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When exotic biocontrol agents travel without passport: first record of *Quadrastichus mendeli*, parasitoid of the blue-gum chalcid *Leptocybe invasa*, in Italy

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Abstract

The larval parasitoid *Quadrastichus mendeli* (Hymenoptera: Eulophidae: Tetrastichinae), a highly effective parasitoid of the invasive blue-gum chalcid *Leptocybe invasa* (Tetrastichinae) was discovered to have been accidentally introduced to Italy, where it was never released. Over the last three years, *Q. mendeli* has become widespread in central and southern Italy, determining in some places the almost complete disappearance of the gall wasp pest. We genetically characterized the parasitoid, confirmed its placement within the genus *Quadrastichus* by phylogenetic analysis, and evaluated the percentage of parasitisation at three localities. We provide other examples of parasitoids that crossed country boundaries without being released, ignoring long and expensive risk assessment aimed at limiting the importation and release of exotic natural enemies.

Key words: biological control, *Eucalyptus*, exotic pest, gall wasps, host tracking, natural enemies, risk assessment, silent invasions, Tetrastichinae.

Introduction

The blue-gum chalcid *Leptocybe invasa* Fisher et La Salle (Hymenoptera: Eulophidae: Tetrastichinae) is a gall wasp of many *Eucalyptus* species. In the early 2000s it was recorded in Italy (Viggiani *et al.*, 2002) and Turkey (Aytar, 2003), and later described for the first time (Mendel *et al.*, 2004). In a span of about 15 years, *L. invasa* has spread to all the five continents (CABI, 2015; Nugnes *et al.*, 2015), where it is detrimental to *Eucalyptus* plantations (Lawson *et al.*, 2012; Zheng *et al.*, 2014). The severe damages caused to eucalyptus trees worldwide urged searching for its natural enemies in the native area (most certainly Australia, where the gall wasp had never been recorded before) and invaded areas. Studies in the invasion range resulted in the identification of several hymenopteran parasitoids. Native *Megastigmus* spp. (Torymidae) were reared from *L. invasa* in Brazil, India, Israel, Italy, Thailand and Turkey (Viggiani *et al.*, 2002; Protasov *et al.*, 2008; Doğanlar and Hassan, 2010; Vastrad *et al.*, 2010; Doğanlar *et al.*, 2013; Sangtongpraow and Charernsom, 2013). *Aprostocetus gala* (Walker) and *Aprostocetus* sp. (Eulophidae Tetrastichinae), *Parallelaptera* sp. (Mymaridae) and *Telenomus* sp. (Platygasteridae) were also found in India (Vastrad *et al.*, 2010). However, most of these parasitoids were found sporadically and did not control the pest, except for *Megastigmus dharwadicus* Narendran et Vastrad and *A. gala* in India, which effectively limited the blue-gum chalcid populations (Ramanagouda and Vastrad, 2015). The situation in Australia is very different, as the blue-gum chalcid is not considered a pest to eucalyptus. This is due to the activity of indigenous natural enemies usually belonging to the subfamily Tetrastichinae, the most effective being *Selitrichodes krycery* Kim et La Salle (Kim *et al.*, 2008), *Selitrichodes neseri* Kelly et La Salle (Kelly *et al.*, 2012), and *Quadrastichus mendeli* Kim et La Salle among several species recorded in the genus *Quadrastichus* (Kim *et al.*, 2008).

Q. mendeli was released starting from 2007 as part of classical biological control programs in Israel first (Kim *et al.*, 2008), then in Kenya (K.E. Mutitu, pers. comm.) and India (Shylesha, 2008**)**. Based on the studies conducted in Israel by Kim *et al*. (2008), the percentage of parasitism of *L. invasa* by *Q. mendeli* reached 73%. After release, *Q. mendeli* was recovered from all sites, suggesting that this species had quickly established in Israel (Kim *et al*., 2008). Introduction to Kenya was instead a failure (Dittrich-Schröder *et al.*, 2014), while hitherto no information about releases in India is available.

