

Odor of the achlorophyllous plant's seeds induce the seed-dispersing ants

Mikihisa Yamada¹, Masaru Hojo², and Akio Imamura¹

¹Hokkaido University of Education - Asahikawa Campus

²Kwansei Gakuin University School of Science and Technology Graduate School of Science and Technology

November 11, 2020

Abstract

Seed dispersal by ants is one of the important means of migration for adherent plants. Although many myrmecochorous plants have seed with elaiosome which is nutritional reward for ants, some seeds without elaiosomes are also dispersed by ant species. We tested seed dispersal by ants using the achlorophyllous and myco-heterotrophic herbaceous plant *Monotropastrum humile*, whose seeds do not have elaiosome, and require a fungal host for germination and ultimately survival. We performed a bioassay using seeds of *M. humile* and the ant, *Nylanderia flavipes*, to demonstrate ant-mediated seed dispersal. We also analyzed the volatile odors emitted from *M. humile* seeds, and conducted bioassays using dummy seeds coated with seed volatiles. Although elaiosomes were absent from the *M. humile* seeds, the ants carried them to their nest. They also carried the dummy seeds coated with the seed volatile mixture to the nest, and left some dummy seeds inside the nest and discarded the rest of the dummy seeds outside the nest with a bias towards locations with moisture conditions conducive to germination. We concluded that seeds of the myco-heterotrophic, herbaceous species were dispersed by the ants, and that seed odors were sufficient to induce directed dispersal even without elaiosomes. The flesh-fruit producing genus *Monotropastrum* have probably evolved from the related anemochorous genus *Monotropa*, which produces capsule fruit. This transformation from anemochory to myrmecochory, presents a novel evolutionary pathway towards ant-mediated seed dispersal in an achlorophyllous plant.

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Author information

Mikihisa YAMADA¹, Masaru K. HOJO², Akio IMAMURA¹

1. Hokkaido University of Education, Asahikawa Campus,

Hokumon-cho 9, Asahikawa, Hokkaido 070-8621, Japan

2. Department of Bioscience, School of Science and Technology, Kwansei Gakuin University, Sanda, Hyogo 669-1337, Japan.

correspondence: Akio Imamura

E-mail: ginryou715@yahoo.co.jp

ORCID ID 0000-0002-5208-1936

Declarations

Acknowledgments

We thank Takafumi Mizuno and Toshiharu Akino for their help with GC-MS analyses. This work was supported by JSPS KAKENHI Grant no. 18H02512. We would like to thank Editage (www.editage.com) for English language editing.

Author contributions

A.I. conceived the study, conducted analyses, and wrote the manuscript. K.Y. conducted experiments. M.K. Hojo performed a gas chromatograph analysis and check the statistical analyses.

Funding

This work was supported by JSPS KAKENHI Grant no. 18H02512.

Conflicts of interest

There are no conflicts of interest to declare.

Data availability statement

All relevant data are within the paper and its Supporting Information files.

Abstract

Seed dispersal by ants is one of the important means of migration for adherent plants. Although many myrmecochorous plants have seed with elaiosome which is nutritional reward for ants, some seeds without elaiosomes are also dispersed by ant species. We tested seed dispersal by ants using the achlorophyllous and myco-heterotrophic herbaceous plant *Monotropastrum humile*, whose seeds do not have elaiosome, and require a fungal host for germination and ultimately survival. We performed a bioassay using seeds of *M. humile* and the ant, *Nylanderia flavipes*, to demonstrate ant-mediated seed dispersal. We also analyzed the volatile odors emitted from *M. humile* seeds, and conducted bioassays using dummy seeds coated with seed volatiles. Although elaiosomes were absent from the *M. humile* seeds, the ants carried them to their nest. They also carried the dummy seeds coated with the seed volatile mixture to the nest, and left some dummy seeds inside the nest and discarded the rest of the dummy seeds outside the nest with a bias towards locations with moisture conditions conducive to germination. We concluded that seeds of the myco-heterotrophic, herbaceous species were dispersed by the ants, and that seed odors were sufficient to induce directed dispersal even without elaiosomes. The flesh-fruit producing genus *Monotropastrum* have probably evolved from the related anemochorous genus *Monotropa*, which produces capsule fruit. This transformation from anemochory to myrmecochory, presents a novel evolutionary pathway towards ant-mediated seed dispersal in an achlorophyllous plant.

