

DESCRIPTION OF A NEW *EUPSOPHUS* SPECIES (AMPHIBIA, LEPTODACTYLIDAE) FROM THE REMNANTS OF MAULINO FOREST, CENTRAL CHILE

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The description of a new species of *Eupsophus*, from Los Queules National Reserve (35° 59' S, 72° 41' W) adds a new endemic taxon to the fauna of telmatobines (Amphibia, Leptodactylidae) of Chile, and extends the geographical distribution of the genus approximately 200 km to the north. The morphological description of the new species includes a karyotype description and molecular data of the mtDNA 12S gene. Chromosome and molecular evidence supports the assignment of the new species to *Eupsophus*, allowing comparisons with congeneric and other lower telmatobine species. The diploid number of the new species is $2n=30$ and it presents the chromosomal XY system of chromosomal sex determination. It differs from *E. vertebralis* and *E. emiliopugini* (both $2n=28$) and groups with *E. calcaratus*, *E. contulmoensis*, *E. roseus*, *E. nahuelbutensis*, *E. insularis* and *E. migueli* ($2n=30$), the last two species also with morphologically differentiated XY chromosomes. This new endemic species reinforces the importance of Los Queules National Reserve as a unique reservoir of the biota of Maulino Forest, central Chile.

Key words: Anura, frog, taxonomy, karyotypes, mitochondrial DNA

INTRODUCTION

The Chilean amphibian fauna is not species-rich, but highly endemic, with 75% of the species restricted to the country (Formas, 1995). This fact might partially reflect the incomplete knowledge of Chilean biodiversity (Simonetti, 2001). In fact, new distributional records and new species of amphibians are described frequently, suggesting this fauna is yet to be fully assessed (e.g. Cuevas & Formas, 2002).

Ongoing sampling of fauna at the coastal Maulino forest of central Chile near Trehualemu, (including the Reserva Nacional Los Queules; 35°59'S -72°41' W; Fig. 1), nearby forest remnants, and the surrounding commercial pine plantations (*Pinus radiata*) have revealed several new taxa for the region, including over 20 species of epigeal coleopterans, three species of small mammals, one lizard and one snake previously unrecorded for this latitude. All these species were previously known to have their northernmost limit more than 200 km south of Reserva Nacional Los Queules (Grez, *et al.*, 2003; Rubio *et al.*, in press; Saavedra & Simonetti, 2000, 2001; Simonetti, 2001).

Among amphibians, we have recorded the presence of two species of leptodactylids, belonging to the genera *Eupsophus* and *Alsodes*. The presence of *Eupsophus* at Trehualemu represents a significant extension of the

distribution of the genus. The nearest record for an *Eupsophus* species is *E. roseus*, at Cordillera de Nahuelbuta (Veloso & Navarro, 1988; Nuñez *et al.*, 1999). Further examination of the specimens from Trehualemu, including karyotypic and molecular data, revealed that they belong to an hitherto undescribed species of *Eupsophus*. Here, we describe this new taxon.

MATERIALS AND METHODS

Twelve specimens of the new species were collected at night, deposited at the Departamento de Biología

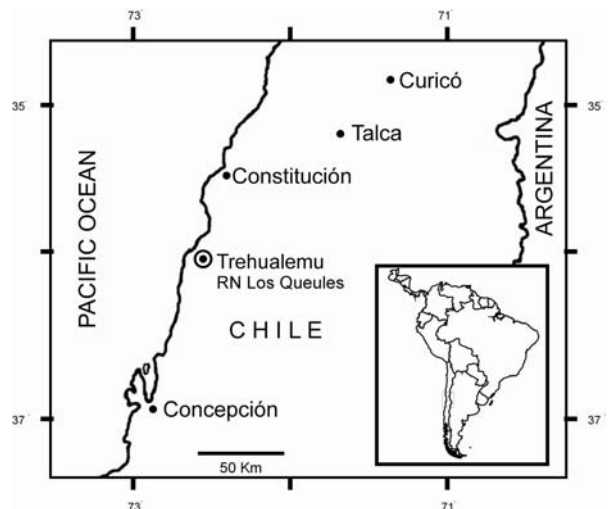


FIG. 1. Map of the type locality of *Eupsophus queulensis*. RN denotes Reserva Nacional Los Queules.

