Research Article

Absence of Cospeciation between the Uncultured *Frankia* Microsymbionts and the Disjunct Actinorhizal *Coriaria* Species

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*Coriaria* is an actinorhizal plant that forms root nodules in symbiosis with nitrogen-fixing actinobacteria of the genus *Frankia*. This symbiotic association has drawn interest because of the disjunct geographical distribution of *Coriaria* in four separate areas of the world and in the context of evolutionary relationships between host plants and their uncultured microsymbionts. The evolution of *Frankia-Coriaria* symbioses was examined from a phylogenetic viewpoint using multiple genetic markers in both bacteria and host-plant partners. Total DNA extracted from root nodules collected from five species: *C. myrtifolia*, *C. arborea*, *C. nepalensis*, *C. japonica*, and *C. microphylla*, growing in the Mediterranean area (Morocco and France), New Zealand, Pakistan, Japan, and Mexico, respectively, was used to amplify glnA gene (glutamine synthetase), dnaA gene (chromosome replication initiator), and the nif DK IGS (intergenic spacer between nifD and nifK genes) in *Frankia* and the matK gene (chloroplast-encoded maturase K) and the intergenic transcribed spacers (18S rRNA-ITS1-5.8S rRNA-ITS2-28S rRNA) in *Coriaria* species. Phylogenetic reconstruction indicated that the radiations of *Frankia* strains and *Coriaria* species are not congruent. The lack of cospeciation between the two symbiotic partners may be explained by host shift at high taxonomic rank together with wind dispersal and/or survival in nonhost rhizosphere.

1. Introduction

The genus *Frankia* comprises nitrogen-fixing actinobacteria that are able to induce perennial root nodules on woody dicotyledonous plants called actinorhizals [1]. The actinorhizal plant families belong to three dicotyledonous orders: Fagales (Betulaceae, Casuarinaceae, and Myricaceae), Rosales (Elaeagnaceae, Rhamnaceae, and Rosaceae), and Cucurbitales (Coriariaceae and Datiscaceae) [2]. Analysis of the molecular phylogeny of members of *Frankia* genus consistently identifies four main clusters regardless of the typing locus used [3]. Three symbiotic *Frankia* clusters containing strains able to establish effective nodules and fulfill Koch’s postulates and one atypical with strains unable to establish effective nodulation on their host plants have been defined among *Frankia* genera. Cluster 1 includes *Frankia* strains in association with Betulaceae, Myricaceae, and Casuarinaceae. Cluster 2 contains *Frankia* nodulating species from the Coriariaceae, Datiscaceae, and Rosaceae families as well as *Ceanothus* of the Rhamnaceae. *Frankia* strains in cluster 3 form effective root nodules on plants from members of the Myricaceae, Rhamnaceae, Elaeagnaceae, and Gymnostoma of the Casuarinaceae.

Symbiotic *Frankia* strains have been only isolated from Fagales (*Frankia* cluster 1) and the families Elaeagnaceae and Rhamnaceae (*Frankia* cluster 3) of the Rosales, while *Frankia* of cluster 2 have still not yet been isolated in culture despite repeated attempts [2]. The position in the *Frankia* phylogenetec tree of cluster 2 relative to the other clusters has varied depending on the marker used. It was proposed at the base using *glnA* and 16S rRNA genes [4, 5], derived with ITS 16S–23S rRNA genes [6] and concatenated gyrB, *nifH* and
2. Materials and Methods