KEYWORDS

directed seed dispersal, elaiosome, myco-heterotrophic plant, myrmecochory, seed volatiles

1 | INTRODUCTION

Various seed and fruit traits such as size and color tend to be correlated, forming what are known as “dispersal syndromes” that may have arisen to attract particular dispersers (Brodie, 2017; Valenta and Nevo, 2020) or may facilitate transport from their parent plants and move by wind or currents (Nilsson et al., 1991; Bullock & Claeke, 2000; Ohnishi et al., 2008; Nathan et al., 2011). Among seed dispersal by animals, dispersal depending on ants is specifically called myrmecochory. Myrmecochory is widespread among angiosperms and ecologically important; over 11,000 species of myrmecochorous plants in 77 families and 334 genera, participate in myrmecochory across various ecosystems (Lengyel et al., 2010). One of the evolutionary significance of myrmecochory is considered as directed seed dispersal. The advantages of the directed ant-dispersal hypothesis are that ant nests maintain moist conditions and the seeds carried to the nest can escape desiccation and have a higher seedling survival (Levey & Byrne, 1993), and the ants bury the seeds at depths where humidity and temperature are suitable for germination (Gibson, 1993).

Seeds of many myrmecochorous plants possess appendages, such as elaiosomes containing various fatty acids and proteins. These are likely to be nutritional rewards for ants who disperse the seeds (Brew et al., 1989; Lanza et al., 1992). If the seeds are carried to an ant nest, the elaiosomes will likely be consumed inside the nest before the seeds are discarded (Culver & Beattie, 1978). Some granivorous ants, such as species of genera *Messor*, *Pheidole*, and *Tetramorium*, lose or abandon their seeds during transportation and thus also disperse seeds without elaiosomes (Retana et al., 2004; Kobayashi, 2009). Various studies have shown that nutritional contents, such as lipids, amino acids, and proteins, in the elaiosome are important for inducing the seed dispersal. In contrast, how seeds without elaiosomes enable efficient dispersal by ants is less investigated, although some chemical signaling might be involved (Youngsteadt et al., 2008). It is also unclear whether the seeds are discarded on suitable sites in a directed manner in species without elaiosomes. In this study, we investigated seed dispersal without specialized nutritional rewards to ants. We also tested the possibility of directed seed dispersal by ants by examining whether abandonment of dummy seeds is directionally biased towards suitable location using an achlorophyllous plant whose seeds do not have elaiosomes.

We used the plant *Monotropastrum humile* (D. Don) H. Hara (Ericaceae) and the ant *Nylanderia flavipes* Smith (Formicidae) to test the hypothesis. *M. humile* is an achlorophyllous and myco-heterotrophic plant that inhabits forest floors in the temperate regions of Asia (Ohashi et al. 2016). The plant is about 10 cm height and does not perform photosynthesis and parasitizes the fungi of *Russula* and *Lactarius* species of Russulaceae (Bidartondo and Bruns, 2001). *M. humile* is a full myco-heterotrophic species (Merckx, 2013). Seeds are smaller than 0.5 mm in length (Uehara & Sugiura, 2017) and require their host fungi for germination, thus, seed destination is a determinant of survival. Their host, the mycorrhizal fungi of *Russula* species, inhabit the shallow areas of the forest floor (Rachel, 2004; Courty et al., 2008), and Imamura and Kurogi (2003) reported that *M. humile* roots inhabit the forest floor to a depth of 5–10 cm. For seed dispersal, *M. humile* uses the insects wandering on the ground when their berries fall to the ground during the fruiting season. Cockroaches (Uehara & Sugiura, 2017) and camel crickets (Suetsugu, 2017) have been reported as their seed dispersers. According to these studies, the *M. humile* berries fall to the ground during the fruiting season, the wandering insects are presumed to consume pulpy flesh around the seeds and consequently contribute to the dispersal of seeds involved in their foraging behavior.