Celular y Genética, Universidad de Chile (DBGUCH), and later compared to congeneric species (*E. roseus*, *E. calcaratus*, *E. migueli*, *E. nahuelbutensis*, *E. insularis*, *E. contulmoensis*, *E. vertebralis* and *E. emiliopugini*) with regard to external morphology, patterns of coloration and karyotypes (Núñez, 2003). In adult specimens of both sexes and unsexed juveniles the following measurements were taken: snout-vent length (SVL), head width at the mid-tympanic level (HW), head length (HL), from the snout tip to posterior margin of the tympanic membrane, tympanic diameter (TD), distance between the anterior edge of the eye opening to the posterior edge of the nostrils (ON), eye diameter (ED), shortest distance between the eyes at the upper part of the head (IOD), tibia length (TL), distance between nostrils (IN), distance from the nostrils to the tip of the snout (NH). All measurements were taken with a digital caliper to the nearest 0.01 mm. The holotype and type series were subsequently transferred to the Museo Nacional de Historia Natural in Santiago (MNHNC), in accordance with Chilean regulations.

CHROMOSOMAL MARKERS

Two adult specimens, male MNHNC 3790 and female MNHNC 3793, were injected with colchicine 1%. Chromosomes were obtained from the intestinal epithelium, cornea and testicles. Intestinal and testicle tissues were subjected to a hypotonic treatment with distilled water and fixed in acetic acid 50%. Squash was performed after fixation. Cornea preparations were obtained by subjecting the eyes to hypotonic treatment with distilled water, and then fixing them in vapours of 100% of acetic acid (Bogart, 1973). The identification of homologous chromosomes was carried out determining the position of the centromeres and measuring the chromosomes with Micromesurer 3.3 (Reeves & Tear, 2000). Chromosome terminology follows Levan *et al.*, (1964).

MOLECULAR MARKERS

Sequence of the 12S gene of mtDNA of *Eupsophus* sp. was compared to putative congeners *Eupsophus vertebralis* (DBGUCH 3342), *E. calcaratus* (DBGUCH 2904) and other closely related species of lower telmatobines (Leptodactylidae): *Batrachyla taeniata* (DBGUCH 2943), *Telmatobius marmoratus* (DBGUCH 3384) and *Caudiverbera caudiverbera* (DBGUCH 3028). DNA was extracted using the phenol-chloroform method (Sambrook *et al.*, 1989). Primers L1091 and H1478 were used for PCR amplifications, after Kocher *et al.*, (1989). PCR products were purified using QIAgen and sequenced in ABI Prism 3100. Sequences were revised with CHROMAS (McCarthy, 1998) and BIOEDIT (Hall, 1999). All sequences were submitted to GenBank (accession numbers AY578817 - AY578822).

Phylogenetic relationships were inferred through PAUP*4.0b8a (Swofford, 2002) and MEGA (Molecular Evolutionary Genetic Analysis, version 1.2; Kumar *et al.*, 2001). Analyses were carried out using Maximum Parsimony (MP) and Neighbour Joining (NJ), using

Kimura two-parameter distances (Kimura, 1980). Internal support for each node was evaluated through bootstrapping (1,000 replicates).

RESULTS

DESCRIPTION OF A NEW SPECIES OF *EUPSOPHUS*

Eupsophus queulensis, new species

Ranita de Los Queules

Los Queules frog

Holotype. MNHNC 3798, adult male, collected by A. Veloso and K. Busse, on November 2003, at the east edge of Reserva Nacional Los Queules, 71 km W Cauquenes, coastal mountain range of the Costa, VII Maule Region (35° 59' S, 72° 41' W) at 450 m elevation, Chile (Fig. 2-3).



FIG. 2. Holotype of *Eupsophus queulensis*. Male MNHNC 3798.



FIG. 3. Variations in the coloration pattern of *Eupsophus queulensis*.

TABLE 1. *Eupsophus queulensis* sp. nov. Morphometric data of the type series (mean, SD and range), and morphometric data of females and juveniles (all data in mm; see codes in methods).

