Underground leaves of *Philcoxia* trap and digest nematodes

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The recently described genus *Philcoxia* comprises three species restricted to well lit and low-nutrient soils in the Brazilian Cerrado. The morphological and habitat similarities of *Philcoxia* to those of some carnivorous plants, along with recent observations of nematodes over its subterranean leaves, prompted the suggestion that the genus is carnivorous. Here we report compelling evidence of carnivory in *Philcoxia* of the Plantaginaceae, a family in which no carnivorous members are otherwise known. We also document both a unique capturing strategy for carnivorous plants and a case of a plant that traps and digests nematodes with underground adhesive leaves. Our findings illustrate how much can still be discovered about the origin, distribution, and frequency of the carnivorous syndrome in angiosperms and, more generally, about the diversity of nutrient-acquisition mechanisms that have evolved in plants growing in severely nutrient-impoverished environments such as the Brazilian Cerrado, one of the world's 34 biodiversity hotspots.

plant carnivory | plant nutrient acquisition | campos rupestres | nitrogen | stable isotopes

Carnivory has evolved at least six times within the angiosperms and, since Darwin's work on the subject in 1875 (1), approximately 20 carnivorous genera distributed among 10 families and four major lineages have been identified (2, 3). A general cost–benefit model predicts that carnivory will be restricted to well lit, low-nutrient, and at least seasonally moist habitats (4), where the major source of nitrogen, phosphorus, potassium, and other nutrients will be derived from captured and digested invertebrates (5). Conditions of high light and moisture are included in the model because the photosynthetic costs of carnivory likely exceed the benefits in shady or dry habitats (4, 6). Recently, this model was extended by including explicit tradeoffs among light, moisture, and nutrient availability through litter which explains the occurrence of carnivory in a variety of habitats with contrasting light and water regimes (7, 8).

Philcoxia comprises three species, all of which grow exclusively in the Campos Rupestres of the Central Brazilian cerrado biome (9, 10), a species-rich mosaic of well lit and low-nutrient rock outcrops and shallow, white sands under a seasonal precipitation regime. According to the cost–benefit model, these conditions are conducive for the evolution of carnivory, and indeed species of the carnivorous genus *Genlisea* (11) are common in this habitat where seepages occur. *Philcoxia* possesses several unusual morphological features similar to some carnivorous species, especially a nonmycorrhizal and poorly developed root system, peltate leaf blades that are inwardly folded at the midvein when immature, stalked capitate glands on the upper leaf surfaces, and leafless scapose inflorescences (2, 9, 10, 12) (Figs. 1 and 2 and Fig. S1). A striking additional feature of *Philcoxia* is the placement of many of its minute leaves (0.5–1.5-mm blade diameter) under the surface of the white sand in which it grows (Figs. 1 *B–D* and 2). The stalked glands produce sticky substances that tightly bind sand grains to the leaf surfaces (Figs. 1*C* and 3).

To be considered carnivorous, a plant must be able to absorb nutrients from dead animals next to its surfaces and have some

feature the primary effect of which is the active attraction, capture, and/or digestion of prey (4). The combination of morphological features, the highly nutrient-impoverished substrate, and the observation of nematodes adhering to the surface of the leaves on herbarium specimens (10) and in the field (Fig. 3) led to the hypothesis that the species of *Philcoxia* are carnivorous, trapping nematodes and perhaps other soil organisms with their leaf glands and absorbing their prey's nutrients (10). To test the carnivory hypothesis, we assayed one of the species, *Philcoxia minensis*, for its capacity to digest and absorb nutrients from nematodes.

Results

To test nutrient acquisition from prey, we fed nematodes labeled with ^{15}N to *P. minensis*. Significant amounts of ^{15}N were traceable in nematode-fed leaves: approximately 5% of the prey ^{15}N was found in the leaves within 24 h and 15% was found after 48 h (Fig. 4*A*), strongly suggesting that nematodes were digested (rather than naturally decomposed) and quickly absorbed by *P. minensis* leaves. Such a pronounced pattern is entirely consistent with carnivory, and the observed rates are relatively high compared with those for other carnivorous species (13).