Although *M. humile* seeds have no elaiosomes and seem to be non-myrmecochorous plants (Suetsugu, 2017; Uehara & Sugiura, 2017), Suetsugu (2017) and Uehara and Sugiura (2017) reported that several ant species, including *N. flavipes*, frequently visited the *M. humile* fruits. In addition, we have observed, by using a fixed-point camera, that *N. flavipes* ants were attracted to the fruits and seeds of *M. humile* and that a part of the seeds, although not many, were certainly carried by them. Since the seeds are coated with a phlegmatic organic layer, it is possible that *M. humile* attract the ants with the fruit- and seed odors and that their seed are carried by the ants. The seeds might be abandoned after the outer components are consumed. Consequently, the seed could be dispersed to the suitable, host-inhabited sites on the forest floor.

To validate directed seed dispersal by ants, it is necessary to track where the seeds are carried to and discarded. However, the seeds of myrmecochorous plants are small and difficult to track in the field. Thus, at first, we performed the bioassay using intact and odorless seeds to verify whether the seed odors of *M. humile* are key signals for dispersal by ants. Afterwards, bioassays were performed using small pieces of the paper coated with the odor component of *M. humile* seeds as dummy seeds. We tested whether the dummy seeds were carried into the nests and were carried out of the nests by the ants.

2 | MATERIALS AND METHODS

2.1 | Plant and ant materials

We used the ant species *N. flavipes*, which is polyphagous and collects plant seeds in the field (Tanaka & Tokuda 2016; Hosoishi et al., 2019). Ten *N. flavipes* colonies were collected from June to July 2019 from the sites at the Yuminariyama mountain and the Hokkaido University of Education Asahikawa Campus in Hokkaido, Japan. All the colonies collected were queenless, consisting of approximately 60 workers and numerous eggs, pupae, and larvae. The colonies of *M. humile* and *N. flavipes* coexist sympatrically at

Yuminariyama mountain and we also collected *M. humile* fruits at Yuminariyama mountain in August 2019 and stored them in a screw tube at 4 .

The ant colonies were kept in a container that consisted of an artificial nest and a foraging arena (Figure 1). The polypropylene box (200 mm length \times 136 mm width \times 68 mm height) was used as a foraging arena. Fluon was applied to the upper 3 cm inside the feeding arena to prevent ant escape. A vinyl tube (10 mm diam. \times 120 mm length) was used as an artificial nest and connected to the side wall of the container at 15 mm height. To prevent the artificial nest from drying out, an absorbent piece of cotton was inserted at the wall-side of the nest and moistened with distilled water. The absorbent cotton was replaced every two weeks.

The feeding arena was divided into four equivalent sections to investigate whether the ants discard seeds in areas with different conditions. The four sections were respectively filled with dry glass beads (1.5-2.5 mm diam.), moistened glass beads, dry cotton wool, and moistened cotton wool.

Glass beads simulated an environment with inorganic substances like sand or small pebbles in the forest floor, which were filled with about 10 g of beads in each section. Cotton wool simulated an environment with organic substances such as fallen and decomposing litter, which were filled with about 0.25 g of cotton in each section. The entrance of the nest was placed at the center of the arena so that the four sections were equally aligned for the ants. The entrance of nest floats was bridged with filter paper at the center of the feeding arena (Figure 1a). In the bioassay, the seeds or the dummy seeds were placed at the center of the arena near where the bridge connected the different sections (Figure 1b).

To moisten two of the sections, 5 mL of distilled water was added every three days. All the containers were kept indoors at 25 . The ants were fed with tuna and honey.

2.2 | Volatile collections and chemical analyses

The volatiles emitted from *M. humile* seeds were collected using solid phase micro extraction (SPME) using a 66- μ m PDMS/DVB fiber (Supelco, Bellefonte, PA, USA). The seeds with pulp were placed in 4-mL glass vials and sealed with aluminum foils. The SPME fiber was inserted into the covered vial and volatiles were sampled for 1 h at room temperature. Blank assays with empty vials were performed before each volatile collection session. Three independent samples were collected and analyzed.

Immediately after sampling, the SPME fiber was injected into the split/splitless injector of a gas chromatograph (GC17A; Shimadzu, Japan) equipped with a DB-WAX column (30 m length \times 0.25 mm inner diameter \times 0.25 μ m film thickness; J & W Scientific Inc., Folsom, CA, USA) and mass spectrometry detectors (QP5000; Shimadzu, Japan) with electron impact ionization (70 eV). The oven temperature was maintained at 40 $^{\circ}$ C for 5 mins, programmed to increase to 220 degC at a rate of 10 degC min^{-1} , and held isothermal for 10 min. The SPME fiber was desorbed in splitless mode for 1 min, while the injector and interface temperatures were 220 degC. Helium was used as carrier gas, and the column head pressure was 100 kPa. Volatile compounds were tentatively identified by the matches with the NIS mass spectral database, and identifications were confirmed by matching mass spectra and Kovat's retention index with those of commercially available authentic standards.

