitation and genetic variation within the economically important *Piper nigrum* (black pepper).
- Three-dimensional (3D) floral morphology imaging using computed tomography will aid in the taxonomic revision and understanding pollination biology of this genus.

***Piper* species (Piperaceae) of Sri Lanka and the diversity of the economically important *Piper nigrum* (black pepper): An overview of what has been done and what is yet to be achieved**

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Abstract: Applying restriction site-associated DNA sequencing (RADseq) and target capture for *Piper* species from species-rich South America and India, Southeast Asia, and Africa will highlight the origin and evolution of Sri Lankan endemics, *P. zeylanicum*, *P. walkerii*, and *P. trineuron*. Looking into the genetic diversity of cultivated *P. nigrum* from different agroclimatic regions and available germplasm in Sri Lanka using RADseq will give an overview of the existing genetic diversity of black pepper, which is economically important and needs genetic improvement. Variation in flower composition (male, female or bisexual) across the spikes and their shape is of major interest to evolutionary and pollination biologists and plant systematists. The 3D shape models of flowers obtained by computed tomography of the wild species of *Piper* from Sri Lanka and cultivated *P. nigrum* will play an important role in revising the taxonomy and understanding the pollination biology of the genus.

Keywords: *Piper*; Taxonomy; RADseq; Phylogenomics; Population genomics; Computed tomography.

INTRODUCTION

Piper L. is one of the most diverse genera among the basal clades of angiosperms and is widespread in tropical wet forests around the world (Dyer and Palmer, 2004), with about 2,000 species (Quijano-Abril *et al.*, 2006). Many members of *Piper* are climbers, although few are shrubs and herbs, and they are abundant in the understory of the tropical wet forests. The monophyly of this large genus has been confirmed by molecular phylogenetic analyses of Piperales (Jaramillo *et al.*, 2004; Wanke *et al.*, 2007; Smith *et al.*, 2008). Species of *Piper* are easily recognized by their swollen nodes, entire, distichously arranged leaves and terminal spikes that become leaf-opposed. The spikes vary in how they are held (erect vs. pendulous) and length, thickness and colour, but they always consist of minute perianth-less flowers subtended by bracts. The small size of the flowers makes it difficult to observe the number and position of the stamens, the main characters of Candolle's

system (1869, 1923), and has thus caused instability in the classification. Indeed, the number of stamens is largely uninformative in Paleotropical *Piper*. In terms of stem anatomy, *P. nigrum* L. has a pattern of vascular bundle arrangement that is similar to that of monocots, whereas the vascular bundle arrangement of *P. colubrinum* Link is similar to that of eudicots (Empari and Sim, 1985).

Trimen (1895) recorded nine Sri Lankan species. A survey done by the Department of Export Agriculture of Sri Lanka revealed the presence of eleven species, namely *Piper nigrum* L., *P. betle* L., *P. longum* Blume, *P. thwaitesii* C.DC., *P. subpeltatum* Willd., *P. argyrophyllum* Miq., *P. sylvestre* Lam., *P. zeylanicum* Miq., *P. trineuron* Miq., *P. attenuatum* Buch.-Ham. ex Miq., and *P. chuyva* (Miq.) C.DC., the last earlier considered a variety of *P. betle* (Samuel, 1981) (Figure 1). The Revised Handbook to the Flora of Ceylon (Huber, 1987) has recognized ten species. Of these, *P. zeylanicum*, *P. walkerii* Miq., and *P. trineuron* are endemics, and *P. nigrum* (black pepper), *P. longum* (thippili), and *P. betle* (betel leaves) are of economic importance in Sri Lanka (Figure 2).

