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Variability of Deltamethrin-Resistant *Metarhizium anisopliae* Aggressive Strains Group to a Population of the Cossid Moth from *Eucalyptus nitens*

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Abstract

Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) was used to examine the genetic variability among *Metarhizium anisopliae* isolates tested to the cossid moth, *Coryphodema tristis*. All the isolates tightly clustered into one or the other of two groups that diverged at 12%. Results suggested that certain genotypes of the fungus, that grouped together, were able to infect moth larvae while others did not. A fragment of 760 bp, which presents high homology with a host-adaptation related protein coding gene, distinguished between aggressive and non-aggressive isolates. Neither mycelial growth nor sporulation rate or presence of known virulence genes was correlated with mortality values. Some isolates, including the most aggressive isolate ARSEF2518, were compatible with deltamethrin. Deltamethrin treatment killed all the larvae after seven days whereas fungal and mixed treatments respectively reached the same mortality after 28 and 21 days.

Keywords

Coryphodema tristis, *Metarhizium anisopliae*, Assays, Compatibility with Deltamethrin, RAPDs

1. Introduction

Coryphodema tristis (Drury) (Lepidoptera: Cossidae) is native to South Africa

and has long been associated with exotic plants such as vines, apples and quince in the Western Cape. It also feeds on multiple native trees. Since 2004, it has been found feeding on *Eucalyptus nitens* in the Carolina-Badplaas-Lothair area in South Africa, resulting in gregarious feeding behavior and extensive damage (Boreham, 2004). Adult females lay eggs on the bark of trees. As the larvae grow, they bore into the cambium, where they cause tunneling which results in severe damage to the trees which often die. The cossid moth has a two-year life cycle in the Western Cape, but the duration of the life cycle in the summer rainfall area is not yet known. Both the main trunks and branches are attacked.

Neither insecticides nor natural enemies are currently in use or registered for the cossid moth. More effective and sustainable control systems should thus be developed, as for example the recent pheromone determination (Bouwer et al., 2015). This study evaluates twelve *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) isolates aggressiveness and their mycelial growth, sporulation rate, virulence genes presence, and insecticide-compatibility. The genetic variability of the assayed population was also determined. A selected isolate, based on the indicated parameters results, was tested in simulated field conditions.

2. Material and Methods

2.1. Bioassays

Initially, infectivity assays were performed. Infested *E. nitens* logs were obtained at Lothair area in South Africa in February 2013, after what *C. tristis* larvae were collected opening these logs with a log hydraulic chopper. Larvae were pre-sorted into separate Petri dishes (60 mm diam.) each containing four larvae, and held at 10˚C in laboratory one week prior to bioassay. All isolates (Table 1) were cultured on PDA at 20˚C for 2 weeks. Conidia were harvested into 0.01% Tween 80 and concentrations of 0, 10^5, 10^7, 10^9 conidia/ml were prepared. Conidial viability > 95% was confirmed by spread-plating 0.1 ml of the 10^5 suspension onto PDA, incubation at 20˚C and germination rate determination after 96 h. In a complete randomized block design at 20˚C and 16:8 h light/dark cycle, each specimen was dipped in the correspondent spore or control suspension decanted into on-ice 5-ml sterile tubes, inverted five times, and transferred to its insect batch into a second series of dishes with 10 ml diet (10 g semi-skimmed power milk, 44 g honey, 20 g agar per 1000 ml distilled water), and sealed with Parafilm (4 larvae/container, 5 replicate containers, 20 larvae/concentration, 4 concentrations, 80 larvae/isolate). Assays were observed every day for 30 days. At each observation, cadavers were removed from the treated arena and placed into cross-referenced dishes with moist filter paper until infection confirmation by sporulation observation. Experiment was repeated twice using insects collected on two occasions and freshly-produced conidia. Daily recorded mortality was corrected for control mortality and mean cumulative mortality was plotted against 30 days.

Table 1. Source, colony growth, sporulation rate and virulence genes presence among the tested twelve *Metarhizium anisopliae* isolates.

