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A new approach for tracking seed dispersal of large plants: soaking seeds with ^{15}N -urea

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Abstract

• **Context** Although various tracking methods have been used in many ecosystems to investigate seed dispersal and seedling recruitment, it is still difficult to measure seed dispersal patterns due to methodological challenges in tracking seed movement away from parent trees and in finding the locations of seedlings.

• **Aim** Here, we aimed to develop a new approach to track seed dispersal by animals in the field.

• **Methods** Our approach involves soaking seeds directly into ^{15}N -urea solutions with different dosages to enrich them isotopically. This new method is expected to create a reliable differentiation between the enriched seeds and natural ones and consequently between the corresponding seedlings.

• **Results** We showed that acorns of *Quercus variabilis* and *Cyclobalanopsis glauca* and seeds of *Pinus koraiensis* soaked in ^{15}N -urea solutions were successfully enriched. We did not find that $\delta^{15}\text{N}$ value of seeds was a linear function of ^{15}N -urea soaking duration. However, with high urea dosage and extending soaking duration, the $\delta^{15}\text{N}$ values in seedlings of

Q. variabilis and *P. koraiensis* were higher than those of normal plants. As expected, ^{15}N isotope was significantly diluted in the growing seedlings germinated from ^{15}N -enriched seeds. Using the ^{15}N -urea soaking method, we successfully located five seedlings of *Q. variabilis* germinated from the enriched acorns in the field.

• **Discussion** The ^{15}N -urea soaking technique is powerful in tracking seed dispersal and seedling recruitment. This new method solves some of the problems inherent in traditional methods for tracking secondary seed dispersal and could further improve the study of seed dispersal ecology.

Keywords Seed dispersal · Seed tagging · ^{15}N -urea soaking · Isotope enrichment · Seedling establishment

1 Introduction

Seed dispersal by animals has significant influences on community ecology, genetic structures, and population dynamics (Jordano and Godoy 2002; Levine and Murrell 2003; Carlo et al. 2007; García et al. 2007; Moore et al. 2007; Yi et al. 2011). However, ecological assessment of seed dispersal has largely been neglected because tracing seed movements from the seed source to the exact locations where the seeds germinate or perish is difficult (Chambers and MacMahon 1994; Wang and Smith 2002; Bullock et al. 2006; Muñoz and Bonal 2011; Hirsch et al. 2012a, 2012b; Davis et al. 2011) and often suffers methodological limitations (Nathan 2006; Galvez et al. 2009; Galetti et al. 2010). Previous studies indicate that the biggest obstacle to track seed dispersal by animals is to develop a method that is simple, effective, labor-saving, and broadly applicable (Takahashi et al. 2006; García et al. 2007; Yi et al. 2008; Carlo et al. 2009; Lemke et al. 2009; Perea et al.

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2011; Hirsch et al. 2012a, 2012b). If a simple and reliable seed-tracking method could be developed, it would facilitate a better understanding of seed dispersal ecology (Will and Tackenberg 2008; Yi et al. 2008; Carlo et al. 2009). Many methods have been used to track seed dispersal by animals and the resulting seed fates, e.g., direct observation (Burnell and Tomback 1985), surgical installation of metal or magnets (Alverson and Díaz 1989; Den Ouden et al. 2005), attachment of colored threads or tags to seeds (Forget 1992; Yi and Zhang 2008; Yi et al. 2011), marking with stable or radioactive isotopes (Winn 1989; Carlo et al. 2009), application of fluorescent microspheres and dye powders (Longland and Clements 1995; Levey and Sargent 2000; Tewksbury et al. 2002; Lemke et al. 2009), genetic techniques based on microsatellites (Godoy and Jordano 2001; García et al. 2007; Smouse et al. 2012), radio transmitters (Hirsch et al. 2012a, 2012b), as well as pit tags (Suselbeek et al. 2013). Although these methods show their own advantages and have been used in many ecosystems for tracking seed dispersal, most of them suffered from deficiencies and limitations (Alverson and Díaz 1989; Forget 1992; Levey and Sargent 2000; Godoy and Jordano 2001; Tewksbury et al. 2002; Yi et al. 2008; Niu et al. 2011; Hirsch et al. 2012a, 2012b). Carlo et al. (2009) recently introduced a new method to track seed dispersal and seed recruitment based on labeling seeds or fruits by foliar-spraying with a solution of ^{15}N -urea during the flowering stage of small herbaceous plants (*Solanum americanum* and *Capsicum annuum*). Although this method is effective and provides ability to find the exact locations that seeds arrive, germinate, and establish in the field, it is not broadly applicable for tree species with large canopies and high trunks because it is very difficult to foliar spray ^{15}N isotope urea solutions to the canopies more than 10 m in height. In addition, foliar sprays applied to wind-pollinated flowers at the time of anthesis could hamper pollination and the subsequent development of seeds and fruits. Our goal in this study was to further develop the ^{15}N stable isotope technique to track seed dispersal of tree species that are tall and have large canopies, e.g., pines and oaks. This method involved direct soaking seeds and acorns into ^{15}N isotope urea solutions with different dosages for several days. The cotyledons or endosperm of the seeds were expected to absorb the urea so that the seeds were ^{15}N enriched, which enables the tracking of seed dispersal and seedling recruitment in the field.