Measurements of leaf nutrient concentration suggest that prey use is critically important as a nutrient source for the growth and survival of *P. minensis* in the nutrient-poor habitats of the Campos Rupestres. Leaf nitrogen content increased substantially by 48 h after the application of nematodes (Fig. 4*B*). Moreover, the dry-weight concentrations of nitrogen and phosphorus in leaves of the species were significantly higher than that of the average among eight neighboring species (nitrogen, 29.2 ± 1.6 mg·g^{-1} vs. 9.4 ± 3.8 mg·g^{-1}; phosphorus, 0.9 ± 0.2 mg·g^{-1} vs. 0.4 ± 0.2 mg·g^{-1}).

Most "historically true" carnivorous plants possess high phosphatase activity on their secretory hairs and emergences (5, 13). We detected the activity of phosphatases on the leaves of *P. minensis* (Fig. 5), suggesting that nematodes are digested directly by the plant, rather than broken down by microbes, as in some pitcher plants (14). We also found a great abundance of nematodes on the leaf blades both in the field and on herbarium specimens, suggesting that *P. minensis* leaves act as a highly effective nematode trap. All nematodes on the leaves of *P. minensis* and the congeneric *Philcoxia bahiensis* in the field were dead at the time of observation—strong evidence that they were neither feeding nor reproducing on the leaves.
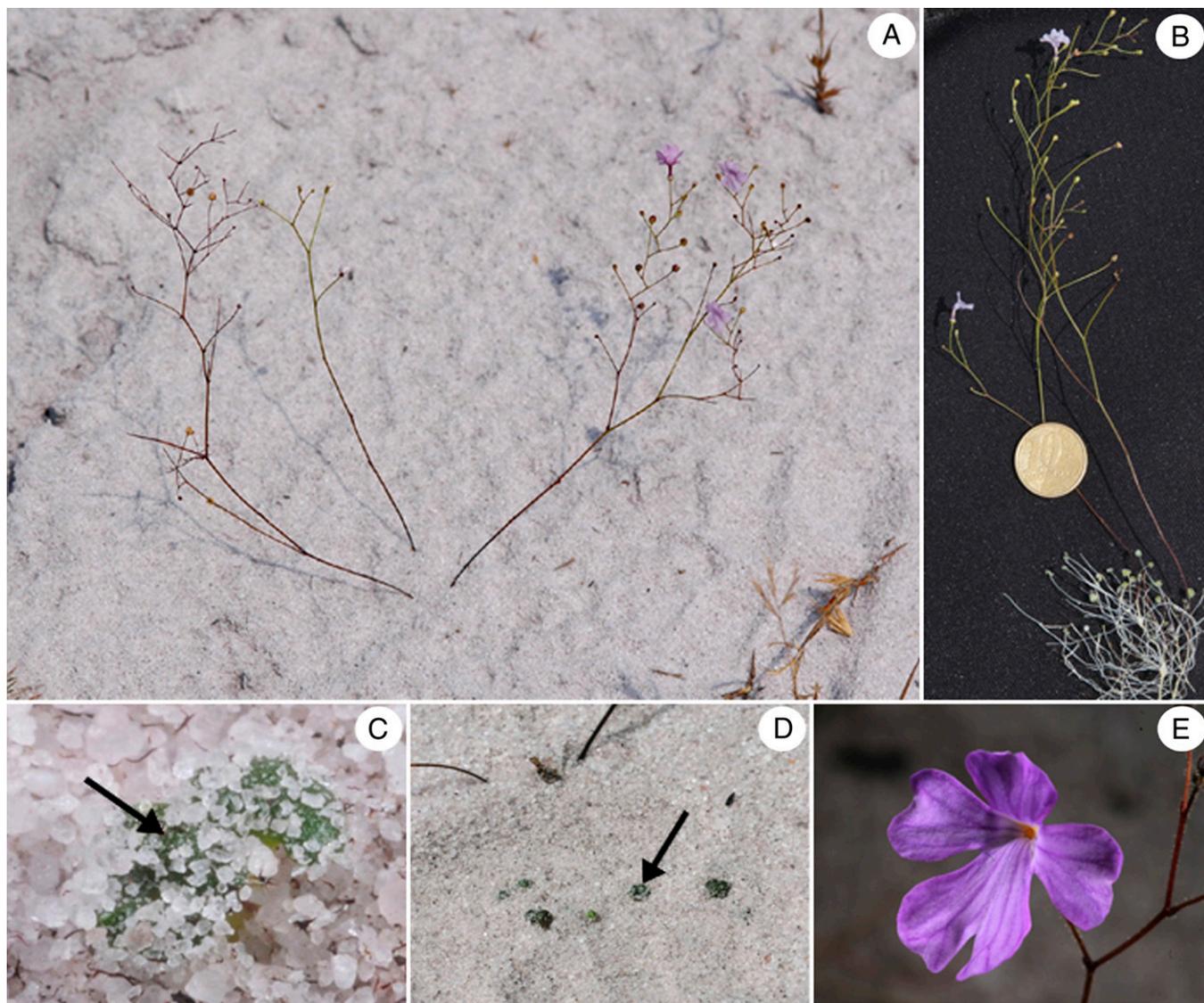
ECOLOGY