2.3 | Bioassay using the *M. humile* seeds

We performed a bioassay using 20 fresh untreated or solvent-treated seeds of *M. humile* . For the treated seeds, 20 fresh seeds of *M. humile* were soaked in ethanol (99.5%, Wako Pure Chemical Industries, Ltd.) for 30 min. Four replicate colonies resulted in a total of 80 seeds for each treatment. Untreated and treated seeds were assayed separately to avoid odor transfer. The colonies were starved (fed with water only) for 72 h before performing the assays. After the seeds were set at the center of the arena, the number of seeds carried to the nest by the ants was recorded every 15 min for 90 min.

2.4 | Bioassay using the dummy seeds of filter paper

We performed a bioassay using dummy seeds made of square bits filter paper of 2 mm x 2 mm. The dummy seeds were soaked with the volatile compounds of the *M. humile* seeds (Table 1). These compounds were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Tokyo Kasei (Tokyo, Japan) with >98.0% purities.

In this dummy-seed assay, 20 pieces of filter paper numerically numbered with a carbon pencil to track the outcome of the dummy seeds were used. The odor treatment in this assay consisted of seven conditions, that is, each of the six odors identified (six conditions) and a mixture of equivalent amounts of the six odors (one condition). Each odor component was diluted to a final concentration of 100 ng / μL using the 99.5% ethanol as a solvent. Twenty pieces of filter paper were soaked with 20 μl odor solution (1 μl for each filter paper). The dummy seeds soaked only with the solvent were used as the control. Each of the odor treatments was replicated six times using the six different colonies, and each colony experienced each treatment only once. The colonies were starved (fed with water only) for 72 h before performing the assays.

The order of the odor treatments that were served to each colony was randomized using the 'sample' function of the base package of R 3.5.2 for Mac OSX (R core team, 2018). Dummy seeds were placed at the center of the arena and the number of dummy seeds carried to the nest by the ants was recorded every 15 min for 90 min. After 72 h from setting the dummy seeds, the number of dummy seeds inside the nest and discarded outside the nest was recorded. For discarded dummy seeds, we also recorded their destination and the numerical number on the dummy seeds.

2.5 | Statistical analysis

All analyses were executed with R 3.5.2 for Mac OSX. The package 'tidyverse' was used for data shaping and arrangement. The factors affecting the number of seeds (or dummy seeds) carried by ants were analyzed using a generalized linear mixed model (GLMM) and likelihood ratio test. We used the zero inflated Poisson distribution with the function `glmmTMB` from the package 'glmmTMB'. In this analysis, colony identity was set as the random effect and the odor conditions were the explanatory variables. Chi-square tests were performed to examine whether the destination of the dummy seeds was biased.

RESULTS

3.1 | Volatile chemical composition of *M. humile* seeds

Chemical analyses of seed volatiles revealed the presence of several substances. By comparing the results with blank analyses, we found eight compounds that were derived from the *M. humile* seeds (Figure 2). Among eight peaks, 6 peaks were identified as isobutyl alcohol, isoamyl alcohol, isoamyl hexanoate, linalool, isobutyric acid, and α -terpineol (Table 1). Peaks 2 and 8 could not be identified from the obtained mass spectra.

3.2 | Bioassay using *M. humile* seeds

The ants carried away the *M. humile* seeds in 90 min. Table 2 shows the number of *M. humile* seeds carried to the nest within 90 min. Nine of the 80 untreated seeds were carried to the nest although none of the treated seeds were carried to the nest (Figure 3, Table S1). GLMM analysis revealed that untreated seeds were carried away significantly more than treated seeds (Table S2).

3.3 | Bioassay using filter paper as dummy seed

Ants did not carry the dummy seeds to the nest in 90 min but did in 72 h (Table S3). A total of 30 out of 840 odor-treated dummy seeds were carried to the nest. Comparing the number of removed dummy seeds for each odor treatment, the six-odor-mixture treatment were carried most to the nest according to the GLMM analysis (Table 2, Figure 4).