2.1. DNA Extraction, PCR Amplification, and Sequencing. Root nodules from naturally occurring Coriaria species (Table 1) were kindly provided by Dr. María Valdés (Escuela Nacional de Ciencias Biológicas, México, DF, México), Dr. Sajjad Mirza (National Institute for Biotechnology Genetic Engineering, Faisalabad, Pakistan), Dr. Warwick Silvester (University of Waikato, Waikato, New Zealand), Dr. Kawther Benbrahim (University of Fes, Fes, Morocco), Dr. Takashi Yamanaka (Forest and Forestry Products Research Institute, Ibaraki, Japan), and Dr. Jean-Claude Cleyet-Mare1 (INRA-IRD, Montpellier, France). Individual lobes were selected, surface-sterilized in 30% (vol/vol) H$_2$O$_2$, and rinsed several times with distilled sterile water. The DNA extraction from single nodule lobes was performed as previously described by Rouvier et al. [26]. Nodule lobes were crushed with sterile plastic mortars and pestles in 300 μL of extraction buffer (100 mM Tris (pH 8), 20 mM EDTA, 1.4 M NaCl, 2% (wt/vol) CTAB (cetyltrimethyl ammonium bromide), and 1% (wt/vol) PVPP (polyvinyl polypyrrolidone)). The homogenates were incubated at 65°C for 60 min, extracted with chloroform-isomyl alcohol (24:1, vol/vol) and the resulting DNA was ethanol-precipitated and resolubilized. The extracted DNA was used for PCR amplification of both bacterial and plant DNA regions using the primers listed in Table 2. The amplifications were then cycle-sequenced in both directions using an ABI cycle sequencing kit (Applied Biosystem 3130). The nucleotide sequences obtained in this study were deposited in the NCBI nucleotide sequence database under the accession numbers given in Table 1.

2.2. Phylogenetic Analysis. Frankia strain Cc13 and Casuarina equisetifolia were used as outgroups in this study because they are physiologically distinct from the group studied yet phylogenetically close. The data sets were completed with homologous sequences present in the databases (Table 1). Alignments of Frankia glnA, dnaA, and IGS nifD-K and Coriaria matK and 18S rRNA-ITS1-5.8S rRNA-ITS2-28S rRNA were generated with ClustalW [27], manually edited with MEGA5.0 [28]. Bacterial and plant sequences were separately concatenated and then used to examine maximum-likelihood cladogram evolutionary relationships of each symbiotic partner using 1000 bootstraps by following the GTR + G base substitution model. The distance between the sequences was calculated using Kimura’s two-parameter model [29]. Phylogenetic trees were constructed using the Neighbor-Joining method [30] with 1000 bootstraps as implemented in MEGA 5.0. In parallel, a Bayesian inference was realized with MrBayes [32] using the GTR + G model and 1,000,000 generations.

A statistical test for the presence of congruence between Coriaria and Frankia phylogenies was evaluated through global distance-based fitting in ParaFit program [33] as implemented in CopyCat [34] and tests of random association were performed with 9999 permutations globally across both phylogenies for each association. An additional statistical test for correlation between geographical distances (obtained using http://www.daftlogic.com/projects-google-maps-distance-calculator.htm) and phylogenetic distances was made using Pearson’s r correlation implemented in the R software [35].