	ARSEF accession no.	Source	Colony area (mean ± SE, mm^2)	Sporulation rate (×10^{11} conidia/mL)	pr1A gene	pr1B gene
1.	798	*Aeneolamia varia* (Homoptera)	2209.30 ± 166.50[ab]	1.546 ± 0.05[c]	+	+
2.	1080	*Helicoverpa zea* (Lepidoptera)	2030.17 ± 85.32[ab]	0.001 ± 0.02[a]	+	+
3.	1489	*Ostrinia nubilalis* (Lepidoptera)	2856.21 ± 110.00[b]	2.586 ± 0.03[d]	+	+
4.	2421	*Nilaparvata lugens* (Homoptera)	1417.58 ± 172.83[ab]	0.004 ± 0.05[a]	+	+
5.	2517	*Deois flavopicta* (Homoptera)	2960.96 ± 86.25[b]	22.160 ± 0.02[h]	+	+
6.	2518	*Conoderus* sp. (Coleoptera)	2983.96 ± 83.25[b]	8.370 ± 0.05[f]	+	+
7.	2786	(Orthoptera: Gryllotalpidae)	2086.50 ± 113.50[ab]	0.995 ± 0.04[b]	+	+
8.	3187	Soil	2818.58 ± 51.62[b]	0.860 ± 0.03[b]	+	+
9.	3621	*Aeneolamia varia* (Homoptera)	2710.21 ± 59.00[ab]	8.469 ± 0.01[f]	+	+
10.	5471	*Eoreuma loftini* (Lepidoptera)	2568.62 ± 15.87[ab]	1.445 ± 0.03[c]	+	+
11.	6546	*Otiorhynchus sulcatus* (Coleoptera)	2487.75 ± 154.95[ab]	13.440 ± 0.05[g]	+	+
12.	7487[a]	*Schistocerca gregaria* (Orthoptera)	2382.85 ± 226.85[ab]	4.920 ± 0.05[e]	+	+

[a]Ex-type isolate. Means with the same letter are not significantly different ($P > 0.05$), by ANOVA followed by Tukey test. Isolates were incubated on PDA plates at 20˚C for 15 days and 16:8 h L:D. + Positive amplicons.

2.2. Compatibility with Deltamethrin

To evaluate *M. anisopliae* isolates sensitivity to deltamethrin (Decis 1.5% w/v; Aventis), potato dextrose agar (PDA) media were amended with deltamethrin at 0, 0.0125, 0.025, or 0.05 μg/ml of active ingredient. Six replicates of each concentration were used for each isolate. Five millimeter diameter mycelial plugs taken from the edge of a two weeks-old colony of each isolate were placed onto PDA plates amended with each concentration. After the plates were incubated at 20˚C for 7 days in the dark, occupied areas were measured. Besides, eight control, eight deltamethrin-treated, eight ARSEF2518-treated, and eight mixed-treated *E. nitens* logs (20 cm long) were placed on emerging cages. Logs were respectively sprayed with 400 ml of sdH$_2$O (negative control), sterile aqueous solution of Decis (0.002%, v/v), sterile aqueous solution of a conidial suspension of 10^9 conidia/ml (1%, v/v), and a mixed treatment solution. Ten *C. tristis* larvae were added to each log. Every week during one month, larvae mortality percentage

was measured on two logs of each type and compared by using ANOVA using SPSS Statistics (version 19).

2.3. Mycelial Growth, Sporulation Rate, and Virulence Genes Detection

To study mycelial growth, six replicate two weeks-old mycelial plugs (0.5 cm diam.) were placed face down at the centre of 60-mm dish containing 10 ml of PDA. After 15 days at 20°C and 16:8 h L:D, occupied areas were traced and measured. Sporulation rate was estimated by collecting conidia with 4 ml of 0.01% Tween 80 at 200 rpm for 30 min at 22°C.