The greatest diversity of *Piper* is concentrated in the Neotropics (ca. 1300 species: 70%), and the Neotropical clade has been estimated to be 65 mya or younger, which indicates that diversification of the Neotropical clades occurred throughout the Cenozoic (Martínez *et al.*, 2015). In India, *Piper* is represented by more than 100 species, most of which (65 species) are confined to the north-eastern region. Evergreen forests of the southern Western Ghats also form a significant centre of diversity for *Piper*, with 18 taxa (17 species and two subspecies) reported from the Kerala region (Sasidharan, 2013). Among these taxa, ten are local endemics with one species categorized as critically endangered (MOE, 2012). In Thailand, 38 species are recorded, out of which eight are popularly used by Thai people as vegetables, spices, decoration and medicines and in traditional ceremonies: *P. betle*, *P. longum*,

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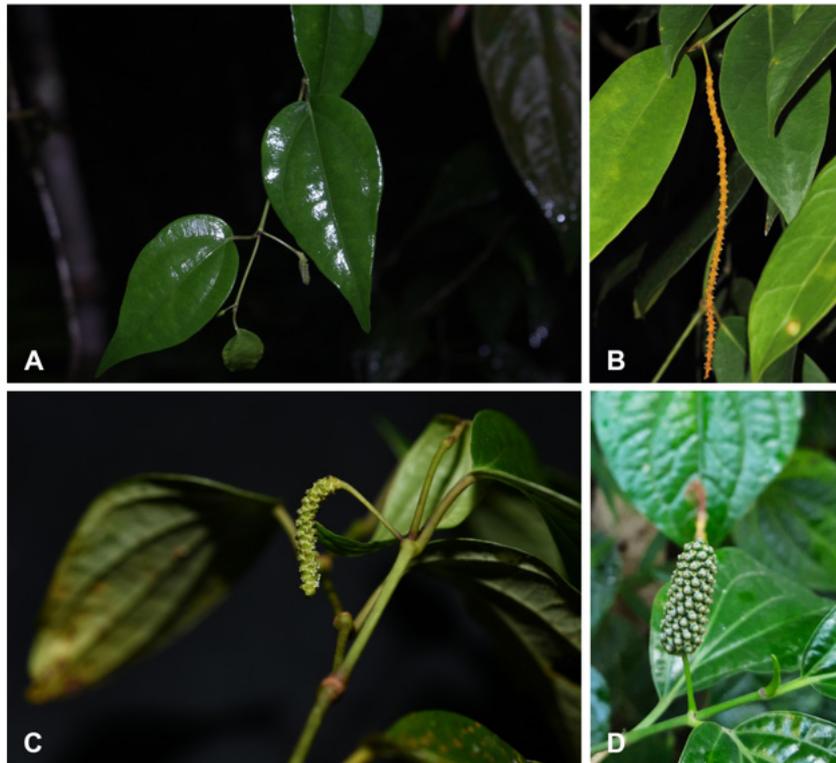


Figure 1: Some *Piper* species of Sri Lanka. A. *P. zeylanicum* B. *P. trineuron* C. *P. sylvestre* D. *P. sarmentosum*.



Figure 2: Cultivated *Piper* species. A. *P. nigrum* B. *P. longum* C. *P. betle* D. *P. betle* inflorescence.

P. nigrum, *P. pendulispicum* C.DC., *P. retrofractum* Vahl, *P. sarmentosum* Roxb., *P. wallichii* (Miq.) Hand.-Maz., and an undescribed *Piper* species (Chaveerach *et al.*, 2006). African *Piper* is estimated to comprise about 15 species found mostly in the tropical regions of East, Central, and West Africa. The most common of these species are *P. guineense* Schumach. & Thonn. and *P. capense* L. f., which are widespread across many African countries. *Piper guineense*, otherwise known as West African black pepper, originates from West Africa and compares favourably with black pepper (*P. nigrum*) as a flavouring for food (Oyemitan, 2017).