3. Results

To avoid taxonomic ambiguities, DNAs from both Coriaria hosts and Frankia microsymbionts were characterized on the same root nodule tissues. The method of DNA isolation from
| Species      | Locality coordinates/altitude (asl) | Nodule labels | Plant sequence accession number | Bacterial sequence accession number | References          |
|--------------|-----------------------------------|---------------|--------------------------------|-------------------------------------|---------------------|
| **Morocco**  |                                   |               | ITSI-ITS2 | mat | glnA | dnaA | IGS nifD-K |                          |
| Oued El Koub, Ouezzane: 35° 01' 879''N/05° 20' 565''E/140 m | CmMs1            | KC796592      | KC796601 | KC796522 | KC796532 | KC796582 | KC796555 | This study                |
|               | CmMs2            | KC796523      | KC796538 | KC796556 | KC796555 | This study |
|               | CmMs3            | KC796524      | KC796584 | KC796557 | KC796555 | This study |
|               | CmMs4            | KC796525      | KC796585 | KC796558 | KC796555 | This study |
|               | CmM1a            | KC796590      | KC796599 | KC796517 | KC796578 | KC796550 | This study |
|               | CmM1b            | KC796518      | KC796579 | KC796551 | KC796555 | This study |
|               | CmM1c            | KC796519      | KC796580 | KC796552 | KC796555 | This study |
|               | CmM2a            | KC796591      | KC796600 | KC796520 | —        | KC796553 | This study |
|               | CmM2b            | KC796521      | KC796581 | KC796554 | KC796555 | This study |
| **C. myrtifolia** | Bab Berred, Chefchaouen: 35° 00' 979''N/04° 58' 092''E/1290 m | CmM1a        |       |       |       |       |       | |
|               |                   | CmM1b        |       |       |       |       |       | |
|               |                   | CmM1c        |       |       |       |       |       | |
|               |                   | CmM2a        | KC796591 | KC796600 | KC796520 | —       | KC796553 | This study |
|               |                   | CmM2b        | KC796521 | KC796581 | KC796554 | This study |
| **France**   | Nyons, 44° 21' 46.50''N/5° 08' 21.82''E/259 m | CmNy1        | KC796598 | KC796603 | KC796531 | KC796591 | KC796564 | This study |
|               |                   | CmNy2        | KC796532 | KC796592 | KC796565 | This study |
|               |                   | CmNy3        | KC796533 | KC796593 | —       | This study |
|               |                   | CmNy4        | KC796534 | KC796594 | KC796566 | This study |
|               |                   | CmNy5        | KC796535 | KC796595 | KC796567 | This study |
|               | Montpellier, 43° 36' 51.48''N/3° 52' 23.97''E/41 m | CmF1         |       |       |       |       |       | This study |
|               |                   | CmF2         | KC796593 | KC796602 | KC796526 | KC796586 | KC796559 | This study |
|               |                   | CmF3         | KC796527 | KC796587 | KC796560 | This study |
|               |                   | CmF4         | KC796528 | KC796588 | KC796561 | This study |
|               |                   | CmF5         | KC796529 | KC796589 | KC796562 | This study |
|               |                   |             | KC796530 | KC796590 | KC796563 | This study |
| **Japan**    |                                   |               |       |       |       |       |       | |
| **C. japonica** | Tosa district, +33° 45' 39.18'', +133° 27' 42, 89''/10 m | CJA          |       |       |       |       |       | This study |
|               |                   | CJB          | KC796594 |       |       |       |       | This study |
|               |                   | CJC          | KC796537 | KC796504 | KC796577 | This study |
|               |                   | CJD          | KC796538 | KC796505 | KC796578 | This study |
|               |                   | CJE          | KC796539 | KC796506 | KC796579 | This study |
|               |                   |             | AF280101 |       |       |       |       | This study |
| **Pakistan** |                                   |               |       |       |       |       |       | |
| **C. nepalensis** | Murree, +33° 5' 15''N 73° 23' 25''E/33.9042''N 73.3903'E/2291.2 m | CnP1         | KC796597 | KC796607 | KC796536 | KC796503 | KC796576 | This study |
|               |                   | CnP2         | KC796544 | KC796508 | KC796584 | This study |
|               |                   | CnP3         | KC796545 | KC796509 | KC796585 | This study |
|               |                   | CnP4         | KC796546 | KC796510 | KC796586 | This study |
|               |                   |             | AF280103 |       |       |       |       | This study |

References: AF280102, AB016459, Yang et al., unpublished (Yokoyama et al., 2000 [19]).
| Species         | Locality coordinates/altitude (asl)                                                                 | Nodule labels | Plant sequence accession number | Bacterial sequence accession number | References             |
|-----------------|--------------------------------------------------------------------------------------------------|---------------|---------------------------------|------------------------------------|------------------------|
| **New Zealand** |                                                                                                  |               |                                 |                                    |                        |
| *C. arborea*    | Hapuku river, North Canterbury, South island: −42°23'42.24"N, +173°41'18.07"E/64 m              | CaNZ1         | KC796595                        | KC796542                           | This study             |
|                 |                                                                                                  | CaNZ2         | KC796543                        | KC796511                           | This study             |
|                 |                                                                                                  | CaNZ3         | KC796544                        | KC796512                           | This study             |
|                 |                                                                                                  |               |                                 |                                    |                        |
|                 |                                                                                                  |               |                                 |                                    |                        |
|                 |                                                                                                  |               |                                 |                                    |                        |
| **Mexico**      |                                                                                                  |               |                                 |                                    |                        |
| *C. microphylla*| Morelos, 99°30', 19°30' /2400 m                                                                 | CmicMx1       | KC796596                        | KC796547                           | This study             |
|                 |                                                                                                  | CmicMx2       | KC796548                        | KC796514                           | This study             |
|                 |                                                                                                  | CmicMx3       | KC796549                        | KC796515                           | This study             |
|                 |                                                                                                  |               |                                 |                                    |                        |
|                 |                                                                                                  |               |                                 |                                    |                        |
|                 |                                                                                                  |               |                                 |                                    |                        |
| **C. intermedia**|                                                                                               | AF280100      |                                 |                                    | Yang et al., unpublished |
| **C. terminalis**|                                                                                               | AY091817      |                                 |                                    | Yang et al., unpublished |
| **C. ruscifolia**|                                                                                               | AY091815      |                                 |                                    | Yang et al., unpublished |
|                 |                                                                                                  | AY091814      |                                 |                                    | Yang et al., unpublished |
|                 |                                                                                                  | AF280104      |                                 |                                    | Yang et al., unpublished |
| **C. sarmentosa**|                                                                                               | AY091816      |                                 |                                    | Yang et al., unpublished |
| **C. papuana**  |                                                                                                  | AY091861      |                                 |                                    | Yang et al., unpublished |
| **Datisca**     |                                                                                                  | AY968449      |                                 |                                    | (Persson et al., 2011 [50]) |
| **glomerata**   |                                                                                                  | AF485250      |                                 |                                    | Zhang et al., unpublished |
| **Casuarina**   |                                                                                                  |                |                                 |                                    | Forrest and Hollingsworth |
| **equisetifolia**|                                                                                               | AY864057      |                                 |                                    | Herbert et al., unpublished |
Table 2: Primers used for PCR amplification and DNA sequencing.