Character	Adult range	Paratypes					Mean±SD		Females		Mean±SD		Juveniles		Mean±SD
		3798	3787	3788	3789	3790	3791	3792	3793	3794	3795	3796	3797		
Code		3798	3787	3788	3789	3790	3791	3792	3793	3794	3795	3796	3797		
SVL	34.00-47.30	39.8	37.20	37.85	40.60	34.00	41.65	39.70	45.50	47.30	26.60	25.70	29.50	27.27±1.99	
HW	14.70-18.60	15.75	14.70	16.00	16.15	15.25	17.00	15.75	18.40	18.60	10.45	9.45	11.60	10.50±1.08	
HL	13.30-16.70	15.40	14.65	13.30	14.60	15.10	13.5	15.35	16.25	16.70	8.45	7.45	7.9	957.95±0.50	
TD	2.05-3.00	2.45	2.25	2.05	2.60	2.30	2.15	3.00	2.25	2.50	1.20	1.30	1.35	1.28±0.08	
ON	2.10-2.95	2.30	2.95	2.50	2.20	2.10	2.25	2.50	2.65	2.55	1.75	1.20	1.90	1.62±0.37	
ED	4.80-6.56	5.50	5.20	4.55	5.40	4.80	5.40	6.35	6.20	6.56	4.75	3.45	4.4	04.20±0.67	
IOD	3.45-4.50	3.85	4.05	3.70	3.90	4.00	3.45	4.25	4.44	4.50	2.95	2.70	2.95	2.87±0.14	
TL	20.55-25.05	20.60	20.80	20.65	20.55	21.60	22.55	21.90	25.05	25.05	14.35	15.60	16.75	15.57±1.20	
IN	3.50-4.70	3.70	3.70	3.85	4.00	3.50	4.65	4.15	4.70	4.70	2.65	2.30	2.90	2.62±0.30	
NH	2.51-4.10	3.45	3.45	2.51	3.15	3.10	4.10	3.20	3.40	3.70	1.70	1.65	2.1	51.83±0.28	

TABLE 2. Comparative chromosomes types in species of *Eupsophus* genus except undescribed *E. nahuelbutensis* karyotype ($2n=30$): $2n$ = diploid number, NF= number of arms, m = metacentric, sm= submetacentric, st= subtelocentric, t= telocentric (Levan *et al.*, 1964). 14* heterochromosome; ** Chromosomes with secondary constriction (nucleolar pair).

Species	Pair															$2n$	NF
	1	2	3	4	5	6	7	8	9	10	11	12	13	14*	15		
<i>E. vertebralis</i>	m	st	m	st	st	st	sm	m	m	m	m	m	m	m	m	28	56
<i>E. emilopugini</i>	m	st	m	st	st	st	sm	m	m	m	m	m	m	m	m	28	56
<i>E. insularis</i>	m	sm	st**	m	t	m	t	t	m	t	m	t	t	m/t*	t	30	44 / 45
<i>E. calcaratus</i>	m	sm	st**	t	m	t	m	m	t	t	t	t	t	m	t	30	46
<i>E. migueli</i>	m	sm**	st	t	t	m	m	m	m	t	m	t	t	m/t*	t	30	44 / 45
<i>E. roseus</i>	m	sm**	st	t	t	m	m	m	m	t	m	t	t	m/m*	t	30	46
<i>E. contulmoensis</i>	m	st	st**	t	t	m	m	t	t	m	t	t	t	m	t	30	46
<i>E. queulensis</i> sp. nov.	m	st	st	t**	t	m	t	m	sm	t	m	t	t	m/t*	t	30	44 / 45

Paratypes. Males MNHNC 3787-3792, females MNHNC 3793-3794, and unsexed juveniles MNHNC 3795-3797, collected by J. L. Celis-Diez and P. C. Guerrero, on September 2002, at the type locality.

Diagnosis. *Eupsophus queulensis* is a medium-sized frog, distinguished by: (1) truncate snout, (2) left epicoracoid superimposed to the right one, (3) xiphisternum without a notch, (4) prominent external and internal carpal tubercles, (5) yellow iris in life, (6) two melanic dots on both sides in the posterior half of the dorsal region, (7) reddish ventral surfaces, (8) an heteromorphic pair of sexual chromosomes, with a Y metacentric chromosome, and (9) secondary constriction at both homologs of the largest telocentric pair of chromosomes.