A new fossil *Piper* from the late Cretaceous of Colombia has been identified and described as *P. margaritae* (Martínez *et al.*, 2015). Previous analyses for Piperales have shown contrasting results for the age of *Piper*, ranging from the Cretaceous (ca. 92 mya, Smith *et al.*, 2008) to the Paleocene (ca. 57 mya, Symmank *et al.*, 2011; appr. 31 mya, Naumann *et al.*, 2013). Molecular phylogenetic analyses have indicated that Piperales are a relatively early divergent clade (Soltis *et al.*, 2000, Jaramillo and Kramer, 2007), with the age of Piperaceae being 91.2 mya, and the crown age of *Piper* being 71.75 mya. *Piper* has been

hypothesized to have dispersed first from the Neotropics to Africa and then to the Pacific and Asia with subsequent dispersal from Asia to Australia and back to Africa (Smith *et al.*, 2008).

Morphology of *Piper*

Although *Piper* is easy to recognize by a combination of vegetative and reproductive characters, the apparent uniformity of its diminutive flowers presents a significant challenge for developing an infrageneric classification. The earliest classifications of Piperaceae emphasizing *Piper* recognized 7-15 genera (Kunth, 1839; Miquel, 1843-1844). The infrageneric taxonomy of *Piper* is currently unresolved, and most local treatments have described species without subgeneric affiliation (Yuncker, 1953, 1972, 1973; Chew, 1972; Yongqian, Nianhe, and Gilbert, 1999). Given their minute size, changes in floral structure may be evolutionarily less important than variation in inflorescence type. It is likely that pollination and dispersal biology have been influenced more by whole inflorescence structure than by individual flower structure (Thies, Kalko, and Schintzler, 1998). In this perspective, modern approaches of imaging hold great promise for finding characters capable of differentiating between species with similar phenotypes (Sedeek *et al.*, 2014) because the most complete way to describe the morphological phenotype is to build a three-dimensional (3D) model (Staedler *et al.*, 2013). The immense amount of data contained in an accurate 3D model can then be mined not only to uncover traits that allow discrimination between closely related taxa but also to explore correlation with other types of data, *e.g.* pollinators (Van der Niet *et al.*, 2010) or crop yield (Jiang *et al.*, 2012).

Molecular phylogenetics of *Piper*

Molecular phylogenetic studies using nuclear ribosomal (nr) ITS DNA and plastid intron *psbJ-petA*, has been useful in examining the monophyly of *Piper* (Jaramillo and Manos, 2001; Jaramillo and Callejas, 2004a, b; Tepe *et al.*, 2004; Jaramillo *et al.*, 2008). So far, no study has examined the phylogenetic relationships of the Sri Lankan species of *Piper*; except for nrITS barcoding work (Jayarathna *et al.*, 2016a). The origin and evolution of *Piper* in Peninsular India were investigated using nrITS and plastid intron *psbJ-petA* (Sen *et al.*, 2019). The plastid markers *ndhF* and *trnL* intron showed a close relationship of the two Malagasy species with the African *P. guineense*, but the sequencing of the nuclear *G3pdh* gene showed some hybridization and introgression with Asian and African species (Palchetti *et al.*, 2020). In addition to DNA sequencing, molecular markers such as RAPD (randomly amplified polymorphic DNA) have been used to characterize genetic diversity among six species of *Piper* (Shivashankar, 2014).

Chromosome number and genome size of *Piper*

Cytological studies of *Piper* species have indicated most wild species are diploids, $2n = 26$ (*e.g.* *P. apiculatum*, *P. trineuron*) and cultivated species, *P. nigrum*, *P. longum*, and *P. betle*, are tetraploids, $2n = 52$ (Mathew, 1958, 1973; Samuel and Bavappa, 1981; Samuel, 1987; Mathew *et al.*,

1999). In addition to these, *P. thwaitseii* is a triploid ($2n = 39$) (Samuel and Bavappa, 1981). Various basic chromosome numbers have been reported for some Indian species such as *P. wightii* Miq. $n = 24$, $n = 28$, *P. attenuatum* $2n = 36$, *P. longum* $2n = 40$, and *P. betle* $2n = 64$ (Viji Stella Bai and Subramaniam, 1985). Chromosome numbers of two Malagasy species, *P. tsarasotrae* Papini, Palchetti, Gori, and Rota ($2n \approx 38$) and *P. malgassicum* Papini, Palchetti, Gori, and Rota ($2n \approx 46$), were recently reported (Palchetti *et al.*, 2020). Information on chromosome number, morphology, and meiotic behaviour would provide useful background for inter- and intraspecific hybridization and crop improvement.