Fig. 1. Morphology of *P. minensis*. (*A*) Individual in its natural habitat at Serra do Cabral, Minas Gerais, Brazil. (*B*) Excavated individual showing leaf arrangement at the base of the plant. (*C*) Close-up of two leaves covered with sand grains. (*D*) Close-up of several leaves (sand brushed away to reveal the subterranean placement of the leaves). (*E*) Close-up of a single flower showing the deeply five-lobed calyx with subequal lobes.

Discussion

The use of prey by plants has been thought to usually not be the most economical way for plants to secure nutrients. This appears to be borne out by the relative rarity of the plant carnivorous syndrome, being present in only approximately 0.2% of flowering plant species (12, 15). The number of known carnivorous plant species could nonetheless represent an underestimate of the true total, because only preliminary tests have been conducted on some putative carnivores such as species of *Dipsacus*, *Geranium*, *Petunia*, and *Potentilla* (15–18). In other cases, carnivory could be cryptic, because of, e.g., the use of microscopic prey, relative rarity or accessibility of the plants, or a mechanism hidden from view. All three of these reasons help explain why only now has carnivory been reported in *Philcoxia*. The present study, and heretofore less conclusive data from other plants with sticky leaves or pitfall-like structures, seem to support the notion that "we may be surrounded by many more murderous plants than we think (19)."

On a global scale, the concentrations of nitrogen, phosphorus, and potassium in carnivorous plant leaves tend to be lower than in leaves from noncarnivorous species (6). However, in their natural habitat, we found a higher nutrient concentration in *P. minensis* leaves compared with its noncarnivorous neighbors, suggesting that carnivory contributes significantly to the mineral nutrition of this species. This is one of the few cases supporting one of the two energetic advantages of carnivory proposed by Givnish et al. (4)—that is, the elevation of photosynthetic rate through an increase in leaf nutrient content. We are currently addressing this question by directly measuring photosynthetic rates under a range of prey concentrations.