As regards some virulence genes detection, PCR amplification of Pr1a protease gene was performed by nested PCR with outer primers METPR1 (5'-CACTCTTCTCCCAGCCGTTC-3'), METPR4 (5'-GTAGCTCAACTTCTGCACTC-3'), and inner primers METPR2 (5'-AGGTAGGCAGCCAGACCGGC-3') and METPR5 (5'-TGCCACTATTGGCCGGCGCG-3'). The template DNA was amplified in a 50 µL PCR reaction volume, consisting of 5 µL of 10X Reaction Buffer, 5 µL of $MgCl_2$ (25 mM), 5 µL of dNTPs (10 mM), 1 µL of each primer (10 µM), 1 µL of DNA solution and 0.5 µL of Super-Therm Taq polymerase. PCR reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems) with an initial denaturation step of 4 min at 95°C. This step was followed by 40 cycles of denaturation at 95°C (60 s), annealing at 58°C (60 s) and elongation at 72°C (2 min). A final extension was conducted for 8 min at 72°C. The second PCR used 1 µl of the first PCR as template. Products were visualized under UV on a 1% agarose gel stained with Gelred (Biotium), run in a Wide Mini-Sub Electrophoresis System at 90V, 400 mA for 30 min and digitalized in a ChemiDoc Gel Documentation System (BioRad). Pr1b protease gene was amplified using the same PCR and conditions but with primers Pr1B1, Pr1B2, and inner primers Pr1B3 and Pr1B4 (Wang et al., 2002).

2.4. Screening of RAPD Markers

To determine the genetic variability of the tested isolates, a total of twenty-one 10-mer random primers (Inqaba Biotech) were screened (Table 2). PCRs were carried out in volumes of 50 µl containing 5 µL of 10X Reaction Buffer, 3 µL of $MgCl_2$ (25 mM), 4 µL of dNTPs (10 mM), 2.5 µL of primer (10 µM), 1.5 µL of DNA solution and 0.5 µL of Super-Therm Taq polymerase. Conditions were 94°C for 4 min, followed by 35 cycles at 94°C (60 s), 47°C (60 s), 72°C (2 min), and then 72°C for 7 min. Electrophoresis was as before but through 2% agarose gels for 90 min. A genetic similarity matrix was calculated using the method of simple matching coefficient. The matrix was used to perform hierarchical cluster analysis based on the un-weighted pair-group method using arithmetic averages using GelQuest and ClusterVis (Sequentix). One diagnostic marker was recovered using the QIAquick gel extraction kit (Qiagen), sub-cloned into the pGEM-T easy cloning vector (Promega), and both stranded sequenced.

Table 2. Twenty-one decamer random primers used for the screening of RAPDs.

Primer name	Sequence (5'-3')
OPE-01	CCCAAGGTCC
OPE-04	GTGACATGCC
OPE-06	AAGACCCCTC
OPE-08	TCACCACGGT
OPE-10	GTGATCGCAG
OPE-15	ACGCACAACC
OPE-16	GGTGACTGTG
OPE-20	AACGGTGACC
PBAM	CGCCTAGGTA
OPA-01	CAGGCCCTTC
OPA-04	AATCGGGCTG
OPA-05	AGGGGTCTTG
OPA-08	GTGACGTAGG
OPA-09	GGGTAACGCC
OPA-14	TCTGTGCTGG
OPA-15	TTCCGAACCC
OPA-16	AGCCAGCGAA
OPA-19	CAAACGTCGG
OPF-02	GAGGATCCCT
PB-07	GACGCAGTCC
OPB-09	TGGGGGACTC

3. Results

Obtained results indicate considerable variation in aggressiveness among isolates, being isolates number 6, 3, 11 and 5 pathogenic whereas the rest of the isolates were not pathogenic. Isolate number 6 (ARSEF2518) presented the highest cumulative mortality value (Figure 1(a)). Mean lethal dose values could not be calculated through probit analysis because pathogenic isolates were only effective at the highest assayed conidia concentration.