Most *Quadrastichus* species are associated with galls, generally as endoparasitoids of Diptera (Cecidomyiidae and Agromyzidae), Hymenoptera (Cynipidae) (Kim *et al*., 2008), and gall-forming eriophyid mites (Vereshchagina, 1961), but the host range includes also Coleoptera (Curculionidae and Buprestidae) (Graham, 1991; La Salle, 1994; Noyes, 2015). This genus also includes one known phytophagous species, *Quadrastichus erythrinae* Kim, which is known to induce galls on *Erythrina* spp. and is itself an invasive pest (Kim *et al.*, 2004; Rubinoff *et al.*, 2010). The systematic position of *Q. mendeli* is controversial, as the validity of the genus *Quadrastichus* has been questioned repeatedly (Kostjukov, 1977; Graham, 1987; Bouček, 1988; Graham and La Salle, 1991).

Classical biological control programmes against exotic pests consists mostly in the release of exotic natural

Table 1. Specimens used for molecular analysis. Sequences downloaded from GenBank are reported in italics.

Code	Species	Host	Locality	Genbank Accession Code		
				28S-D2	COI	ITS2
*Qm*_1	*Quadrastichus mendeli*	*Leptocybe invasa*	Portici, Italy	KU133481	KU133509	KU133495
*Qm*_2	*Quadrastichus mendeli*	*Leptocybe invasa*	Portici, Italy	KU133482	KU133510	KU133496
*Qm*_3	*Quadrastichus mendeli*	*Leptocybe invasa*	Portici, Italy	KU133483	KU133511	KU133497
*Qm*_4	*Quadrastichus mendeli*	*Leptocybe invasa*	Ercolano, Italy	KU133484	KU133512	KU133498
*Qm*_5	*Quadrastichus mendeli*	*Leptocybe invasa*	Ercolano, Italy	KU133485	KU133513	KU133499
*Qm*_6	*Quadrastichus mendeli*	*Leptocybe invasa*	Ercolano, Italy	KU133486	KU133514	KU133500
*Qm*_7	*Quadrastichus mendeli*	*Leptocybe invasa*	SG Cremano, Italy	KU133487	KU133515	KU133501
*Qm*_8	*Quadrastichus mendeli*	*Leptocybe invasa*	SG Cremano, Italy	KU133488	KU133516	KU133502
*Qm*_9	*Quadrastichus mendeli*	*Leptocybe invasa*	SG Cremano, Italy	KU133489	KU133517	KU133503
*Qm*_10	*Quadrastichus mendeli*	*Leptocybe invasa*	Roma, Italy	KU133490	KU133518	KU133504
*Qm*_11	*Quadrastichus mendeli*	*Leptocybe invasa*	Roma, Italy	KU133491	KU133519	KU133505
*Qm*_12	*Quadrastichus mendeli*	*Leptocybe invasa*	Roma, Italy	KU133492	KU133520	KU133506
*Qm*_13	*Quadrastichus mendeli*	*Leptocybe invasa*	Roma, Italy	-	KU133521	-
*Qm*_14	*Quadrastichus mendeli*	*Leptocybe invasa*	Roma, Italy	-	KU133522	-
*Qm*_15	*Quadrastichus mendeli*	*Leptocybe invasa*	Roma, Italy	-	KU133523	-
Qe_1	*Quadrastichus erythrinae*	*Erythrina* sp.	Hawaii, USA	KU133493	*FJ872114*	KU133507
PnS1	*Pnigalio soemius*	*Holocacista rivillei*	Latina, Italy	*FJ812243*	*EF507495*	*GU361592*
Neo11	*Neochrysocharis formosa*	*Liriomyza sativae*	Sumitomo, Japan	KU133494	KU133524	KU133508
LI_CN_5	*Leptocybe invasa*	*E. globulus*	Hubian, China	*KP143991*	*KP233994*	*KP143966*
LI_IT_5	*Leptocybe invasa*	*E. camaldulensis*	Portici, Italy	*KP143973*	*KP233976*	*KP143947*
Bs_1	*Baryscapus silvestrii*	*Bactrocera oleae*	Portici, Italy	*KP233970*	*KP233995*	KU133525

enemies from the pest's native area. Up until 1998, there was no *ad hoc* regulation about the release of exotic natural enemies. Nevertheless, despite the numerous introductions carried out over the last century, very few negative effects had been reported (van Lenteren *et al.*, 2003; 2006). In the wake of such rare failures, and with the need to preserve indigenous biodiversity, a methodology to carry out a risk assessment for the introduction of exotic natural enemies was suggested (van Lenteren *et al.*, 2006) This methodology establishes some parameters (dispersion, establishment and host range of a beneficial organism) to be assessed before deciding whether to introduce an exotic beneficial species (van Lenteren *et al.*, 2006). Following an European directive, several Countries began enacting laws to regulate the introduction of exotic enemies. The Italian Ministry of Agricultural, Food and Forestry Policies, for example, made risk assessment procedures mandatory by the legislative decree 84/2012, article 7-bis.

