Variability in deer diet and plant vulnerability to browsing among forests with different establishment years of sika deer

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Abstract

Increased ungulate browsing alters the composition of plant communities and modifies forest ecosystems worldwide. Ungulates alter their diet following changes in availability of plant species, however, we know little about how browse selection and plant community composition change with different stages of deer establishment. Here, we provide insight into this ~~field~~ area of study by combining multiple approaches: comparison of the understory plant community, analysis of records of browsing damage, and DNA barcoding of sika deer feces at 22 sites in forests in northern Japan varying in when deer were first established. The coverage of vegetation ~~coverage~~ and number of plant species were only lower at sites where deer were present for more than 20 years, while the difference in plant coverage among deer establishment years varied among plant species. Deer diet differed ~~among~~ across~~the~~ establishment years, but was more affected by the site, thereby indicating that food selection ~~of~~by deer could change over several years after deer establishment. Plant life form and plant architecture explained the difference in plant coverage a~~mong~~cross establishment years, but ~~a~~ large variability was observed in deer diet within the two categories. Integrating these results, we categorized 98 plant taxa into six groups that differed in vulnerability to deer browsing (~~the damage~~ degree of damage and ~~the~~ cover~~age~~age). The different responses to browsing among plant species inferred from this study could be a first step in predicting the short- and long-term responses of forest plant communities to deer browsing.

Introduction

A recent global conservation issue is the drastic increase in ungulate populations that has caused degradation in forests ecosystems. For example, high densities of red deer (*Cervus elaphus*) in Europe and New Zealand, moose (*Alces alces*) and white-tailed deer (*Odocoileus virginianus*) in North America, and sika deer (*C. nippon*) in Japan are caused by recent introductions or environmental changes (Côté et al., 2004; Cooms et al., 2003; McCullough et al., 2009, Tape et al. 2014, Kaji & Iijima 2017). Increased deer browsing ~~has been reported to~~

41 alter~~s~~ the composition of plant communities and decrease~~s~~ the diversity of plant species within

42 one or two decades in areas such as North America, and <u>the </u>Netherlands (Akashi &

43 Nakashizuka, 1999; Russel et al. 2001, Maesako & Takatsuki 2015, Ramirez et al. 2019).

44 However, ~~the ~~temporal changes in <u>the </u>plant community in response to browsing have not been

45 fully assessed as most studies are based on a comparison of the vegetation before and after

46 ungulate population control or exclusion by fencing. These studies have shown that the recovery

47 of vegetation often takes ~~more than ~~decades after a decrease in deer density<u>,</u> or ~~can even cause~~

48 ~~irreversible~~<u>the</u> degradation of forest vegetation <u>may be irreversible</u> (Mysterud, 2006; Tanentzap

49 et al., 2012; Tamura, 2016).

50 Deer populations are usually managed using estimated densities and perceived carrying

51 capacity (Kaji et al., 2010). The estimation of deer densities is technically demanding, ~~which~~

52 requir~~es~~<u>ing</u> extensive field sampling or elaborate population models (Millspaugh et al., 2009),

53 while recent studies show improvement and new developments in population estimation (Iijima

54 2020). Moreover, deer impacts can be low or high at the same density, depending on forage

55 availability and browsing history, which is critical to the regeneration of plant species

56 (Tanentzap et al., 2012). Recent studies have emphasized the need to monitor not only deer

57 density but also its impact on ~~the ~~<u>the </u>ecosystem~~s~~, including ~~the ~~vegetation (Stout et al., 2013;

58 Iijima & Nagaike, 2015). Plant responses to grazing are known to differ among species which

59 may be explained by plant architecture and traits, due to food selection by deer, tolerance to

60 herbivory, and competitive interactions among plants (Augustine & deCalesta, 2003, Hanley et

61 al., 2007; Diaz et al., 2007; Nishizawa et al., 2016). As plant persistence under browsing pressure

62 may vary among plant species, ~~not only~~<u>both</u> ~~the ~~food selection by deer ~~but also~~<u>and</u> ~~the ~~plant

63 abundance ~~is required~~<u>must be considered</u> to understand ~~the ~~plant vulnerability to deer browsing.