| Gene primers | Sequence (5'-3') | Amplicons approximate size (bp) | References |
|--------------|-----------------|-------------------------------|------------|
| **glnA**     |                 |                               |            |
| DB41         | TTCTTCATCCAGCCCGT 500 |                               | (Clawson et al., 2004 [4]) |
| DB44         | GGCTCGGCATGAAGGT 700 |                               |            |
| **dnaA**     |                 |                               |            |
| F7154_dnaAF  | GAGGARTTCACCAACAGCTCTCAT 700 |                               |           |
| F7155_dnaAR  | CRGAAGTGCTGCCGATCTTT 500 |                               | Bautista et al. unpublished |
| **IGS nifD-K** |                  |                               |            |
| F9372_nifD1 5 | GTCATGCTCGCGCTGCGNG 700 |                               | This study |
| F9374_nifK1 5 | GTCATTCTCCGGTAyTCCA 700 |                               | This study |
| F9373_nifD2 5 | ACCGGCTACGAGTTCGCNCA 700 |                               |           |
| F9375_nifK2 5 | TGGCACACGCTGACCAGNG 700 |                               |           |
| **18S-ITS1-5.8S-ITS2-28S** | |                               |            |
| ITS1         | TCCGTAAGTGAACCTGCGG 700 |                               | (White et al., 1990 [52]) |
| ITS4         | TCTCTCGGCTTTATGATGTC 400 |                               |           |
| **F9030-CJ-ITSF** |                |                               |            |
| F9030-CJ-ITSF| AGCCGGACCCCGCGAGGCAGTT 400 |                               | This study |
| F9031-CJ-ITSR| CGACGGTCGTAAGCGACGCCCA 700 |                               |           |
| **matK**     |                 |                               |            |
| F9249-matKF  | ACATTTAAATATGTCGAG 700 |                               | This study |
| F9250-matkR  | TGCATATACGTATCAGCAATA 700 |                               |           |

root nodules used in this study yielded PCR-amplifiable DNA for both bacterial and plant PCR target sequences in all cases. However, in several instances it was easier to amplify Frankia than Coriaria DNA, which may have been mostly due to the specificity of the primer sets used. Thus, in this study, new primers were designed (Table 2).

For the bacterial microsymbionts, the average uncorrected \( p \)-distances (proportion of differences between sequences) were computed for each region and were found to be relatively small for dnaA \( (p = 0.0378) \), intermediate for glnA \( (p = 0.0625) \), and high for IGS nifD-K region \( (p = 0.0833) \). Blast analyses of the individual genes permitted assigning them all to Frankia cluster 2. Nearly 3000 nucleotides were obtained by concatenating sequences of the three DNA regions.

Sequences variation for Coriaria species was small based on matK gene \( (p = 0.0205) \) compared to ITS1-ITS2 sequences \( (p = 0.0423) \). By concatenating matK and ITS1-ITS2 region, a composite sequence of 1500 nt was used for phylogenetic inference.