Description of the holotype. Adult male of medium size, head as long as broad (HL/HW = 0.98), slightly more than 1/3 of the SVL (HL/SVL = 0.38), laterally spaced narines, canthus rostralis almost flat, short and truncate snout, NH/ON = 1.35, prominent tympanic annulus, tympanic membrane covered with skin, vomerine teeth separated in the half line behind the lateral border of the elliptic coanes, robust forearms, nuptial pads on first finger. Hind limbs slender, tibio-tarsal articulation surpasses the anterior border of the eye orbit when hind limb is flexed toward the head. Relative length of toes: 4 > 3 > 5 > 2 > 1. Inner metatarsal tubercle present, outer metatarsal tubercle absent, interdigital membrane absent. Relative length of fingers : 3 > 1 > 4 = 2. Inner and external carpal tubercles present. Left epicoracoides superimposed to the right one, xiphisternum without notch, smooth dorsal and ventral skin, and a cutaneous tarso-metatarsal fold present, smooth plantar and palmar surfaces. Measurements are summarized in Table 1.

Coloration in alcohol. Small gray dots along the edge of the upper jaw. Gray dorsal surface with dark stripes, extending from behind the eyes to the dorsum, on both sides of the mid-line. A conspicuous white mid-dorsal strip, extending from the tip of the snout to the vent. Posterior half of the dorsum with irregular black spots, forming a geometric figure on both side of the mid-line. Two symmetrical black spots in the dorso-lumbar area. Postorbital black spots extending laterally to the proximal end of the arms. Ventral pale yellow with black pigmentation in the throat region. Anterior extremities with small black spots. Posterior limbs with transverse black lines.

Coloration in life. The coloration in life contrasts with that of the preserved animals. Dark spots are more

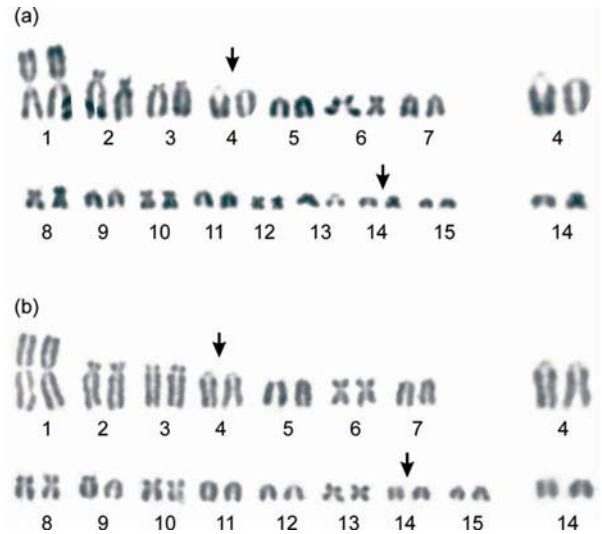


FIG. 4. Male (a) and female (b) karyotypes of *E. queulensis*. Arrows indicate the sexual chromosomes and the nucleolar chromosome pair. In the frames, these chromosomal markers are identified.

defined and the gray coloration presents a reddish tinge. Also, the iris of the live animal has a yellow upper part and brown lower part. The eyelid edge is blue-gray.

Variation in the coloration pattern. Other male and female specimens do not show a yellowish dorsal mid-line (Fig. 3).

Karyotype. Diploid number is $2n=30$ with a fundamental number (NF) of 45 in males and 44 in females (Table 2, Fig. 4). The sexual pair is heteromorphic with a metacentric Y chromosome. The largest pair of telocentric chromosomes is the nucleolar pair, depicting a secondary pericentromeric constriction.

Molecular markers. A mtDNA 12S fragment of 319 base pairs was amplified. Maximum Parsimony (MP) and Neighbour Joining analyses gave the same tree topology. MP resulted in a single tree of 70 steps, with a Consistency Index of 0.86 and a Retention Index of 0.66. In this tree, *E. queulensis* shares a clade with *E. calcaratus* and *E. vertebralis*, apart from species of different genera. Within this clade, *E. queulensis* is the sister species of *E. calcaratus*, with *E. vertebralis* as the sister taxon of that clade (Table 3, Fig. 5).

Natural history. Field observations were carried out at the Reserva Nacional Los Queules, nearby forest fragments and surrounding pine plantations. Within native Maulino forest, individuals were found only in moist habitats including shallow streams, soil cavities, leaf lit-

TABLE 3. Genetic distances among species studied. In bold are the averaged distances estimated using Kimura 2 parameter model (below diagonal) and in italics is the standard error estimated by 1000 bootstrap replicates (above diagonal).