In 2013, samples of a species that could be identified as *Q. mendeli* were collected from *L. invasa* galls on *Eucalyptus camaldulensis* Dehnhardt in Italy, although this parasitoid was never officially released. In the present study, we aimed at: 1) genetically characterizing the collected specimens by using ribosomal and mitochondrial DNA markers; 2) evaluating the phylogenetic relationships of this species with other *Quadrastichus* and with closely related genera; 3) assessing the effectiveness of *Q. mendeli* in controlling populations of *L. invasa* in some Italian areas. Finally, we critically evaluated the importance of this finding in the context of current regulations on the introduction of exotic beneficial species.

Materials and methods

In 2013, trees of *E. camaldulensis* over 10 years old infested with galls of *L. invasa* were sampled from different localities in Italy. Following the first finding of *Q. mendeli* in Portici, in 2014 and 2015 the monitored area was expanded to 10 localities covering four Regions in central and southern Italy (including Sicily). Leaves and sprouts harbouring galls were placed in sealed boxes and stored at room temperature (25 ± 3 °C) awaiting emergence of adult pest and parasitoid wasps. Soon after their emergence, wasps were killed in 95% ethanol and morphologically identified following the original description (Kim *et al.*, 2008).

Molecular characterization and phylogenetic analyses of *Q. mendeli*

DNA was extracted from 15 whole single individuals (table 1) by a non-destructive Chelex and proteinase K method modified as in Gebiola *et al.* (2009), and eventually treated and card mounted as in Gebiola *et al.* (2012). Three genes were sequenced: the mitochondrial cytochrome c oxidase subunit I (COI) and two ribosomal genes, the expansion segment D2 of the 28S ribosomal subunit (28S-D2) and the Internal Transcribed Spacer 2 (ITS2). The COI gene was amplified using the forward primer C1-J-2183 (Simon *et al.*, 1994) paired with COII primer C2-N-3400, a reverse complement of C2-J-3400 (Simon *et al.*, 1994) (5'-TCAATATCATTGATGTCCAAT-3'); 28S-D2 was amplified with primers D2F and D2R (Campbell *et al.*, 1993). PCR reactions and cycling conditions for COI were 1 min of initial denaturation at 94 °C, 40 cycles step at 94 °C for 30 sec, 48 °C for 1 min e 30 sec and

72 °C for 2 min, the amplification was completed by holding for 7 min at 72 °C and for 28S-D2 was set as described in (Gebiola *et al.*, 2009). For the amplification of ITS2, primers ITS2F (Campbell *et al*., 1993) and ITS2rev-Trich (Stouthamer *et al*., 1999) were used in PCR reactions as in Gebiola *et al.* (2010).

PCR products were checked on a 1.2% agarose gel stained with ethidium bromide and directly sequenced. All fragments with chromatograms showing ambiguous peaks were cloned. Amplicons were ethanol precipitated, ligated into the pGEM-T Easy plasmid vector (Promega), and cloned into *Escherichia coli* TOP10 competent cells (Invitrogen) according to the manufacturer's instructions. Transformants were PCR-screened with universal M13 vector primers, and inserts of the expected size were sequenced.

Chromatograms were assembled using BioEdit 7.0 (Hall, 1999) and edited manually. COI sequences were virtually translated to amino acids to detect frameshift mutations and nonsense codons using EMBOSS Transeq http://www.ebi.ac.uk/Tools/st/emboss_transeq/, and aligned manually, whereas ITS2 and 28S-D2 sequences were aligned using the G-INS-I algorithm in MAFFT 7 (Katoh and Standley, 2013). All sequences were deposited in GenBank with accession numbers reported in table 1.