All studied sequences were analyzed independently to test for incongruence between the data sets for each symbiotic partner. Similar topologies have been generally observed between phylogenetic trees inferred from glnA, dnaA, and IGS nifD-K sequences for Frankia and from matK and ITS sequences for Coriaria regardless of the used phylogenetic methods (not shown).

The topologies of the trees obtained for the two symbiotic partners were not congruent (Figure 1). Moreover, global distance-based ParaFit analysis recovered mostly random associations between Frankia and Coriaria host plant species \( (p = 0.33) \) and rejected cospeciation hypothesis. On the microbial side, the New Zealand microsymbionts were at the root (Group A); then three groups emerged, group B comprising the Pakistani, Mexican, and Mediterranean symbionts from France, group C comprising microsymbionts from Morocco, and then group D comprising French and Japanese microsymbionts as well as the Dg1 reference sequence obtained initially from a Pakistani soil. On the host plant side, group 1 at the root comprises New Zealand and South American sequences, while group 2 comprises the Japanese, Mediterranean, and Pakistani sequences.

On the other hand, no significant correlations were found for Frankia symbionts \( (r^2 = 0.772; \text{Fgenetic dist} = (\text{geog dist} \times 5.830E^{-06}) + 2.541E^{-02}) \) nor for the Coriaria host plants \( (r^2 = 0.883; \text{Fgenetic dist} = (\text{geog dist} \times 2.023E^{-06}) + 6.460E^{-03}) \) (data not shown).

4. Discussion

Cospeciation has been postulated to have occurred in some Frankia actinorhizal host plants, in particular in the Casuarina-Frankia cluster 1b [18] but not in Alnus-infective and Elaeagnus-infective Frankia strains where many isolates able to fulfill Koch’s postulates have been obtained. To test if cospeciation was general or an exception, it was decided to study uncultured Frankia microsymbionts and representative Coriaria hosts, a lineage where no Frankia isolate exists and where geographic discontinuities may have limited dispersion. DNA sequences were obtained from root nodules collected from New Zealand (C. arbores), Pakistan (C. nepalensis), Japan (C. japonica), Mexico (C. microphylla), and France and Morocco (C. myrtillo) and multiple molecular markers were analyzed for phylogenetic inference.
Paleontological data based on macrofossils and pollen fossils have brought several authors [36–40] to conclude that the Coriariaceae had a Laurasian origin (North America and Eurasia). There have been a few dissenting opinions, in particular those of Croizat [41] and Schuster [42] who considered that Coriaria originated in Gondwana and migrated to the Northern Hemisphere. However, such paleontological studies are not very convincing, as it is recognizably hard to ascribe fossils to a given family and even more so to a given genus. Thus, several authors have been surprised by the results of molecular phylogeny positioning Coriariaceae close to the Datiscaceae. Molecular approaches would thus give support to a Gondwanan origin.

Yokoyama et al. [19] proposed that Coriaria species had emerged 59–63 million years ago, which is coherent with the date of 70 million years proposed by Bell et al. [25], considerably older than that proposed (30 million years) by the same authors for the Casuarinaeae.

Topology and clustering of Coriaria phylogeny obtained in the current study are similar to those obtained by Yokoyama et al. [19], while the position at the base of the host plant species from New Zealand, C. arborea, and the South American C. ruscifolia and C. microphylla species was contrary to that of Yokoyama et al. [19] who found the Eurasian species at the base using rbcL (a large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase) and matK (maturase K) genes. The present study suggests that the Coriaria ancestor may have emerged between Asia and NZ and then dispersed worldwide and that the Asian lineage may have given rise relatively recently to the Mediterranean species, while the NZ lineage gave rise to the North American species (Figure 2).

Previous studies had concluded that Frankia cluster 2 had a low genetic diversity [6, 7, 16] but these studies had been focused on only part of the full diversity of the symbiotic Coriaria-Frankia, essentially in North America and Mediterranean. In this work we aimed to expand the scope of the study to the worldwide diversity and phylogeny of microsymbionts of Coriaria species. Four microbial subgroups were identified that did not fit to the geographic range of the host plants, while two host plant subgroups were identified. The position of subgroup A containing microsymbionts of New Zealand C. arborea at the base of Frankia cluster 2 is in agreement with previous study [16]. In view of previously
reported data, members of cluster 2 Frankia studied here were found to have relatively higher sequences variation (p-distance = 0.0625) than those reported by Vanden Heuvel et al. [16] (p = 0.00454) based on the same 460 nt of the glnA gene.