P. bahiensis and *Philcoxia goiasensis*, the other two species of *Philcoxia*, may well be carnivorous also, because they occur in the same type of nutrient-poor sands and exhibit the same basic morphology as *P. minensis* (9). Moreover, on herbarium specimens, their leaves bear the remains of nematodes (10). All three species are narrow and apparently very rare endemics, possibly at least in part because of the specific set of microenvironmental conditions under which they occur in the Campos Rupestres. For example, *P. minensis* is found in relatively low flat areas, where water likely persists after rain. All shrubs and cacti avoid these areas, although they are common elsewhere in the immediate
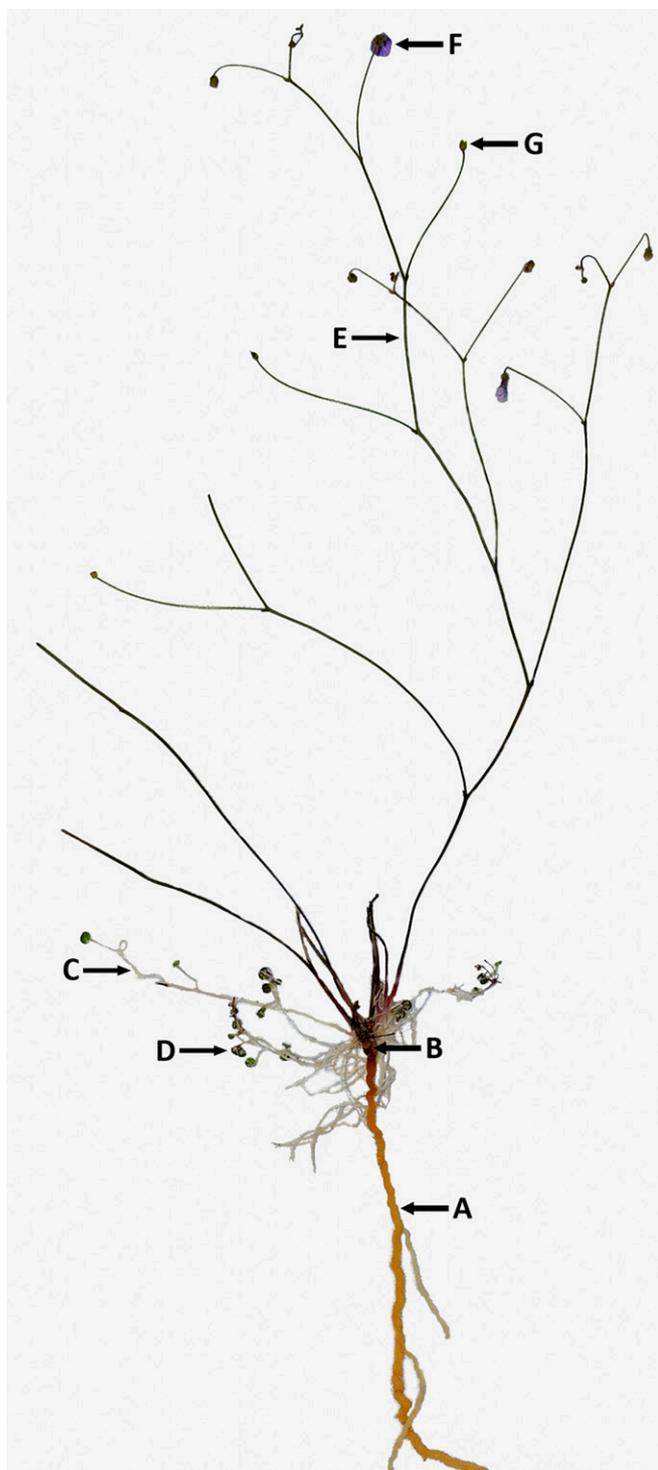
Fig. 2. Detailed morphology of *Philcoxia minensis*. (*A*) The simple and poorly developed root system. (*B*) The short upright stem. (*C*) Lateral stem/petiole. (*D*) Leaf blade. (*E*) Inflorescence branch. (*F*) Flower. (*G*) Fruit.
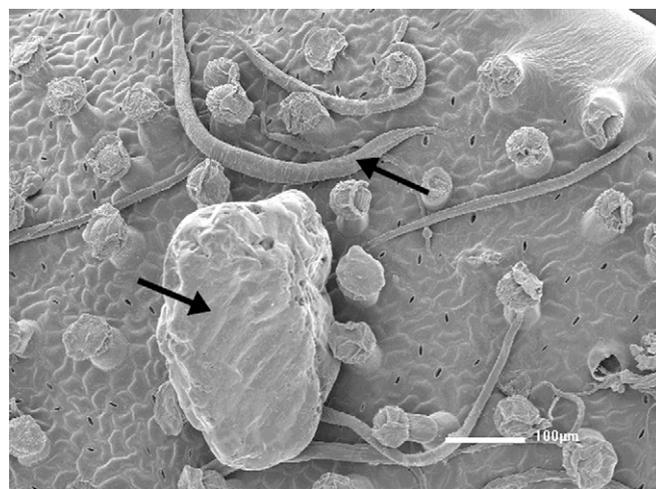