Phylogenies were reconstructed using maximum likelihood (ML) in RAxML 7.0.4 (Stamatakis, 2006) on a concatenated ITS2-28S-COI alignment. A GRT+G+I evolutionary model was used, as selected by jModeltest (Posada, 2008). ML trees were obtained after 1000 multiple inferences on the original alignment, starting from a random most parsimonious tree, and default initial rearrangement settings and number of rate categories. ML branch support was based on 1000 rapid bootstrap pseudoreplicates, and clades were considered supported when bootstrap > 70%. In order to clarify the placement of *Q. mendeli* within *Quadrastichus*, all species of *Quadrastichus* available in GenBank were included. As outgroups, we selected taxa representative of Eulophidae subfamilies among those available in GenBank [*L. invasa*, *Baryscapus silvestrii* Viggiani et Bernardo for Tetrastichinae, the thelytokous *Neochrysocharis formosa* (Westwood) for Entodoninae and the thelytokous *Pnigalio soemius* (Walker) for Eulophinae]. If any of the three chosen markers were not available for these taxa, we sequenced them as described above for *Q. mendeli* (see table 1).

Evaluation of *Q. mendeli* effectiveness

Ten trees of infested *E. camaldulensis* were sampled at each of several locations (table 2), galls on leaves and sprouts collected from 10 branches/tree were placed in a sealed box, and emerged wasps were collected daily. *Q. mendeli* was considered present when at least one female was collected. The mean percentage of parasitisation was calculated as in Ovruski *et al.* (2004), that is, dividing the total number of emerged parasitoid by the sum of emerged parasitoids and hosts. All data are presented with standard errors.

Results

Spreading of *Q. mendeli*

Following the first record in October 2013 from Portici, *Q. mendeli* was later found in more localities of Campania Region, and in other Regions of Central (Lazio) and Southern (Apulia) Italy. Detailed record data are reported in table 2. Only female specimens were collected at all localities.

Molecular and phylogenetic analyses

COI sequences were obtained for *Q. mendeli* and for *N. formosa*. Sequences of 28S-D2 and ITS2 were obtained for *Q. mendeli*, *Q. erythrinae* and *N. formosa*. We also sequenced ITS2 for *B. silvestrii*. No intraspecific variation was recorded for either 28S-D2, ITS2 or COI. As all *Q. mendeli* specimens were identical at each gene, only one taxon was included in the phylogenetic analysis. Within the Tetrastichinae clade, two main clades were highly supported: one with *Aprostocetus monacoi* Viggiani that is sister to *L. invasa*, and one including all *Quadrastichus* species with the exception of *Quadrastichus haitiensis* (Gahan), which renders the genus paraphyletic (figure 1). Furthermore, a species available in Genbank as *Q. mendeli* from India resulted to be closely related with *Q. erythrinae*.

Table 2. Records of *Q. mendeli* in Italy. *Localities in which percentage of parasitisation was calculated. +Localities sampled only once.

Locality	Region	Coordinates (m a.s.l.)	First sampling	Presence	First record
Santa Maria al Bagno+	Apulia	40°07'N 17°59'E (4)	26.VII.2014		-
Gallipoli*+	Apulia	40°04'N 18°00'E (2)	28.VIII.2015	X	28.VIII.2015
Portici	Campania	40°48'N 14°21'E (29)	31.V.2013	X	23.X.2013
Ercolano	Campania	40°48'N 14°22'E (44)	03.VI.2014	X	03.VI.2014
San Giorgio a Cremano*	Campania	40°49'N 14°20'E (56)	03.VI.2014	X	28.IX.2014
Nola	Campania	40°52'N 14°31'E (58)	30.V.2014		-
Roma*	Lazio	41°54'N 12°21'E (79)	06.V.2014	X	04.VI.2015
Roma	Lazio	41°57'N 12°27'E (63)	06.V.2014	X	27.VIII.2015
Roma	Lazio	41°54'N 12°21'E (79)	06.V.2014	X	27.VIII.2015
Costa Saracena+	Sicily	37°18'N 15°07'E (22)	01.VIII.2013		-