We designed our experiments to address the following five questions: (1) Can any kind of seeds be isotopically enriched in $\delta^{15}\text{N}$ values through urea solution soaking?; (2) What is the optimal dosage of ^{15}N -urea and soaking duration for seed isotope enrichment?; (3) Do enriched seeds reliably produce seedlings with $\delta^{15}\text{N}$ values higher than those of normal seedlings?; (4) What is the optimal stage to sample seedlings for ^{15}N isotope enrichment analysis?; and (5) Is it possible to accurately discriminate seedlings produced by ^{15}N -enriched seeds against those germinated from natural seeds in the field?

2 Materials and method

2.1 ^{15}N isotope enrichment in seeds and seedlings

In autumn of 2011, we soaked seeds of *Pinus koraiensis* (Pinaceae) and acorns of *Quercus variabilis* (Fagaceae) and *Cyclobalanopsis glauca* (Fagaceae) in ^{15}N -urea solutions in Henan University of Science and Technology, Luoyang, China, to see if they and their seedlings were successfully enriched with the ^{15}N isotope. Three dosage treatment solutions (0, 1, and 2 mmol/L) of ^{15}N -urea (98 atom%; Isotec, Sigma-Aldrich, St. Louis, Missouri, USA) were used. Sound and healthy-looking seeds of the three tree species were used. Trees of *Q. variabilis* and *C. glauca* produce large acorns rich in starch, whereas seeds of *P. koraiensis* are small and rich in fat. *P. koraiensis* was included to test whether seeds rich in fat can successfully absorb the ^{15}N -urea solution. Acorns of *Q. variabilis* exhibit no dormancy and germinate immediately at or even before seed fall (Yi et al. 2012a). In contrast, acorns of *C. glauca* and seeds of *P. koraiensis* remain dormant through winter until the next spring. This design further allows us to test whether differences in seed dormancy affects urea solution absorption. We selected 100 seeds and acorns of each tree species and separately soaked them into each dosage solution of ^{15}N -urea. Thus, 300 propagules of each tree species were used for the urea soaking experiments. Seeds and acorns with pericarps attached were half submerged in the solutions, and the solutions were stirred everyday to ensure normal respiration and metabolism of seeds/acorns. After 10 days of soaking, 15 seeds or acorns (seed coat and pericarps were excluded) of each species in each dosage solution were collected and divided into three individual samples. Each sample contained 1–3 g dry mass of five acorns or seeds and were ground for measurement of ^{15}N enrichment. At the same time, 20 acorns of *Q. variabilis* in each dosage solution were randomly selected and planted into an organic soil in plastic trays with 50 grids (length \times width \times height: 4 cm \times 4 cm \times 6 cm) to see if seedlings were enriched with ^{15}N . To determine how long the isotopic signal lasted in the growing seedlings, the aboveground parts of three seedlings were individually harvested for ^{15}N isotope analysis when the seedlings reached 5–6 cm (first stage), 8–10 cm (second stage), and 12–15 cm (third stage) in height (see below), respectively. We then repeated the above procedures after 20 and 30 days of soaking to determine ^{15}N enrichment both in the acorns and seedlings, respectively. To investigate if the ^{15}N -urea soaked seeds of *P. koraiensis* can reliably produce seedlings with enriched $\delta^{15}\text{N}$ values, we planted 15 seeds soaked in 0, 1, and 2 mmol/L ^{15}N -urea for 20 days in the plastic trays, respectively (this procedure allows us to save money for isotope analysis). When the seedlings reached the maximum of the first flush (see below), the aboveground parts of three seedlings at each dosage solution were individually harvested for ^{15}N isotope analyses.