64 It has been shown that deer change their diet following changes in vegetation availability

65 over several decades or even ~~in ~~short periods (Brown & Doucet, 1991; Bruinderink &

66 Hazebroek, 1995; Takahashi & Kaji, 2001). They can also change their consumption based on

67 food availability at the patch scale including neighboring plants (Bergval et al. 2006, Rautio et al.

68 2012). Investigation of the diet of ungulates in addition to ~~their ~~<u>observed </u>browsing damage

69 would be effective ~~to reveal~~<u>in revealing</u> deer-food selection because browsing damage may

70 include damage caused by ~~the ~~untargeted ungulate species. Conventional methods ~~for ~~<u>of </u>diet

71 analysis, including analysis of gut contents or microscopic analysis of feces, require~~s~~

72 considerable training and extensive reference plant collection~~s~~ (Takatsuki, 1978; Takatsuki &

73 Fuse, 2010), and the absence of a unique epidermal structure in plant species leads to low

74 taxonomic resolution (Pompanon et al., 2012). Conversely, DNA barcoding with feces is

75 reported to have a high taxonomic resolution and is a useful and fast technique to estimate

76 herbivore diets<u>,</u> including those of ungulates (Valentini et al., 2009; Raye et al., 2011; Ando et

77 al., 2013, Kress et al., 2015, Nakahama et al., 2020). However, whether it can be used for

78 quantitative evaluation is still under consideration (Pompanon et al., 2012). <u>However, ~~I~~</u>it is

79 important to combine multiple approaches for a robust evaluation of ungulate diet.

In forests in Japan, the population of sika deer, (hereafter referred to as deer) has drastically increased in the last two decades, and its distribution range has expanded by more than 2.5 times in the past 36 years from 1978 to 2015 (Fig. 1a, the Ministry of Environment 2015). Especially in the northern Tohoku region of Japan, deer were almost extirpated in the early 1900's but the population has increased rapidly since 1950 (Takatsuki, 2009b), and its distribution range has expanded, especially during the 2010's (the Ministry of Environment, 2015). With the growing demand to address the serious threats posed by sika deer, techniques for population monitoring that can be applied to low deer densities are required during the initial stage of establishment in these areas. Recent studies have introduced a novel approach to evaluate the abundance of sika deer in low-density populations, such as methods to distinguish between feces of sika deer and Japanese serow (Aikawa et al., 2015), and the acoustic monitoring of male abundance (Enari et al., 2017). However, there is a lack of knowledge about the response of plant communities in the initial stage of deer establishment. Information on the response of plant species to browsing ~~from~~ after the initial stage of deer establishment would be informative for ~~reforming effective~~ conservation efforts ~~al management~~ to maintain plant community diversity. The years since deer establishment exhibits geographic variability due to the recent expansion of ~~the~~ deer in the Tohoku region. This provides a promising opportunity to compare food selection among different establishment years of deer, and examine plant vulnerability to browsing from the initial stage of deer establishment. We investigated the understory plant community and deer diet at 22 sites in forests in northern Japan, that vary~~ies~~ in year of deer establishment. We predicted that ~~the~~ deer diet and plant community composition would vary among deer establishment years. We evaluated plant vulnerability based on both deer-food selection (~~damage~~ degree of damage) and vegetation (plant cover~~age~~) among plant species in response to deer establishment.