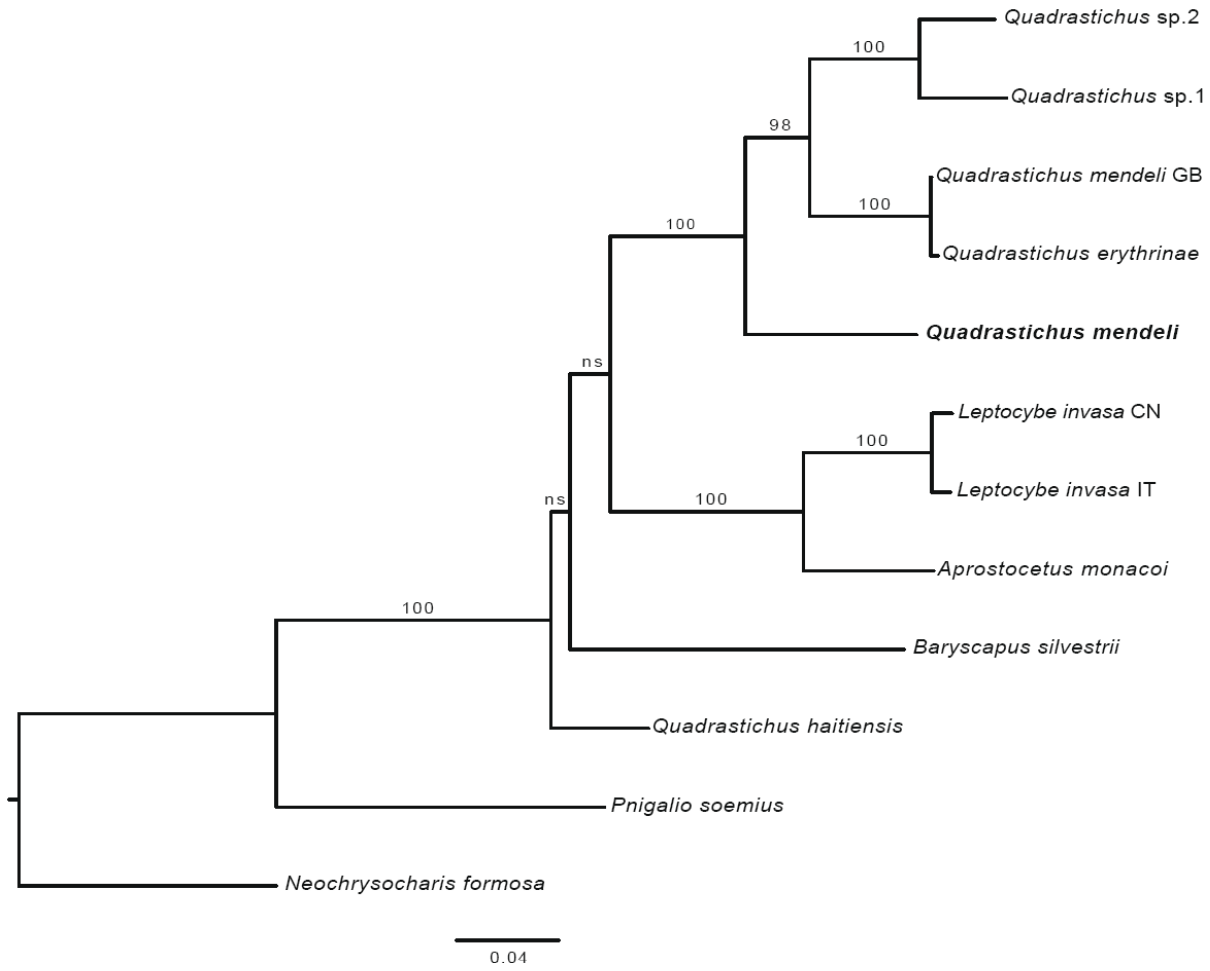

Figure 1. ML consensus tree for the concatenated 28S-D2-ITS2-COI dataset, bootstrap values > 70% are shown above branches. ns = not significant.

Evaluation of *Q. mendeli* effectiveness

The mean percentage parasitisation was 30.2 ± 8.10% (ranging from 0 to 61.1%) in Rome, it ranged from 0 to 100% with a mean of 38.0 ± 8.96% in Gallipoli, and from 27.3% to 100% with a mean of 50.5 ± 6.16% in San Giorgio a Cremano. It was not possible to calculate the percentage of parasitisation in Portici due to the nearly complete disappearance of *L. invasa* galls shortly after its first record.