Among the twelve isolates, isolates number 2, 3, 6, 8, 9, 10 and 11 growth was not significantly reduced by the tested deltamethrin concentrations (Figure 1(b)). Deltamethrin treatment killed all the larvae after 7 days. Treatment with ARSEF2518 fungus and mixed treatments respectively reached the same mortality percentage after 28 and 21 days (Figure 1(c)).

Neither mycelial growth (Pearson correlation coefficient (P) = 0.516, F = 0.086) nor sporulation rate (P = 0.360, F = 0.250) data were correlated with cumulative mortality values. Tested virulence genes were present among all the isolates (Table 1).
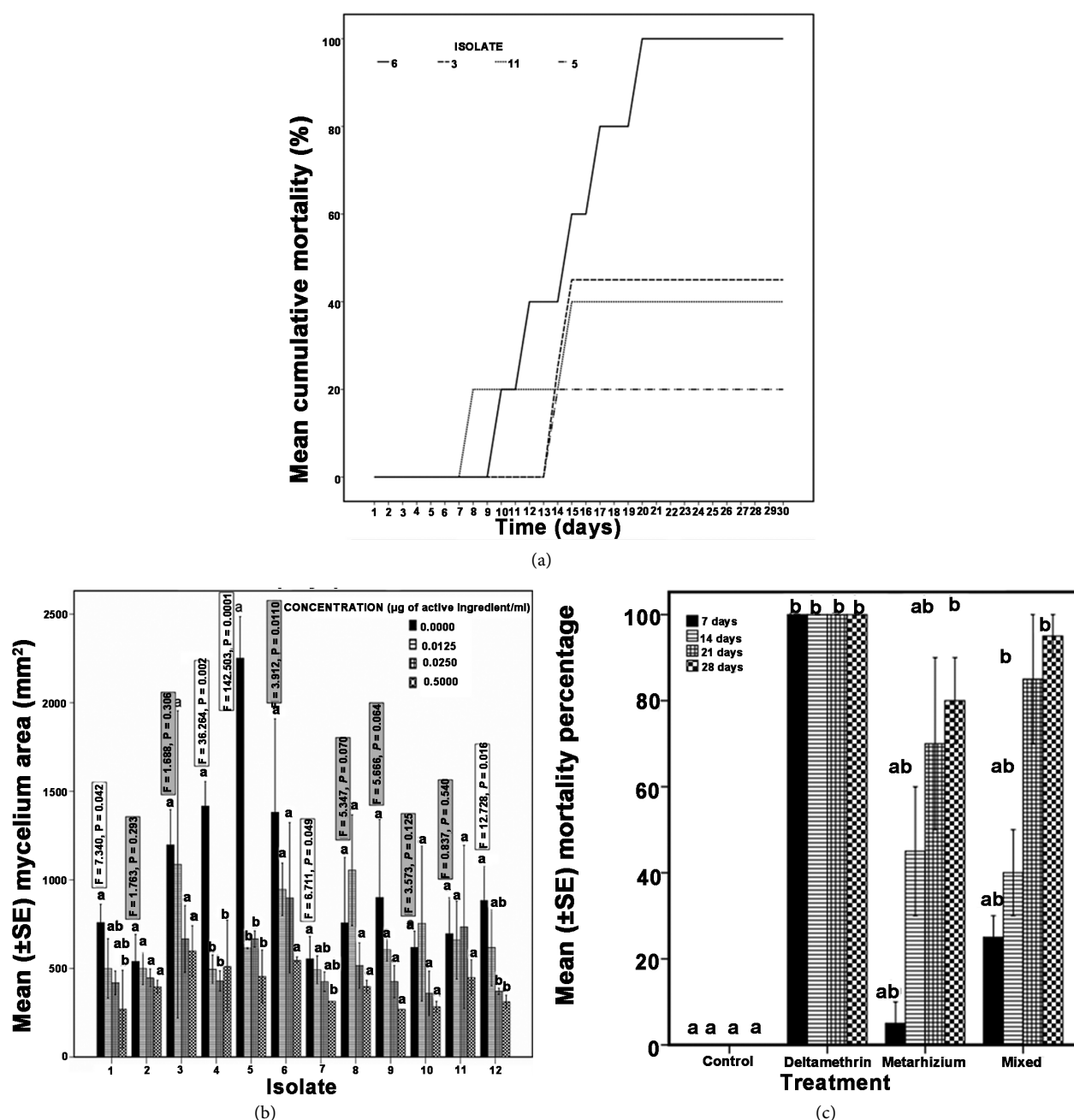
(a)