Fig. 3. Scanning EM image of the upper leaf surface shows the abundance of nematodes, stalked glands, and adherent sand grains. Arrows point to nematodes and sand grains.

are phylogenetically well separated from one another (20), and each employs distinct methods of prey capture. Molecular phylogenetic data place *Philcoxia* solidly within the Gratioleae (10), a tribe of mainly terrestrial herbs and small shrubs, but also many aquatics (22). This raises the question whether the origin of
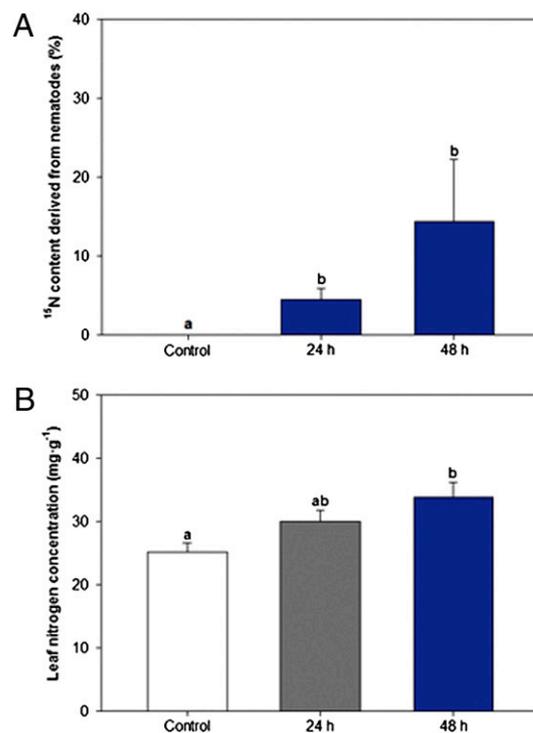

Fig. 4. Nitrogen absorption in *P. minensis*. (*A*) ^{15}Nitrogen absorption shown as mean values with standard error bars ($n = 8$). Data show a significant difference in the isotope signature of the plants at 24 h and 48 h after placement of the nematodes onto the leaves compared with the control. No difference was observed between the 24-h and 48-h treatments as a result of the substantial variation observed among the samples of the latter. (*B*) Average nitrogen absorption shown with standard error bars ($n = 8$). Data show a tendency of increase at 24 h and a significant increase at 48 h after placement of the nematodes onto the leaves compared with the control ([a,b]$P < 0.05$ between treatments).

vicinity, and only a few plants such as sedges co-occur, suggesting a highly specialized niche.

The present study provides evidence for carnivory in the Plantaginaceae, a family of approximately 92 genera and nearly 2,000 species (20). This family is a member of the order Lamiales, along with the well established carnivorous families Byblidaceae and Lentibulariaceae (2, 20, 21). The three clades
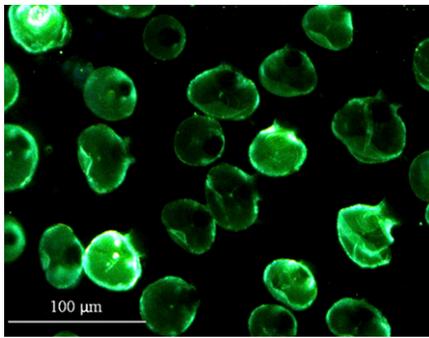
Fig. 5. Glandular trichomes of *P. minensis* stained with ELF97 phosphatase substrate. Green fluorescence demonstrates phosphatase activity on the surface of the trichomes.

Philcoxia was terrestrial or aquatic, the answer to which could have significant implications affecting the cost–benefit model for the evolution of carnivory. Although the phylogenetic placement of *Philcoxia* suggests a terrestrial origin for the genus (10), the Gratioleae are still poorly sampled for phylogenetic data and its genera ill-defined. Thus, assessing the aquatic versus terrestrial origin of *Philcoxia* will require additional phylogenetic study.