2.2 Assessing seedling recruitment in the field

To investigate whether the ^{15}N soaking method is suitable for seed dispersal and seedling assessing, we conducted a field experiment using ^{15}N -enriched acorns of *Q. variabilis* soaked in 2 mmol/L ^{15}N -urea solution for 20 days. In December 2011, 600 ^{15}N -enriched acorns were selected and released at two seed stations ($2 \times 2 \text{ m}^2$) in Tianchi Mountain Natural Reserve, Luoyang, China (average elevation 1,400 m, $33^\circ 45' - 33^\circ 85' \text{N}$, $111^\circ 75' - 112^\circ 45' \text{E}$), with 300 acorns in each station. Seed removal rate and seed fate were not checked. Previous studies in the experimental area show that small rodents (*Sciurotamis davidianus*, *Apodemus peninsulae*, *Niviventer confucianus*, *Apodemus agrarius*, and *Tscherskia triton*) usually disperse acorns less than 25 m (Yi's unpublished data); therefore, one-year seedlings (6–10 cm high) of *Q. variabilis* were located around each seed station (radius $\approx 30 \text{ m}$) and were sampled for ^{15}N isotope analysis in the spring of 2012. The epicotyl was sampled from each seedling and was cleaned and dried for ^{15}N isotope analysis. The enriched seedlings were identified by comparing with the natural ^{15}N values in nature (Craine et al. 2012).

2.3 Stable isotope analysis

All samples (seeds, acorns, and seedlings) were cleaned using distilled water and air-dried to constant weight in an oven at 70°C for 48 h. They were ground finely and placed in an isotope ratio spectrometer for isotopic analysis using elemental analyzer/continuous flow isotope ratio mass spectrometry. Samples were analyzed for stable nitrogen isotope abundance at the Laboratory of Stable Isotope Spectrometer, Chinese Academy of Forestry Sciences (Beijing). Interface between element-analysis meter and spectrometer was Flash EA1112 HT (Thermo Finnigan, USA). Operation condition: oxidizing furnace temperature was 900°C , reducing furnace was 680°C , and pillar temperature was 40°C . The resulting N_2 was purified in a vacuum line and injected in a Finnigan MAT Delta V advantage spectrometer (Thermo Fisher Scientific, Inc., USA) fitted with double inlet and collector systems. The results are expressed in $\delta^{15}\text{N}$ relative to the standards in the conventional δ per mil notation with a standard deviation of 0.2 per mil:

$$\delta^{15}\text{N} = \left[\left(\frac{^{15}\text{N}/^{14}\text{N}}{^{15}\text{N}/^{14}\text{N}} \right)_{\text{sample}} / \left(\frac{^{15}\text{N}/^{14}\text{N}}{^{15}\text{N}/^{14}\text{N}} \right)_{\text{standard}} - 1 \right] \times 1000$$

Where $^{15}\text{N}/^{14}\text{N}$ are the isotopic ratios of sample and standard (atmospheric nitrogen).

2.4 Data analysis

We conducted all statistical analyses in SPSS 16.0. Two-way ANOVA was used to test the effects of soaking duration and urea dosage on ^{15}N enrichment in the seeds or acorns of *Q.*

variabilis, *C. glauca*, and *P. koraiensis*. Three-way ANOVA was applied to detect the effects of soaking duration, urea dosage, and sampling stage on ^{15}N enrichment in the seedlings of *Q. variabilis*. Because the effects of soaking duration and sampling stage were not evaluated, difference in the ^{15}N enrichment in seedlings of *P. koraiensis* in response to urea dosage was tested using one-way ANOVA. A nonparametric Mann–Whitney test was used to compare the difference in ^{15}N enrichment in the seedlings of *Q. variabilis* collected from the field.