Of the 30 dummy seeds carried, 17 were discarded (Figure 5). The rest of the seeds carried was left inside the nests. The chi-square test was performed on the six categories and included the four compartments, the empty area, and the nest, and there was a significant bias ($P = 0.0002$). When we performed the chi-square

test on four compartments of the dummy seeds, glass beads, water-moistened glass beads, cotton wool, and water-moistened cotton wool, the distribution was also significantly biased ($P = 0.0032$) and most of seeds were discarded on moistened glass beads (Figure 5).

4 | DISCUSSION

4.1 | Dispersal of elaiosome-less seeds by ants

In this study, we first verified that *M. humile* seeds without elaiosomes surely attracted ants. We have confirmed that 9 out of 80 untreated seeds were carried to the ant nest, whereas no treated seeds were carried (Table S1). We also found that by using dummy seeds coated with the odor components of *M. humile* seeds, those dummy seeds treated with a mixture of 6 major components were carried to the nest by the ants, although the dummy seeds treated with the solvent were not carried away (Table S3). Among the 6 main odor components, linalool and α -terpineol were also identified as floral scents of *M. humile*, but the other 4 components were not identified (Kubo & Ono, 2014). These results indicate that *M. humile* seeds attracted ants by their seed or fruit specific odor mixture. In the pepper family plant, *Peperomia macrostachya*, the mixture of seed odors efficiently attracts the ant, *Camponotus femoratus* (Youngstead et al., 2007). Since the volatile seed odors would have disappeared in 72 h, the dummy seeds were abandoned outside the nest. Similarly, the seeds are likely to be abandoned after the outer fruity coating components are consumed. Therefore, even in the absence of elaiosomes, some plants, including *M. humile*, could disperse their seeds through the ants using a specific blend of odor components.

Hence, ants are thought to participate in the seed dispersal of *M. humile*, in addition to herbivorous insects, such as cockroaches and camel crickets (Suetsugu 2017; Uehara & Sugiura 2017), and the seed dispersal by ants could be beneficial for the achlorophyllous species. With regards to the seed dispersal by ant, the seed-discards or left-inside-the-nest indicated that dispersal by ants should be important for plant seeds of such achlorophyllous plants as *M. humile*. The plant does not depend solely on the myrmecochory, as Suetsugu 2017 reported many of seeds have been detected from the herbivorous insects' excretion. Because the number of seeds carried in our assay is relatively small, myrmecochory of the species could be one aspect of their zoochory.

4.2 | Implication by the biased discard of dummy seeds

Although we could not track where the genuine seeds discarded because of their small-size, the author M. Y. observed a part of the genuine seeds carried into the nests are left in a corner of the nest (data not shown). Thus, the ant nest could be one of the seed directions. The dummy seeds coated with mixed odors were carried into the nest and subsequently discarded out of the nest by the ants. Although 13 dummy seeds were left inside the nest (17 were discarded outside), it appears that seeds staying in the nest can also be considered as dispersed since *N. flavipes* nests are located under litter or on rotting trees (Kallal & LaPolla, 2012). The direction of discards was biased, and the dummy seeds were discarded mostly onto the water-moistened glass beads. In the ant species *Myrmica rubra*, it is known that workers discard inert items and corpses in different destinations (Diez et al., 2012). In our study, it was also observed that ant corpses were dumped into cotton wool compartments, where the dummy seeds were not dumped. These results suggests that the ants differently use the discard destinations depending on what they are discarding, and the seeds could be discarded specific locations by the ants.

Mycorrhizal fungi of *Russula* species as the hosts of *M. humile* inhabit the top shallow layer of the forest floor (Rachel, 2004; Courty et al., 2008), and Imamura and Kurogi (2003) reported that *M. humile* roots inhabit 5-10-cm depth as parasites on Russulaceae fungi. Thus, where the ant nests are located and where the seeds are discarded (moist soil near the surface) is considered to be close to their hosts and to be suitable for germination, growth and survival of the *M. humile* seeds although the mycorrhizal roots have not been quantified before.