The 4C content of four New World and five Old World *Piper* species ranged overall from 2.73 pg in the tetraploid *P. longum* to 9.62 pg in the octoploid *P. nigrum* (Samuel *et al.*, 1986). Genome size reports are also available for a few more species from India (Chikkaswamy *et al.*, 2007).

Phytochemical survey of *Piper*

Piper is one of the most important genera of medicinal plants (Mgbeahuruike *et al.*, 2017; Salehi *et al.*, 2019; Kumar *et al.*, 2020). Species have been reported to possess various pharmacological activities such as insecticidal, antibacterial, anti-inflammatory, antithyroid, antitumor, and hepato properties. *Piper nigrum*, which is known as the king of spices, contains the major pungent alkaloid piperine that also possesses medicinal properties (Srivastava and Sing, 2017).

The chemical composition and morphology of the Sri Lankan *Piper* species were investigated for utilizing their valuable traits for crop improvement. *Piper nigrum*, *P. betle*, *P. longum*, *P. chuvya*, *P. siriboa* L., *P. sylvestre*, *P. zeylanicum*, and *P. walkeri* were examined in morphological and phytochemical studies (Jayarathna *et al.* 2016b) using the specimens collected from natural habitats and cultivation. Thirteen morphological and anatomical characters were subjected to cluster analysis to infer species relationships. According to the phenogram in Jayarathna *et al.* (2016b), the taxa were divided into two main clusters: *P. longum*, *P. zeylanicum*, *P. sylvestre*, *P. nigrum*, and *P. walkeri* formed one cluster, whereas *P. chuvya*, *P. betle*, and *P. siriboa* formed a second cluster. This study has played an important role in the crop improvement of *P. betle*. Leaves of *P. betle* are considered to be rich in phenolic compounds with antiproliferative, antimutagenic, antibacterial, antidiabetic, and antioxidant properties. Studies on *P. betle* have reported that it contains important chemical constituents such as chavibetol, chavibetol acetate, and caryophyllene (Arambewela *et al.*, 2011; Shah *et al.*, 2016). *Piper longum* is used as an important ingredient in traditional medicine in Asia and the Pacific islands and is widely cultivated in Sri Lanka for medicinal purposes. The dried spikes and roots are used in a decoction for acute and chronic bronchitis, fever, and cough (Kumari and Yakandawala, 2008).

Economically important *Piper* species

Cultivated *Piper nigrum*: the pepper of commerce comprises dried mature berries of *Piper nigrum*, the second most widely traded spice worldwide after cinnamon. Black pepper has been considered to have its origin in the Western Ghats of Kerala, India, and may be an allotetraploid (hybrid) due to normal pairing of chromosomes at meiosis (Mathew, 1998). Based on morphological and biosystematic studies, it was suggested that three species, namely *P. wightii*, *P. galeatum* (Miq.) C.DC, and *P. trichostachyon* (Miq.) C.DC, are among the putative parents of *P. nigrum* (Neema, 2008; Lekhak *et al.*, 2014). In the past, forests were continuous, which might have led to overlapping species ranges and facilitated natural crossing, and such polyploid hybrids might have originated many times at different locations, gradually leading to the establishment of large populations (Abbott *et al.*, 2013). The allotetraploids once formed are effectively genetically isolated from the parents by their different ploidies.