Discussion and conclusions

Over the past 15 years, starting likely from Australia, *L. invasa* has spread at an extraordinarily fast rate to the other four continents, a speed that has few precedents (Wylie and Speight, 2012; Zheng *et al.*, 2014). The damages induced by this gall wasp are especially significant in Countries where *Eucalyptus* species are cash crops (Sánchez, 2003; Branco *et al.*, 2006; Costa *et al.*, 2008; Aquino *et al.*, 2011). *Q. mendeli* is considered the most effective parasitoid to contain the blue-gum chalcid (Kim *et al.*, 2008). We report it here for the first time from Southern and Central Italy, and Europe. By genetically characterizing this species, we confirmed that *Q. mendeli* belongs to a *Quadrastichus* clade, and also highlighted some inconsistencies. For example, *Q. haitiensis* does not cluster within the *Quadrastichus* clade, confirming that the placement of *Q. haitiensis* is still uncertain, as pointed out by LaSalle (1994) who provisionally placed *Tetrastichus haitiensis* Gahan in *Quadrastichus*. Furthermore, the only *Q. mendeli* ITS2 sequence available in GenBank is nearly identical to *Q. erythrinae*, indicating a case of morphological misidentification.

We only collected female specimens, confirming the thelytokous status of *Q. mendeli* (Kim *et al.*, 2008). Thelytokous species have a greater potential for biological control (Silva *et al.*, 2000), but the efficiency of the control by *Q. mendeli* is mainly due to its short life cycle. Indeed, developmental time (egg-adult) of *Q. mendeli* lasts about 30 days (Kim *et al.*, 2008), a period about five times shorter than that of its gall wasp host (132 days) (Mendel *et al.*, 2004). Wherever we found it, *Q. mendeli* showed mean percentages of parasitism varying from 30.2% to 50.5%. Although the parasitism is lower than in Israel (up to 73%) (Kim *et al.*, 2008), the parasitoid seems to be able to control *L. invasa* because in some places where the pest was abundant up until two years ago, galls have become very rare. Even though the distribution of the parasitoid is still patchy, as it is absent in some of the sampled ar-

eas (table 2), we predict that *Q. mendeli* will shortly reach other neighbouring countries, as happened already for other parasitoid species. For example, the eulophid *Semielacher petiolatus* (Boucek), released only in Israel (Argov and Rössler, 1996) and Morocco (Nia *et al.*, 1997) to control the citrus leafminer *Phyllocnistis citrella* Stainton, was recorded in Italy shortly after (Mineo and Mineo, 1999; Siscaro *et al.*, 1999; Viggiani, 2001). Similarly, the parasitoid *Thripoctenus javae* (Girault) (=*Thripobius semiluteus* Boucek) was found in Spain (Beltrá and Soto, 2011) just some years after its official release in Israel and Italy (Wysoki *et al.*, 1996; Viggiani and Bernardo, 1996) to control the greenhouse thrips *Heliothrips haemorroidalis* (Bouche). More generally, several cases are known of parasitoids that have host-tracked their hosts, either simultaneously or after the invasion of the pest. *Cirrospilus talitzkii* Boucek and *Pnigalio mediterraneus* Ferriere et Delucchi, parasitoids of *Cameraria ohridella* Deschka et Dimic, have tracked the horse chestnut leafminer in Italy and all over Europe (Radeghieri *et al.*, 2002; Gebiola *et al.*, 2014). In 2010 *Psyllaephagus bliteus* Riek (Encyrtidae) was recorded soon after the first finding of its host, the psyllid *Glycaspis brimblecombei* Moore (Caleca *et al.*, 2011). Similarly, in less than one year, two eulophids, the phytophagous *Ophelimus maskelli* (Ashmead) and its parasitoid *Closterocerus chamaelon* (Girault) were found in California (Burks *et al.*, 2015a; 2015b).

This work remarks the ability of both pests and beneficials to bypass human delimitations and efforts in keeping them tight. Although the monophagy of this parasitoid assures the absence of non-target effect, the introduction and inoculation of *Q. mendeli* in accord to European directives and national laws would have required a long and expensive procedure of risk assessment. We argue for the need of acknowledging that natural enemies often cross political boundaries and spread independently from human will, ignoring the timing of laboratory assessments.

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