Species	1	2	3	4	5	6
1. <i>Batrachyla taeniata</i>		<i>0.022</i>	<i>0.020</i>	<i>0.019</i>	<i>0.017</i>	<i>0.015</i>
2. <i>Caudiverbera caudiverbera</i>	0.136		<i>0.026</i>	<i>0.027</i>	<i>0.024</i>	<i>0.019</i>
3. <i>Eupsophus calcaratus</i>	0.111	0.178		<i>0.012</i>	<i>0.016</i>	<i>0.019</i>
4. <i>Eupsophus queulensis</i>	0.096	0.182	0.044		<i>0.015</i>	<i>0.019</i>
5. <i>Eupsophus vertebralis</i>	0.087	0.156	0.072	0.068		<i>0.015</i>
6. <i>Telmatobius marmoratus</i>	0.072	0.112	0.103	0.102	0.065	

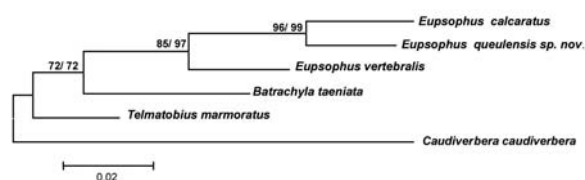


FIG. 5. Maximum Parsimony tree based on 12S mtDNA sequences for telmatobine species. The numbers indicate bootstrap values for the groupings. Bootstrap values are indicated on each node of the tree (MP/ML respectively) after 1000 pseudoreplicates.

ter and under logs. No individual was recorded in surrounding pine plantations. Vegetation in the Maulino forest is dominated by a deciduous tree, *Nothofagus glauca*, along with evergreen trees such as *Persea lingue*, *Gevuina avellana*, *Aetoxicum punctatum* among others (see Bustamante *et al.*, in press). Vocalizations, composed of a low frequency sound followed by an abruptly finishing crescendo, were heard from September to January. Male and female pairs were found together with egg masses or recently hatched embryos in soil cavities near streams. Also, larvae at stage 35-36 (Gosner, 1960) were found in shallow waters. Eggs have large amounts of yolk; neither eggs nor larvae exhibit pigmentation. In gravid females, eggs are visible through the skin at the lower part of the abdomen. These features are shared with other *Eupsophus* species (Díaz, 1986).

Geographic distribution. *Eupsophus queulensis* is known only from the type locality.

Etymology. The new species is named after the Reserva Nacional Los Queules, one of the few protected areas aiming to conserve the unique biota of the coastal Maulino forest.

DISCUSSION

Eupsophus is an endemic genus which comprises eight species: *E. calcaratus*, *E. contulmoensis*, *E. emiliopugini*, *E. insularis*, *E. migueli*, *E. nahuelbutensis*, *E. roseus* and *E. vertebralis*. As such, it is the most diversified genus of leptodactylids in the *Nothofagus* forests (Núñez, 2003). Some species have their geographical range restricted to their type locality, e.g., *E. contulmoensis* (Monumento Nacional Contulmo, Cordillera de Nahuelbuta), *E. nahuelbutensis* (Parque Nacional Nahuelbuta, Cordillera de Nahuelbuta) and *E. insularis* (Isla Mocha; Formas 1995). Others have broader distributions, and exhibit geographic replacement with latitude, such as *E. roseus* by *E. calcaratus* and *E. vertebralis* by *E. emiliopugini* (Núñez *et al.*, 1999). The geographic distribution of *E. queulensis* ought to be assessed by sampling beyond the Trehualemu area.