(b)

(c)

Figure 1. (a) Mortality of *Coryphodema tristis* larvae after inoculation with *Metarhizium anisopliae* isolates (curves correspond with four aggressive isolates at the highest tested conidia concentration 10^9 conidia/ml). (b) Colonized area of twelve *M. anisopliae* isolates grown for one week at 20°C on potato dextrose agar (PDA) media amended with deltamethrin (Decis 1.5% w/v; Aventis) at 0, 0.0125, 0.025, or 0.05 μg of active ingredient/ml. (c) Larvae mortality percentage when infesting *E. nitens* logs (20 cm long) respectively sprayed with four treatments. See experimental details. Means with the same letter are not significantly different ($P > 0.05$), by ANOVA followed by Tukey test. Isolates details given in Table 1.

Aggressive isolates grouped together in the topology of the 21 decamer primers (Table 2)-based consensus tree (Figure 2). Cluster analysis of the banding patterns revealed two tightly clustered groups among the *M. anisopliae* isolates. Group 1 consisted of six isolates with a mean similarity of 88.6%, and group 2 consisted of six isolates with a mean similarity of 89%. Group 1 and group 2
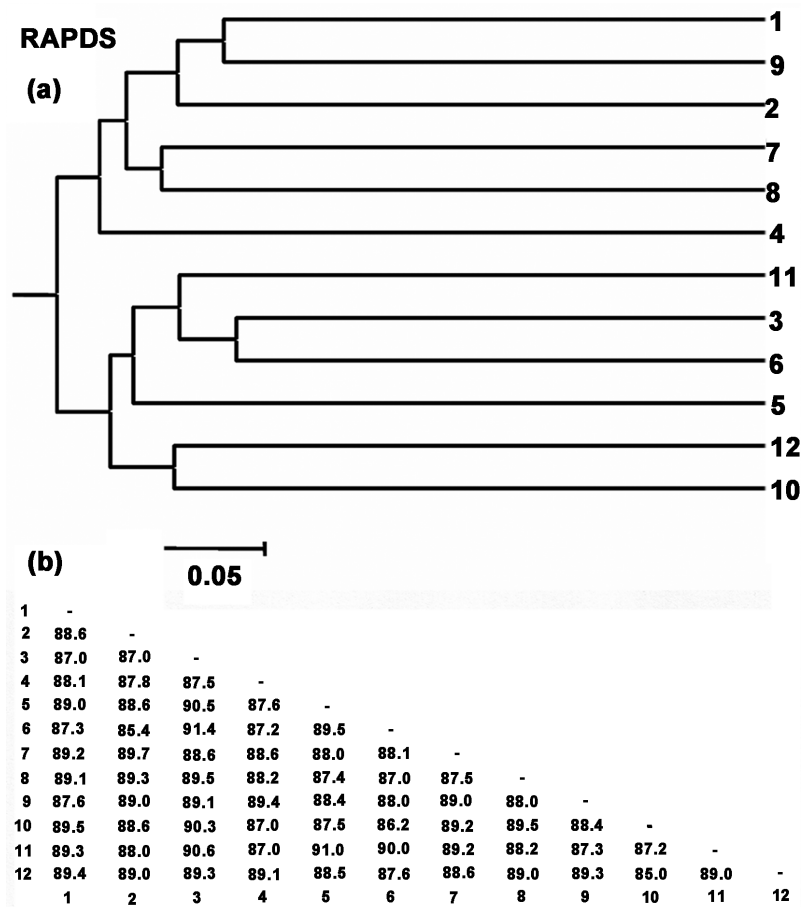
RAPDS