Materials & Methods

Study sites

This study was conducted from 2016 to 2017 at 22 cool-temperate deciduous-forest sites at an average ~~altitude~~ elevation of 410 m dominated by oaks such as *Quercus crispula*, and *Q. serrata* in the northern Tohoku region in Japan (Table S1, Fig. 1b). We focused on this type of vegetation distributed in low-~~altitude~~ elevation forests because this allowed us to replicate multiple sites across the region. According to the Japanese Ministry of the Environment, Japan is divided into 5 km meshed units, which are categorized into ~~either of the~~ five categories: 1) deer established before 1978 (1978 year site), 2) deer established during 1979-2003 (2003 year site), 3) deer established during 2004-2011 (2011 year site), 4) deer established during 2012-2014 (2014 year site), and 5) not established. We replicated 4-9 sites for each category of 1) to 4). The estimated deer density of the 5 km meshed unit that contained each site was higher in older deer establishment sites, while it varied in recent establishment sites (Table S1). Although deer density may vary between years, we selected sites where deer have been constantly observed in the two years before the survey according to the Tohoku Regional Forest Office (2017;)

120   (approval number: 29-392, 29-487, 28-446). Although sites 18-22 are located in meshed units
121   categorized as 5), deer were observed in these sites in the two years before the survey, and thus,
122   these sites were categorized as 4). We did not include sites in category 5) because our main
123   objective was to compare the food selection of deer among establishment years and we cannot
124   evaluate this in sites without deer.
125
126   *Vegetation survey of forest understory*
127   To investigate the plant community of the site (500m × 500m), we set 20 random
128   replicates of 2 m × 2 m plots in the forest floor including both ridges and valleys at each site in
129   early September. Surveys were conducted in either 2016 or 2017. Plants with more than 5%
130   coverage were recorded at 5% intervals, and those with less than 5% coverage were scored as
131   1% following the method used by Sakata and Yamasaki (2015). We included flowering plants
132   that were < 130 cm in height. We excluded ferns because few little browsing damage were was
133   found on ferns. However, it is notable that some unpalatable ferns can become dominant in
134   intensively browsed forests (Cretaz & Kelty 1999, Ishida et al. 2008), which may affect the plant
135   community. We categorized each plant species into each life form (annual, perennial, vine, tree,
136   shrub). We also categorized forb species into eachby plant architecture (prostrate, erect, rosette,
137   rosette [seasonal], tussock) according to the indices described by Asano (2005). Note that the
138   categorization of woody species of plant architecture is identical with the categorization of life
139   form.
140
141   *Browsing damage investigations*
142   To investigate the browsing damage of deer among across plant species, we measured the
143   damage ofto focal plant species at each of the 22 sites in early September. We first assessed the
144   morphology characteristics of the browsing damage to exclude damage by other herbivores such
145   as rabbits (rabbits leave clean cuts, while deer leave rough or torn cuts). Although we cannot
146   completely exclude the possibility that the browsing damage was made by Japanese serow
147   (*Capricornis crispus*), we only found feces of deer in our sites, as described in the feces
148   sampling section below. Therefore, the browsing damage found in our survey is was likely to
149   reflect the damagecaused by deer. We selected 273 species (86 families) as focal plant species to
150   cover the plant species that appeared in the vegetation survey in of all sites. We randomly
151   selected 10 individuals in the forest understory for each plant species which were present by
152   walking around the whole area of each site including the 20 plots of the vegetation survey. Each
153   plant individual was separated in more than 3 m. We aimed to evaluate browsing damage in the
154   growing season of the plant, which is during spring and summer of the current year. Each plant
155   species was categorized into eachby life form and plant architecture. For each plant unit (ramet
156   for forbs and current-year shoots for woody plants and vines), the damage level was visually
157   estimated and classified into the following four levels: CL1, no damage; CL2, 1-10% damage of
158   the plant; CL3, 11-50% damage of the plant; CL4, 51-100% damage of the plant. Subsequently,
159   the values for all four levels were added and divided by 10 (i.e., total number of plants or ramets:

160   N) to calculate the mean damage grade (MDG) (Sakaguchi et al. 2012) for each plant species at

161   each site, as shown in the following equation:

162   MDG =(0*CL1+5*CL2+30*CL3+75*CL4)/N

163   We estimated the damage level by assuming that the plant unit was in the averaged size

164   of the undamaged plants observed at each site. Although this method may lead to biased

165   evaluation among plant life forms, the broad classification (i.e., four categories) would reflect the

166   relative damage, which is comparable across plant taxa.