Eupsophus is characterized by medium-sized species, eggs and larvae without pigmentation and polymorphism in colour pattern. Morphologically, features that distinguish *E. queulensis* from *E. roseus* are a left epicoracoid superimposed to the right in the former,

while in *E. roseus* the opposite is true: the right being superimposed to the left. Iris colour in *E. queulensis* is yellow in the upper part and brown in the lower part, whereas it is orange in *E. roseus*. It differs from *E. calcaratus* by its truncate snout. It also differs from *E. insularis* in dorsal coloration, which is brown with irregular yellow spots as compared to the dark spots found in *E. queulensis*. The new species differs from *E. migueli* in dorsal coloration, in its two conspicuous spots at both sides of the half line in the lumbar region, and in presenting an unnotched xiphisternum. *Eupsophus queulensis* differs from *E. nahuelbutensis* in the colour pattern of the ventral region, which is whitish with dark-brown pigmentation in *E. nahuelbutensis* (Núñez, 2003). Finally, it differs from *E. contulmoensis* in presenting prominent external and internal carpal tubercles. Also, the ventral coloration of this last species is brilliant yellow (Formas, 1992).

Karyotypically, *Eupsophus* species can be grouped according to their diploid number (Formas, 1980; Iturra & Veloso, 1989; Formas, 1991; Cuevas & Formas, 1996, Núñez 2003). A first group is comprised by *E. emiliopugini* and *E. vertebralis* ($2n=28$) and a second one includes *E. calcaratus*, *E. contulmoensis*, *E. migueli*, *E. roseus* and *E. nahuelbutensis* ($2n=30$; Díaz & Veloso, 1979; Formas, 1991, 1992; Núñez, 2003), to which *E. queulensis* should be added. *Eupsophus queulensis* differs from its congeneric species in the location of the secondary constriction (Table 2). Three of these (*E. insularis*, *E. migueli* and *E. queulensis*) have sexual heteromorphic chromosomes in the same stage of differentiation, with an X telocentric and a Y metacentric chromosome, the male being the heterogametic sex. *Eupsophus roseus* also has a pair of sex chromosomes but with the same morphology (X and Y metacentrics) that can only be determined by banding techniques (Iturra & Veloso, 1989). Despite having the same $2n=30$ and sex chromosomes, *E. queulensis* differs from *E. migueli* because the former has a telocentric shape of the nucleolar pair (AgAsNOR) while in *E. migueli* is sub-telocentric.

Based on external morphology, karyotype and molecular data, the specimens collected at Trehualemu can be assigned to *Eupsophus*. Despite the reduced number of species evaluated, phylogenetic analysis of mitochondrial DNA confirms that the new species described here belongs to the genus *Eupsophus*; within this genus *E. queulensis* is closer to *E. calcaratus* than to *E. vertebralis*. High bootstrap values support this assertion (Fig. 5). Similarly, the differentiation of a pair of sexual chromosomes, shared with *E. insularis* and *E. migueli*, suggests a narrow phylogenetic relationship with these two species whose chromosomal system of sex determination (XY-type) is in the same stage of differentiation (Iturra & Veloso, 1989; Cuevas & Formas, 1996).

The Maulino coastal forest contains a high number of endemic and threatened species such as *Gomortega keule* (Gomortegaceae), *Pitavia punctata* (Rutaceae) and *Berberidopsis corallina* (Berberidopsidaceae)

among plants, *Dromiciops gliroides* (Microbiotheriidae), *Oncifelis guigna* (Felidae), *Pudu pudu* (Cervidae) among mammals, and *Megalometides discors* (Curculionidae) among insects. This unique biota is protected in the Reserva Nacional Los Queules.

The finding of more southern species, typical of the Valdivian forest, such as *D. gliroides*, *Geoxus valdivianus* and *Irenomis tarsalis* (Muridae), *Tachymenis chilensis chilensis* (Colubridae), *Liolaemus cyanogaster cyanogaster* (Iguanidae), *Protosphindus bellus* (Sphindidae) and *Pteroderes tuberosa* (Ulodidae) along with a new species of *Eupsophus*, previously regarded as a southern genus, suggests that the Maulino forest is the current northernmost limit of the Valdivian fauna, and also reinforces the importance of this reserve for the conservation of this unique biota (Simonetti, 2001; Saavedra & Simonetti, 2001; Grez et al., 2003). In Trehualemu, pine plantations are the dominant landscape feature, while native forest is restricted to small scattered remnants. The 145 ha Reserva Nacional Los Queules is one of the protected areas aiming to conserve the Maulino forest, which faces a high rate of destruction and replacement (Grez et al., 1997). The absence of *E. queulensis* from the extensive pine plantations suggest that, if restricted to native forest, *E. queulensis* could be threatened due to the scanty surface of native forest remnants.

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