In Sri Lanka, although pepper cultivation is concentrated in the Kandy, Matale, Kegalle, Kurunegala, Badulla, Monaragala, and Ratnapura districts, and it is found in ten of the 25 districts. The area of pepper cultivation has been estimated to be around 39,284 hectares (statistics from Dept. of Export Agriculture 2018). Germplasm collections of *Piper nigrum* in Sri Lanka hold several local selections (MB12, GK49, DM7, IW5, KW27, and a few others selected based on their yield and quality) and two introduced varieties, Panniyur (from India) and Kuching (from Malaysia), introduced in 1970. Three artificial hybrids between the introduced variety Panniyur and local selections have high yields and high oleoresin and piperine content. Genetic diversity in the germplasm of black pepper has been investigated by various molecular markers such as EST-SSR (Wu *et al.*, 2016) and nuclear microsatellites (Joy *et al.*, 2011; Kumari *et al.*, 2019). The quality of black pepper apart from appearance and diversity is judged by both odour and pungency (Kay, 1970). The degree of pungency and flavour is primarily determined by the intrinsic characteristics of the variety or cultivar, and quality attributes can be improved by the selection and propagation of suitable strains (Purseglove *et al.*, 1981). The black pepper cultivars of Malabar, Lampong, and Sri Lanka have high volatile and non-volatile solvent extracts (Nambudiri *et al.*, 1970). The piperine content of Sri Lankan pepper is higher (7–15%) when compared with the commercial Indian and Malaysian varieties (Jennings and Wrolstad, 1965).

***Piper betle*:** in addition to the extensive and well-established domestic market, betel has gained a significant position in the export market since 1974 (Arambewela *et al.*, 2011). Betel leaves are closely associated with many cultural events and rituals, where they are traditionally offered as a mark of respect and auspicious beginnings. Betel leaves have been used for chewing purposes along with other condiments. Six cultivars of *P. betle*, namely ‘Galdalu,’ ‘Mahamaneru,’ ‘Kudamaneru,’ ‘Ratadalu,’ ‘Nagawalli,’ and ‘Malabulath’ are cultivated in Sri Lanka (Arambewela *et al.*, 2011).

***Piper longum*:** this is naturally distributed in the wet and intermediate zones and included in the ten largest important herbal materials in Sri Lanka. A recent study using morphological characters identified three distinct phenetic groups, which highlights the importance of the correct identification of the specimen for ayurvedic treatments (Kumari and Yakandawala, 2008).

Application of next-generation sequencing in *Piper*

For non-model plants such as black pepper, next-generation sequencing (NGS) technologies offer a great opportunity for rapid access to genetic information (Lister *et al.*, 2009; Hawkins *et al.*, 2010). High-throughput sequencing of the *Piper nigrum* (black pepper) root transcriptome has been reported (Gordo *et al.*, 2012). Transcripts were functionally annotated and represent the first sequences derived from the transcriptomes of *P. nigrum*. The complete plastid genome of *P. kadsura* (Choisy) Ohwi was sequenced and includes 131 genes, including four unique rRNA plus 30 tRNA and 79 protein-coding genes (Lee *et al.*, 2015). In addition, *de novo* assembly and characterization of fruit transcriptome in *P. nigrum* is also available (Hu *et al.*, 2015). Recently, comparative transcriptome analysis of several plant tissues including leaves, roots, and spikes has been reported for *P. longum* L (Dantu *et al.*, 2021). A reference genome of black pepper (*P. nigrum*) comprised 761.2 Mb sequences assembled into 26 pseudochromosomes provides insights into the molecular basis of species-specific piperine biosynthesis (Hu *et al.*, 2019), the major alkaloid responsible for the pungency and flavour of black pepper. These genomic studies are important for further evolutionary investigations and biological and agronomic research on *Piper*.