3 Results

3.1 ^{15}N enrichment in acorns/seeds

Our soaking experiments showed that the dosage of ^{15}N -urea solution had significant effects on ^{15}N enrichment in the acorns of *Q. variabilis* ($F=17.680$, $\text{df}=2$, $P<0.001$) (Fig. 1). The dosage of 2 mmol/L ^{15}N -urea solution resulted in much higher ^{15}N in acorns than those of 0 and 1 mmol/L solutions (all $P<0.001$). However, we found no significant influence of soaking duration on ^{15}N enrichment in the acorns ($F=2.520$, $\text{df}=2$, $P=0.108$) (Fig. 1). Similarly, we detected significant effects of urea dosage on ^{15}N enrichment in the acorns of *C. glauca* and seeds of *P. koraiensis* ($F=23.338$, $\text{df}=2$, $P<0.001$; $F=3.366$, $\text{df}=2$, $P=0.057$) (Fig. 2). Soaking duration showed no effect on ^{15}N enrichment in seeds or acorns of these two tree species ($F=1.064$, $\text{df}=2$, $P=0.366$; $F=1.928$, $\text{df}=2$, $P=0.174$) (Fig. 2).

3.2 ^{15}N enrichment in seedlings

Three-way ANOVA indicated significant effects of soaking duration, urea dosage, and sampling stage on ^{15}N enrichment in the seedlings of *Q. variabilis* ($F=9.159$, $\text{df}=2$, $P<0.001$; $F=31.884$, $\text{df}=2$, $P<0.001$; $F=4.713$, $\text{df}=2$, $P=0.013$) (Fig. 3). We also found a significant interaction effect between any two of the three factors (all $P<0.05$). Multiple

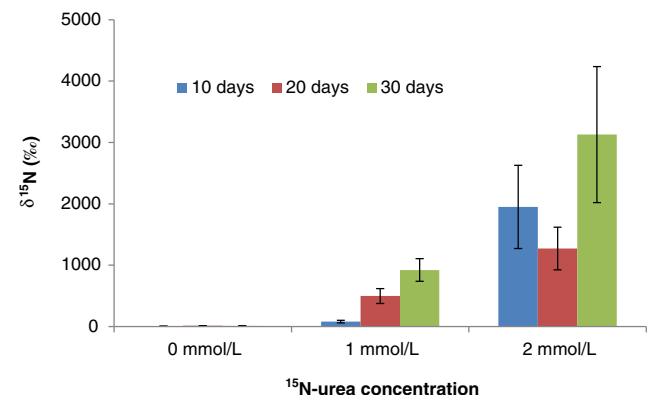
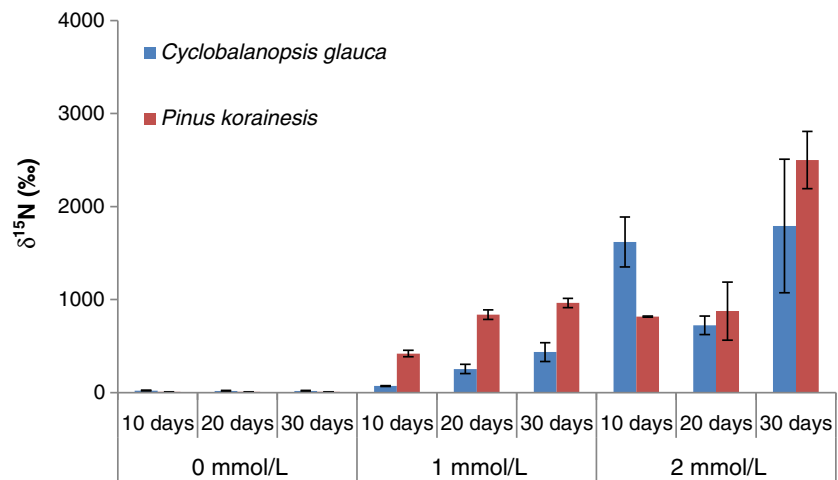


Fig. 1 The effects of urea dosage and soaking duration on ^{15}N enrichment in the acorns of *Q. variabilis*. Data are expressed as mean \pm SE