167

168   *Sampling feces and diet analysis using DNA barcoding*

169   We prepared a trnL P6 loop region reference database comprising 297 flowering plant

170   species that occurred at our study sites (Table S2); 167 species obtained from a database in a

171   previous study (Nakahama et al. 2020), 71 species from the National Center for Biotechnology

172   Information (NCBI), and 59 species were newly sequenced in this study (see Appendix 1 for

173   detailed methods).

174   To evaluate the diet of deer, we searched for deer feces at each site and when available,

175   fecal pellets were sampled from 5 - 10 dung piles (10 pellets on average per dung pile) found in

176   each site during late July to early September and stored at -20 °C. We collected feces from 16

177   sites (Table S1). Note that for site Tazawa, feces were collected in November. Of these, we

178   chose five fresh fecal pellets from 3-5 dung piles collected at each site. Each five pellets

179   correspond to one fecal sample, yielding 63 fecal samples in total. These pellets were checked

180   whether they were deer feces using a recently developed loop-mediated isothermal amplification

181   method targeting the cytochrome b gene in the mitochondrial DNA of deer (Aikawa et al. 2015).

182   The food plant DNA was extracted from each fecal sample, obtained the sequences of the plant

183   DNA, and identified the plant taxon in each fecal sample using the reference database described

184   above (see Appendix 2 for detailed methods).

185

186   *Data analyses*

187   We conducted multiple analyses of data obtained from each survey, as summarized in

188   Table S3. All the generalized linear mixed model (GLMM) analyses were conducted using the

189   lme4 package (Bates et al. 2011) and car package (Fox and Weisberg 2011) of R 3.3.2 (R

190   Development Core Team 2016). In all the GLMM analyses described below, the significance of

191   the variables ~~were~~ was determined using a likelihood ratio test, compared to the chi-square

192   distribution. When the effect of a variable was significant ($P < 0.05$), we tested for differences

193   among categories within a variable using Tukey's honestly significant difference (HSD) test on

194   least-squared means with the overall type I error rate at 5% using the "lsmeans" package (Lenth

195   2015) of R. All the permutational multivariate analysis of variance (PERMANOVA) analyses

196   and non-metric multidimensional scaling (NMDS) analyses were performed based on Bray-

197   Curtis dissimilarity index. In all the PERMANOVA analyses we described, we also tested

198   whether the multivariate dispersion regarding the group centroid differed among the main effects

199   by using the betadisperser function in VEGAN package in R (Oksanen et al. 2019), and it was