Taken together, our results satisfy the various conditions for carnivory in *P. minensis*, specifically via trapping, enzymatic digestion, and subsequent nitrogen uptake by specialized underground leaves. We also report a unique capturing strategy, as, to our knowledge, no other carnivorous species with adhesive traps has been described with subterranean leaves. Moreover, we document a case of a plant that traps and digests nematodes with adhesive leaves. Nematode digestion has been extensively described in fungi (23, 24) but is rare in plants. The discovery of carnivory in *Philcoxia* and its unique trapping method highlight the diversity of nutrient-acquisition mechanisms among plants in severely nutrient-impoverished environments, as shown in similar systems elsewhere in the world (25). Avenues for further investigation of carnivory in *Philcoxia* include the mechanism of prey attraction and details of prey capture by the traps, the influence of seasonal variation on carnivory, and the species composition and ecology of the nematode prey.

Materials and Methods

Leaf Nutrient Concentration Analysis. Leaf samples were collected at the Serra do Cabral, Minas Gerais, and were oven-dried at 60 °C for 3 d. Samples were then ground and sent to Centro de Energia Nuclear na Agricultura at the University of São Paulo for nutrient analysis. Nitrogen concentration was determined by using an automatic nitrogen and carbon analyzer (ANCA-SL; Europa Scientific) with a magnetic sector of low resolution, coupled to an IRMS 20–20 mass spectrometer (Europa Scientific). After digesting the leaf samples with nitric and perchloric acid, phosphorus concentration was determined by using the malachite green method (26) with a light spectrophotometer (Multiskan Spectrum; Thermo Fischer Scientific). Comparative analyses were made for *P. minensis* (Plantaginaceae) and eight other flowering plant species, each from one of the following families: Clusiaceae, Cyperaceae, Ericaceae, Eriocaulaceae, Fabaceae, Poaceae, Velloziaceae, and Xyridaceae. Data were compared by using a Mann–Whitney rank sum test with SYSTAT 13.0 software (Systat).

Nutrient Uptake from Nematodes. Plants were collected from the field and grown in 5-L pots (one plant per pot) for 5 mo in the greenhouse before the experiment. NA22 *Escherichia coli* bacteria were grown in CGM-1020-SL-N media (Cambridge Isotope Laboratories) for ^{15}N labeling. After growing to stationary phase, the bacteria were washed in S Buffer (27), resuspended in the same buffer, and placed in NGM plates without peptone. Nematodes of the model species *Caenorhabditis elegans* (27) were fed the isotope-labeled bacteria. After exhausting the bacteria, the nematodes were washed with M9 Buffer (27) and placed over the leaves of *P. minensis*, where they were left at 25 °C for 2 d. Eight plants were fed with ^{15}N-labeled nematodes, and leaves were harvested 24 and 48 h after nematode addition. Plants with unlabeled nematodes and those without nematodes were used as controls. Leaves were then oven-dried, cleaned, and analyzed for the detection and rate of ^{15}N uptake. The leaves were carefully washed twice in milli-Q water, which eliminated all possible nematode remains, and then left at 60 °C for 3 d. The dried samples were then sent for isotope analysis at Centro de Energia Nuclear na Agricultura, and the nitrogen stable-isotope composition was determined with the automatic nitrogen and carbon analyzer as described earlier. Data were compared with a Kruskal–Wallis one-way ANOVA in SYSTAT.

Determination of Phosphatase Activity. Leaves of *P. minensis* were kept intact and assayed with ELF97 phosphatase substrate (Invitrogen/Molecular Probes) as described previously (13, 21, 28). Samples were incubated in a substrate solution of 250 μM ELF97 phosphatase substrate in milli-Q water at room temperature for 15 min. Leaves were then screened for green fluorescence in an epifluorescence microscope [Optiphot-2 or Eclipse E800 with UV-2A filter (Nikon); excitation, 330–380 nm; dicromatic mirror, DM 400 nm; emission filter, BA 420 nm]. Fluorescence was documented with a Nikon FDX-35 digital camera. Unstained samples were used to check for possible autofluorescence.

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