Fig. 2 The effects of urea dosage and soaking duration on ^{15}N enrichment in the propagules of *C. glauca* and *P. koraiensis*. Data are expressed as mean \pm SE



comparisons further demonstrated that ^{15}N values in the seedlings sampled at the third stage (12–15 cm high) were much lower than those sampled at the first and second stages ($P=0.003$; $P=0.145$, respectively) (Fig. 3), indicating that seedlings 8–10 cm in height are optimal to be sampled

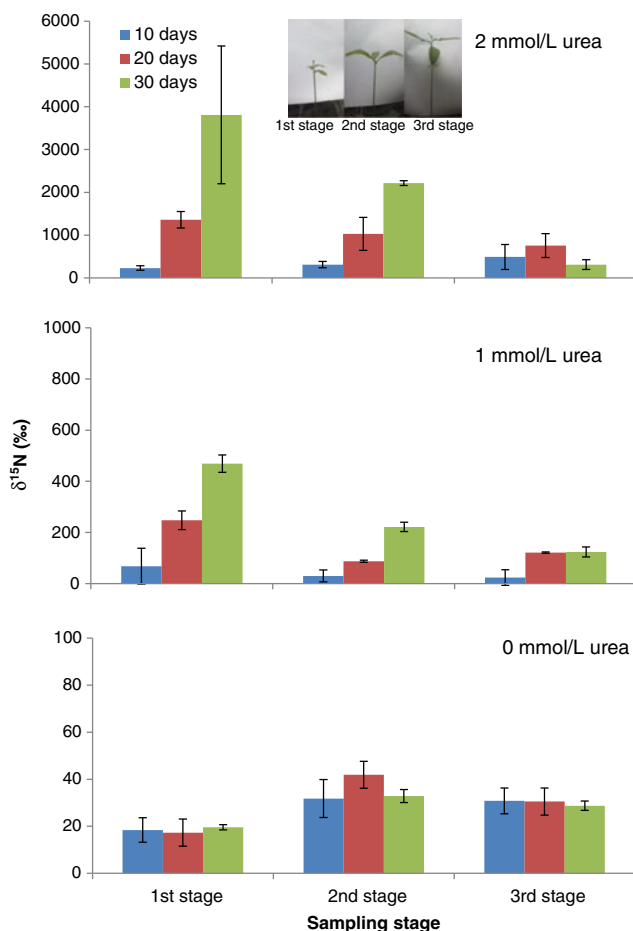


Fig. 3 The effects of urea dosage, soaking duration, and sampling stage on ^{15}N enrichment in the seedlings of *Q. variabilis*. Embedded images indicate three seedling sampling stages. Data are expressed as mean \pm SE

for ^{15}N isotope analysis. Although no effect of soaking duration on isotope level in acorns was observed, seedlings produced by acorns soaked for 30 days exhibited much higher ^{15}N values than those soaked for 10 and 20 days, respectively ($P<0.001$; $P=0.015$) (Fig. 3). Multiple comparison tests also indicated that seedlings produced by acorns soaked in 2 mmol/L dosage solution exhibited much higher ^{15}N values than those soaked in 0 and 1 mmol/L dosage solutions, respectively (all $P<0.001$). We found a reliable and gradual ^{15}N enrichment in *P. koraiensis* seedlings produced by seeds soaked in 0, 1, and 2 mmol/L dosage urea solutions for 20 days ($F=179.837$, $df=2$, $P<0.001$) (Fig. 4).

3.3 ^{15}N -enriched seedlings in the field

In the spring of 2012, we located 28 seedlings of *Q. variabilis* around the two seed stations (radius ≈ 30 m) through extensive search by two people for 2 days. Stable nitrogen isotope analyses indicated that five of them showed much higher $\delta^{15}\text{N}$ values ($867.28 \pm 177.89\text{‰}$) than normal seedlings ($10.89 \pm 0.47\text{‰}$) ($Z=3.449$, $P=0.001$) (Fig. 5), indicating that these five unnatural seedlings definitely were originated from the enriched acorns we released in the winter of 2011. The average distance between the enriched seedlings and the corresponding seed stations was 11.9 ± 4.0 m. We failed to detect seedlings from the other 595 ^{15}N -enriched acorns possibly because they were either consumed, larder hoarded, or perished.