The rapid expansion of NGS has yielded a powerful array of tools to address biological questions at a scale that was not possible until a few years ago. Restriction-site-associated DNA sequencing (RADseq; Baird *et al.*, 2008; Wang *et al.*, 2012) and whole transcriptome shotgun sequencing (WTS; Wang *et al.*, 2009) have quickly become the predominant genomic methods used in evolutionary studies. As a simple NGS derivative of a more traditional marker-based approaches, such as AFLP (amplified fragment-length polymorphisms; Miller *et al.*, 2007), RADseq in particular has emerged as the gateway genomic approach for most non-model species. It is also referred to as “genotyping by sequencing” (Narum *et al.*, 2013), a term that includes other genomic fragment-sequencing approaches. In addition, high-throughput target capture (hyb-seq) is a general class of methods that achieve genome partitioning through selective enrichment of specific subsets of the genome prior to NGS. Hyb-seq (sometimes referred to as exon-capture sequencing) consists of enriching genomic libraries for regions of interest (nuclear or organellar), such as highly conserved regions or more variable, low-copy orthologous (Weitemier *et al.*, 2014) or functional loci (Moore *et al.*, 2018). Sequencing 424 genes from over 130 herbarium specimens of *Erythroxylum* species demonstrated the efficacy of hyb-seq with highly degraded DNA (White *et al.*, 2021). In land plants, the hyb-seq method has recently become a standard

procedure for generating large amounts of sequence data for phylogenomics of non-model organisms (Gernandt et al., 2018; Medina et al., 2019).

High throughput DNA sequencing technologies and analysis algorithms provide an opportunity to study in detail genome-wide variability across groups of individuals, adding significant power to evolutionary investigations. In particular, RADseq (Baird et al., 2008) was established as a powerful tool for interspecific comparisons across a large number of loci in non-model organisms (Cariou et al., 2013). RADseq had been used in phylogenetics of tribe Shoreae, Dipterocarpaceae (Heckenhauer et al., 2018) and the European shrub willows (*Salix* L subgens. *Chamaetia* and *Vetrix*; Wagner et al., 2018). In *Heliosperma pusillum*, RADseq has been successfully used to examine parallel ecological divergence (Trucchi et al., 2017), whereas in radiating New Caledonia *Diospyros* this method could be used to evaluate substrate effects on speciation (Paun et al., 2016). In *Nicotiana* (Solanaceae), RADseq was employed to evaluate the possible interactions between chromosome number and genome size changes, which are both controlled by epigenetic mechanisms (Chase et al., 2022).

RADseq analyses of wild and cultivated species of Sri Lankan *Piper* will contribute to the taxonomic revision of this genus. Inclusion of selected species from South America, India, and Southeast Asia will help to detect the origin and timing of the endemic radiation of *P. zeylanicum*, *P. trineuron*, and *P. walkerii*. However, RADseq requires good quality DNA, in general, isolated from fresh or silica-gel dried plant material, which becomes disadvantageous if herbarium specimens are expected to be used for phylogenomics. Investigations at the population level for *P. nigrum* using RADseq will give an overview of the genetic diversity of this cultivated, economically important species across the different agroclimatic regions, which will help selection for high-yielding varieties and thus the economy.

Computed tomography of inflorescence

In addition to the genetic diversity, it is important to investigate the floral structure and composition of bisexual flowers across the spikes of *P. nigrum*, which plays a decisive role in the yield of black pepper. Application of computed tomography to obtain the 3D structure of flowers in spikes of the wild and selected cultivated *P. nigrum* accessions (composition of male, female and bisexual flowers in the spikes) will provide an overview of pollination and fruit set in the spikes. A detailed study of inflorescences of the wild species will help to establish a phylogenetically based infrageneric classification of the genus *Piper*.

CONCLUSION

The NGS-based technique, RADseq, has been suggested as a powerful tool for intra- and interspecific comparisons across a large number of loci in non-model organisms, delivering clear phylogenetic information. This technique will enable to resolve phylogenetic relationships among the *Piper* species. It will also help to date the origin of the endemic taxa as well as to identify new species. In addition, the use of this technology at the population level in *P.*

nigrum will give a clear overview of the genetic diversity of black pepper growing in different agroclimatic regions in Sri Lanka and thus be an aid for crop improvement. Further examination of looking into floral morphology of the wild species using 3D computed tomography may help in the taxonomic revision of *Piper*. In addition computed tomography of the inflorescence (spikes) in cultivated *P. nigrum* will provide insights into pollination biology and fruit set to improve the yield of black pepper. Studies on chromosome and genome size can play an important role in black pepper breeding programs.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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