200 revealed that ~~the~~ composition differed among the main effect. In all K-means clustering analyses,
201 the number of groups was determined using gap statistics (Tibshirani et al. 2001).
202
203
204 -Vegetation survey-
205   We conducted the following six vegetation analyses. First, to evaluate how plant species
206 diversity differed among deer establishment years, generalized linear models (GLMs) were
207 applied. We set the number of species and the Shannon-Wiener diversity index per site as
208 response variable, establishment year as an explanatory variable with Poisson and normal
209 distributions, respectively. Second, to test whether the composition of understory species differed
210 among deer establishment years, we performed a PERMANOVA. We used the data matrix of the
211 273 plant species coverage of each site (the coverage of the 20 plots were added), and we
212 included deer establishment years of each site as an explanatory variable. For this analysis, the
213 ADONIS function in the library VEGAN in R was used. We used NMDS to examine understory
214 species composition. To consider spatial structures, we also used distance-based Moran's
215 Eigenvector Maps (dbMEM) on detrended data and x,y coordinates using the adespatial R
216 package (Dray et al. 2021). Significance of the spatial vectors was assessed using ANOVA and
217 forward selection was carried out to identify significant dbMEM vectors following Borcard et al.
218 (2018). Third, to investigate how vegetation cover differed among deer establishment years, we
219 used GLMMs with Poisson distribution. The sum of vegetation coverage of all plants per plot
220 was set as response variable and deer establishment year as an explanatory variable. Site nested
221 within surveyed year was included in the model as random intercept to deal with unknown
222 effects of climate and resource variations among sites and surveyed years of the study. Fourth, to
223 test whether the plant coverage differed among deer establishment years for each life form and
224 plant architecture (excluding woody species), GLMMs with Poisson distribution were applied.
225 ~~Plant c~~Coverage ~~foo~~of~~r~~ each life form and plant architecture per plot was set as the response
226 variable and deer establishment year as an explanatory variable, and site nested within surveyed
227 year was included in the model as random intercept. Fifth, to explore whether the patterns of
228 variation in ~~the~~ coverage among deer establishment years differed among plant life forms and
229 plant architectures, a PERMANOVA was performed. For 58 plant species that were present in at
230 least one site of each establishment year in the vegetation survey, coverage of each plant species
231 for each establishment year was averaged over sites, yielding four coverage values per species.
232 Subsequently, the effects of life form on the coverage of the four combined coverage values were
233 tested. For the 30 species excluding woody species, the effect of plant architecture on the
234 coverage of the four combined establishment years was tested. The coverage for each
235 establishment year was averaged over the sites. Finally, a K-means cluster analysis of the
236 coverage of the four establishment years combined for 58 plant species was performed to
237 identify groups of plants with similar patterns of variation in the coverage among deer
238 establishment years. The K-means clustering was set to define two statistically distinguishable
239 groups.

240

241   -Browsing damage of plants-

242   We conducted the following three analyses for deer browsing damage. First, to test
243   whether ~~the~~ damage level was affected by ~~the~~ plant coverage, GLMM with normal distribution
244   was applied. The squared transformed MDG (mean damage grade) of each species was set as a
245   response variable and plant coverage as an explanatory variable. Site nested within surveyed year
246   was included in the model as random intercept. Second, to explore whether patterns of variation
247   in the MDG among deer establishment years differed among plant life forms and plant
248   architectures, a PERMANOVA was performed. For 34 plant species that were present in at least
249   one site of each establishment year, the effects of life form and plant architecture (excluding
250   woody species) on the MDG of the four combined establishment years was tested. MDG of each
251   plant species for each establishment year was averaged over sites, yielding four MDG values per
252   species. Finally, a K-means cluster analysis of the MDG of the 34 plant species was performed to
253   identify groups of plants with similar patterns of variation in the damage level among deer
254   establishment years. The K-means clustering was set to define three statistically distinguishable
255   groups. Additionally, we listed 16 species that were absent in the 1978 year sites but were
256   browsed in sites within the other three establishment years because these plants may have
257   disappeared in the 1978 year sites due to browsing.

258

259   -DNA barcoding of feces-

260   We conducted the following three analyses for the DNA barcoding analysis. First, we
261   performed a PERMANOVA to test whether diet composition differed among deer establishment
262   years. We used the data matrix of the proportion of reads (i.e., number of sequence reads of each
263   plant taxa/ the total number of sequence reads) of 98 plant taxa contained per sample. The effect
264   of site was considered as a block effect by including site as a strata function in the library
265   VEGAN in R. To consider spatial structures, we also used dbMEM on detrended data and x,y
266   coordinates. Significance of the spatial vectors was assessed using ANOVA and forward
267   selection was carried out to identify significant dbMEM vectors. In temperate deciduous forests,
268   season~~s~~ of the year influences plant development stage and consequently food availability to deer
269   (Takatsuki, 1986; Dumont et al., 2005, Nakahama et al., 2020); therefore, we also conducted the
270   same analysis described above by excluding samples collected in Tazawa site in November. An
271   NMDS was performed using the data matrix of the proportion of reads of each plant taxa to
272   visualize the diet composition distances among fecal samples. Second, to test whether the
273   proportion of reads differed among deer establishment years for each life form and plant
274   architecture, GLMMs with normal distribution were applied. The proportion of reads of each
275   plant taxa was set as _the_ response variable and deer establishment year as explanatory variable,
276   and site nested with surveyed year was included in the model as random intercept. Third, to
277   explore whether patterns of variation in the proportion of reads of each plant taxa among deer
278   establishment years differed among plant life forms and plant architectures, a PERMANOVA
279   was performed. The 98 plant taxa detected in the analyses were categorized ~~into each~~by life form