4 Discussion

4.1 Effectiveness of isotopic urea soaking method

Our experiments clearly showed that ^{15}N isotope was successfully incorporated into the acorns and seeds of *Q. variabilis*, *C. glauca*, and *P. koraiensis*. ^{15}N enrichment could be

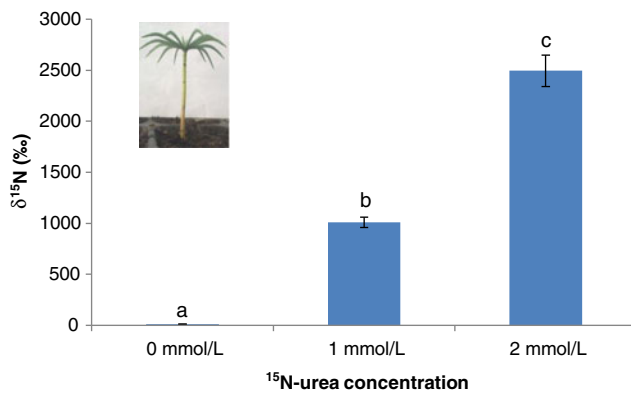


Fig. 4 The effects of ^{15}N -urea dosage on ^{15}N enrichment in the seedlings of *P. koraiensis*. Different letters on the histograms indicate significance at $\alpha=0.05$ level. Embedded image indicates seedling sampling stage. Data are expressed as mean \pm SE

expected in the seedlings of other tree species through urea soaking because we observed a very high enrichment of stable N isotope in the seedlings of *Q. variabilis* and *P. koraiensis* in the present study. We found that ^{15}N enrichment in the seeds and seedlings were urea dosage and sampling stage-dependent. While, the significant effect of soaking duration on ^{15}N enrichment in seedlings but not in acorns can possibly be explained by the absorption of ^{15}N from the pericarps by *Q. variabilis* seedlings because acorns were not shelled when sowing. Higher concentration of ^{15}N -urea and longer duration of soaking would generate higher levels of ^{15}N enrichment both in seeds and seedlings. These findings address the first four questions we addressed in the introduction. According to the results of our experiments, we recommended a treatment of 2 mmol/L ^{15}N -urea dosage and 20-day soaking duration for labeling seeds and tracking seedling recruitment in the field. However, ^{15}N isotope tended to be diluted in the growing seedlings, echoing the results of Carlo et al. (2009). Therefore, sampling seedlings in proper development stage is crucial for ^{15}N isotope analysis. Taking ^{15}N -urea concentration and seedling morphological performance into account, we suggest that

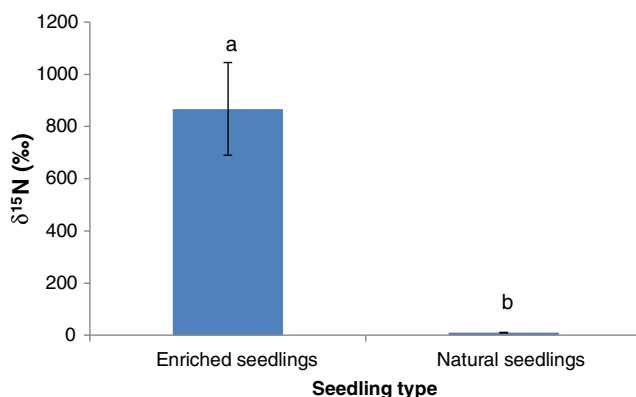


Fig. 5 Difference between $\delta^{15}\text{N}$ values of enriched seedlings and background seedlings collected in the field. Different letters on the histograms indicate significance at $\alpha=0.05$ level. Data are expressed as mean \pm SE

8–10 cm high seedlings are ideal for sampling and ^{15}N analysis. Compared with the natural ^{15}N values in nature (Craine et al. 2012), we successfully tracked several seedlings produced by ^{15}N -enriched acorns of *Q. variabilis* in the field, verifying the feasibility of ^{15}N -urea soaking to tag seeds for tracking their dispersal and recruitment. Compared to the urea foliar-spraying method (Carlo et al. 2009), soaking seeds directly in ^{15}N -urea circumvented some methodological problems encountered when trying to label propagules of large plants with high trunks and canopies that are difficult to reach.