The dummy seeds were left inside the nest or most frequently discarded onto the water-moistened glass beads section. This observation can support two seed-dispersal hypotheses: 1) seeds relocated to the moist envi-

ronments including ant nests can escape desiccation and have enhanced germination and seedling survival; 2) seeds relocated to ant nests can also escape predators (modified from Hanzawa et al 1988).

Our results suggest that seed dispersal of *M. humile* by ants is directional. Directed seed dispersal and its ecological significance have rarely been verified in achlorophyllous herbaceous species (reviewed by Giladi, 2006). With regards to myco-heterotrophic plants, large parts of them produce dust-like and anemochorous seeds, which require host fungus in their germination, and which could disperse over long distances (Merckx, 2013). Thus, there is a trade-off of seed dispersal between distance and direction. Since the genus *Monotropastrum*, producing fleshy fruit, is thought to evolve from the relative and anemochorous genus *Monotropa*, producing capsule fruit (Bidartondo & Bruns, 2001), this speciation is considered as from anemochory (distance dispersal) to zoochory including myrmecochory. Therefore, the verification of the role of myco-heterotrophic herbaceous species in the temperate forests of Asia, and of the role of seeds without specialized rewards for ants, presents a new aspect of directed seed dispersal of achlorophyllous and myco-heterotrophic plants involving ants.

5 | CONCLUSION AND CAVEATS

We verified that reward-less seeds of achlorophyllous and myco-heterotrophic *M. humile* were certainly dispersed by ants. A part of the seeds was left in the ant nests and the other part of the seeds could be discarded outside the nest directionally although we could not evaluate the fate of genuine seeds but evaluated that of dummy seeds. In future studies, we must track the fate of the genuine seeds and investigate spatial distribution of the plants and the ant nests to validate the directed seed dispersal of the achlorophyllous plant *M. humile*. An expected way of bioassay is to copy various conditions of the forest floors and to examine the directed dispersal by ants in detail.

ACKNOWLEDGMENTS

We thank Takafumi Mizuno and Toshiharu Akino for their help with GC-MS analyses. This work was supported by JSPS KAKENHI Grant no. 18H02512. We would like to thank Editage (www.editage.com) for English language editing.

Author contributions

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Conflict of interests

There are no conflicts of interest to declare.

Data availability

All relevant data are within the paper and its Supporting Information files.

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Peak No.	Components	Molecular formula	Molecular weight	Retention Index
1	Isobutyl alcohol 99.0%	C ₄ H ₁₀ O	74.1	1073
3	Isoamyl alcohol 99.0%	C ₅ H ₁₂ O	88.2	1208
4	Isoamyl hexanoate 98.0%	C ₁₁ H ₂₂ O ₂	186.3	1480
5	Isobutyric acid 99.0%	C ₄ H ₈ O ₂	88.1	1573
6	Linalool 98.0%	C ₁₀ H ₁₈ O	154.2	1588
7	α-terpineol 98.0%	C ₁₀ H ₁₈ O	154.2	1725
-	Mixture of six components			

Table 1 Odor components identified by GC/MS analysis. We used samples of these components in our bioassay.

Peak numbers correspond to those in Figure 2

Table 2 Results of the generalized linear mixed model (GLMM) and likelihood ratio test on the number of dummy seeds carried by ants with a zero-inflated Poisson distribution. The colony identity was set as the random effect. The odor component was the explanatory variable.

Conditional model	Variance	Std. Div.		
(Intercept)	8.52e-12	2.92e-06		
Components	Estimate	Std. Error	z value	Pr(> z)
Isobutyl alcohol	-22.325	3.17e+04	-0.001	0.999
Isoamyl alcohol	-0.866	7.78e-01	-1.113	0.266
Isoamyl hexanoate	-0.866	7.78e-01	-1.113	0.266
Isobutyric acid	-22.325	3.17e+04	-0.001	0.999

Linalool	-0.228	5.54e-01	-0.411	0.681	
α - terpineol	-0.920	7.50e-01	-1.226	0.220	
Mixture of six components	1.370	2.31e-01	5.927	< 0.001	***
Ethanol as control	-22.325	3.17e+04	-0.001	0.999	
	Estimate	Std. Error	value	Pr(> z)	
Estimate of Zero-inflation model (Intercept)	-1.52	1.14	-1.33	0.18	

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