(a)

(b)

1	-										
2	88.6	-									
3	87.0	87.0	-								
4	88.1	87.8	87.5	-							
5	89.0	88.6	90.5	87.6	-						
6	87.3	85.4	91.4	87.2	89.5	-					
7	89.2	89.7	88.6	88.6	88.0	88.1	-				
8	89.1	89.3	89.5	88.2	87.4	87.0	87.5	-			
9	87.6	89.0	89.1	89.4	88.4	88.0	89.0	88.0	-		
10	89.5	88.6	90.3	87.0	87.5	86.2	89.2	89.5	88.4	-	
11	89.3	88.0	90.6	87.0	91.0	90.0	89.2	88.2	87.3	87.2	-
12	89.4	89.0	89.3	89.1	88.5	87.6	88.6	89.0	89.3	85.0	89.0	-
	1	2	3	4	5	6	7	8	9	10	11	12

Figure 2. Dendrogram (a) generated from genetic similarity coefficients (b) obtained from presence or absence of DNA bands in twelve *Metarhizium anisopliae* isolates and based on the unweighted pair-group method using arithmetic averages (UPGMA). Matrix calculated from bands yielded by twenty-one primers. Isolates details given in Table 1.

isolates diverged at the 12% similarity level. One primer with reproducible amplified band of 760 bp unique to the aggressive isolates group was selected.

4. Discussion

This is the first study investigating the aggressiveness of *M. anisopliae* to *C. tristis*. There was considerable variation in aggressiveness among isolates, presenting isolate number 6 (ARSEF2518) the highest cumulative mortality value. It is interesting to note that some Coleoptera or Homoptera-derived isolates showed more aggressiveness than some isolates from Lepidoptera, supporting that the original host is not a reliable indicator of higher virulence in some entomopathogenic fungi (Varela & Morales, 1996; Devi et al., 2008; Romón et al., 2017). Entomopathogenic fungi and deltamethrin have been used in integrated management research to control ticks (Bahiense et al., 2008) and *Triatoma infestans* (Forlani et al., 2013), with similar mortality rates among individual and mixed treatments and with no negative effect on certain isolates of the fungus after its

combination with deltamethrin at the usual or higher doses than those applied in the field. As expected, considering previous studies, no synergistic effect was found in the combined treatment probably because entomopathogenic fungi present high detoxification abilities (Forlani et al., 2013).

Random amplified polymorphic DNA (RAPD) markers are polymorphic DNA sequences that are amplified using PCR with decamer primers. This procedure is useful, simple, fast, requires only trace amounts of template DNA, and it does not require prior genome information. RAPD-PCR has been used in a number of surveys of *M. anisopliae*. In general, these studies have shown that the species consists of numerous genotypes and is considered very diverse. Correlations of distinctive groups of isolates defined by host (Bridge et al., 1997), geographical range (Leal et al., 1994), morphology (Bidochka et al., 1994) and/or pathogenicity (Fegan et al., 1993) have been demonstrated previously. In the present study, the nucleotide sequence of the cloned diagnostic RAPD-marker fragment from OPA-08 has been deposited in the GenBank database under accession numbers KJ543536-KJ543539, and it presents 96% homology with a protein mRNA from *Metarhizium robertsii* related with host adaptation (Hu et al., 2014). Biocontrol agent development requires isolation in pure culture of the microorganism, its identification, efficacy laboratory trials, pilot trials under field conditions, implementation of formulation and biosafety studies. The phase of screening for proper isolates is very time-consuming and expensive and there is a need for faster and easier methods. The development of fingerprinting of higher aggressiveness could be valuable. Our results suggest that the isolate ARSEF2518 appears to have the best potential among the tested isolates for future evaluation and development as fungal biocontrol agent to *C. tristis*. However, further laboratory and field experiments are needed. More studies should be directed to determine effects of different formulations and storage on conidial viability, and to test its ability and the other aggressive isolates in controlling *C. tristis* populations in the field.

Acknowledgements

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