4.2 Pros and cons of the soaking method

Our study on these three tree species suggests that isotope marking by ^{15}N -urea soaking is useful to investigate seed dispersal and seedling recruitment. Although this method omits the exact seed movement provided by animals during the dispersal process in the field, it is more labor-saving and less costly compared to molecular analyses to identify the mother trees (Godoy and Jordano 2001; Carlo et al. 2009). Therefore, our method permits the exact location of the seedling establishment to be determined, which facilitates the measurement of dispersal distance from the parent plants. This new isotope marking method overcomes the methodological bottlenecks that “large plants or trees will require large volumes of urea solutions, and canopies are obviously hard to reach” (Carlo et al. 2009). ^{15}N -urea solution soaking method also avoids the influence of weather conditions and manual application procedures on the variations in $\delta^{15}\text{N}$ values that urea foliar-spraying methods usually face. Although this method is not very suitable for tracking seed dispersal of many individuals within the same site, distinction among seeds and seedlings can be made by the dosage level provided (Carlo et al. 2009). Moreover, searching for enriched seedlings in large areas is expected to be much easier than searching for enriched seeds passed through avian passage (Carlo et al. 2009). Compared to the traditional tracking methods (Iida 1996, 2006; Yi and Zhang 2008; Yi et al. 2011; Canner and Spence 2011), the ^{15}N -urea solution soaking method provides a nondestructive approach to track seed dispersal, which will authentically reflect the seed dispersal processes because the traditional methods always involve surgical manipulations on seeds (e.g., inserting magnets or transmitters, drilling holes, and attaching transmitters) (Alverson and Díaz 1989; Winn 1989; Forget 1992; Den Ouden et al. 2005; Pons and Pausas 2007; Yi and Zhang 2008; Yi et al. 2011). These manipulations frequently lead to unexpected seed death, fungal contamination, seed weight modification, and seed consumption (Alverson and Díaz 1989; Den Ouden et al. 2005; Iida 1996, 2006; Canner and Spence 2011; Hirsch et al. 2012a, 2012b). Furthermore, our isotope tagging method shows an advantage over the traditional methods (Yi et al. 2008; Niu et al. 2011; Hirsch et al. 2012a, 2012b) that it does not add any visual cues to the ^{15}N -enriched seeds, which resembles the natural seeds and prevents animals from using

these cues for recovering the cached seeds. This method also has several shortcomings: (1) it requires much effort to locate all potential seedlings over a wide area after seed dispersal, and therefore (2) it is not well appropriate for long distance dispersal by frugivory, (3) although it may be powerful to study “secondary dispersal” such as scatterhoarding by rodents and similar processes, the urea soaking method does not permits the study of dispersal of plants that obtain seed removal directly from the canopies (like frugivory). Therefore, ^{15}N -urea soaking approach is expected to have a great potential for providing insights into seed dispersal and seedling recruitment manipulated by scatterhoarding animals.

4.3 Broad application of tracking seed dispersal

Although seeds of the three tree species are different in the germination schedule (dormant or nondormant), chemical composition (e.g., tannin), seed coat thickness, and morphological size, ^{15}N isotope was ubiquitously enriched in seeds and seedlings of *Q. variabilis*, *C. glauca*, and *P. koraiensis*. These facts imply that ^{15}N enrichment is unlikely to be affected by seed trait. Traditional seed-tracking methods are often limited by the size of seeds, and thus, only seeds of large size are selected for labeling and tracking (Sone and Kohno 1996; Mack and Druliner 2003; Yi et al. 2008, 2012b). Given that seed species of any size can successfully germinate and establish, our method makes it possible to track dispersal and recruitment of both small- and large-seeded plant species. Although our method may not efficient to study “natural” removal rates of seeds, especially those seeds contained within fleshy fruits, it is expected to be ideal for “seed addition” experiments to track seed dispersal of some tree species (Nathan and Mueller-Landau 2000; Clark et al. 2007). Our study verified the validation of ^{15}N -urea soaking in tracking seed dispersal and seedling recruitment of a number of tree species and provided the confidence to distinguish between the seeds added and those arrived naturally. We believe that this promising method will improve the seed-tracking efficiency of seed dispersal ecology.

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