Molecular clock dating suggests that Frankia genus has emerged much earlier, 125 Myr bp before the appearance of angiosperm fossils in the Cretaceous period and the extant actinorhizal plants [4]. Normand et al. [5] using the 4% divergence in the 16S rRNA between cluster 2 and other Frankia lineages as equivalent to 50 MY/1% distance [17] concluded that the genus Frankia had emerged long before the extreme dicotyledonous lineages. These authors proposed Frankia cluster 2 as the proto-Frankia as nonsymbiotic ancestor of 62–130 Myr bp [43] and 100–200 Myr bp [5]. Since the distance in the 16S rRNA gene between cluster 1a (Frankia alni) and cluster 1b is less than 1%, the date of emergence of the Casuarina-infected lineage has been proposed to be less than 50 million years [5]. Thus the Casuarina/Frankia 1b lineage is considerably younger than the Coriaria/Frankia lineage and would have had less time to migrate out of its cradle and mingle with other hosts in its new territories and lose the cospeciation signal.

Symbiotic partnership often tends to become obligatory, as in the case of Casuarina host plants, where Frankia is only present in soils close to the host plant [44], which means that the bacterium loses autonomy and becomes dependent on its host. Speciation of the host could then lead to synchronous speciation of its microsymbiont unless dispersal through long-distance carriers such as winds or migratory birds occurred or if there is survival of Frankia cluster 2 in the rhizosphere of nonhosts as was recently demonstrated for Alnus glutinosa in Tunisia [45]. The numerous transitions seen in the Frankia phylogenetic tree from one continent to another would reinforce the idea.

Yokoyama et al. [19] concluded from their study of the Coriaria species phylogeny that the Eurasian species had diverged earlier and are more diverse than other groups, but that nevertheless the origin of the genus could have been in North America, whence the South America and the Pacific species could have originated. Our study brings us to suggest a third possibility, Oceania, which could also be the origin of this actinorhizal symbiosis, which can be concluded from phylogenetic inferences positioning both bacterial and host plant partners as at the base to Frankia-Coriaria symbiosis. Another element that would support this hypothesis is the large number of extinct species there; according to Yokoyama et al. [19] New Zealand would be home to 8 of the 17 existing species. A similar argument has often been made to establish Sub-Saharan Africa as the cradle of humankind [46] or Mexico for maize [47].

Comparison of both the plant and the microbe phylogenetic topologies did not show any evidence for cospeciation of Frankia microsymbionts and their Coriaria host species. The results obtained in this study suggest that Frankia microsymbionts hosted currently by Coriaria species had probably dispersed globally as a proto-Frankia, a free living and nonsymbiotic ancestor. In parallel, the proto-Coriaria then diversified into the extant Coriaria species that appear to have been retreating given their scattered distribution, a trend...
possibly reinforced recently due to man uprooting because of the toxicity of the fruits for mammals [48, 49]. It can thus be hypothesized that *Coriaria* appeared in the Pacific Islands more than 70 million years ago and presumably was symbiotic from the start, before dispersing over all continents as they drifted apart. The *Coriaria* species diversified in their different biotopes, as they saw the appearance of other plants hosting the same microsymbiont of *Frankia* cluster 2 such as Datisca canadensis, *Rosaceae*, *Ceanothus*, or even nonhost species such as *Alnus glutinosa* that was recently found to host *Frankia* cluster 2 in its rhizosphere [45]. Members of these alternative host plant species cooccur sympatrically with *Coriaria* such as *Ceanothus* and *Purshia* species in Mexico and *Datisca cannabina* in Pakistan. These *Frankia* cluster 2 host plant species have more extended geographic distribution and overlap in some instances *Coriaria*’s disjunct area and as a result can compensate *Frankia* microsymbionts remoteness, which would thus obscure the cospeciation signal. Cospeciation may also occur but subsequently is lost after bacterial mixing and fitness selection in the presence of “indigenous” and “dispersal” symbionts.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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