280 and plant architecture. We analyzed the effects of life form and plant architecture (excluding the
281 woody species) on the proportion of reads of the four establishment years combined for the 98
282 plant taxa. The proportion of reads for each establishment year was averaged over sites. Finally,
283 a K-means cluster analysis of the number of reads of the 98 plant taxa was performed to identify
284 groups of plants with similar patterns of variation in the damage level among deer establishment
285 years. The K-means clustering was set to define three statistically distinguishable groups.
286

Results

288 *Vegetation coverage of understory plant community*
289 We detected 273 species (86 families) during the vegetation survey at 22 sites. In the
290 1978-year sites, deer browsing damage and feces were abundant, and the understory vegetation
291 was scarce (Fig. 1). The number of plant species significantly differed among deer establishment
292 years (χ^2= 40.99, P <0.001, N=22, d.f=3), and it was lower in the 1978 -year sites than other
293 sites (Fig. 2a). Conversely, the Shannon-Wiener diversity index did not differ among the
294 establishment years (Fig. 2b, χ^2=7.75, P=0.05, N=22, d.f.=3). The sum of vegetation cover
295 significantly differed among deer establishment years (χ^2= 48.55, P < 0.001, N=440, d.f.=3),
296 and the 1978-year sites were lower than the other sites, and the 2014-year sites were higher than
297 the other sites (Fig. 2c). Plant coverage differed among deer establishment years within life
298 forms and plant architecture (Table 1). In perennials, the coverage at the 2011-year sites was
299 higher than that in the 1978-year sites (Fig. S1a). For vines, cover was lower in 1978-year sites
300 than that in the 2014-year sites. For shrubs, cover was higher in the 2014-year sites than that in
301 the other sites, and was higher in the 2003-year sites than that in the 1978-year sites. In the
302 category of plant architecture, it was higher in the 2011-year sites than that in the 2003-year sites
303 for prostrate plants (Fig. S1b). For tussock, cover was lower in the 1978-year sites than that in
304 the other sites.
305 Plant community composition differed among the establishment years (R^2 = 0.21, P =
306 0.001, Fig. 3). We did not detect spatial correlation, as none of the vectors (MEMs) were
307 statistically significant. The 1978-year sites were plotted separately from other sites. The plant
308 coverage of the four establishment years combined differed among life forms (R^2 = 0.13, P =
309 0.012), and plant architecture (R^2 = 0.24, P = 0.029). The clustering analysis classified species
310 into the following two groups: 1) plants whose coverage decreased in sites with longer
311 establishment years, and 2) plants whose coverage did not decrease in sites with longer
312 establishment years but was highest in the 1978-year sites (Table S4, Fig. 4a).
313

314 Browsing damage of plants
315 Browsing damage w~~ereas~~ detected on 174 plant taxa in total across all sites. Plant
316 coverage had a weak positive effect on MDG (MDG = exp (0.0027*coverage + 1.109), χ^2 = 4.15,
317 P = 0.04, N=724). The MDG of the four combined establishment years did not differ either
318 between life forms (R^2 = 0.18, P = 0.06), or plant architecture (R^2 = 0.18, P = 0.06). The
319 clustering analysis classified 34 species into three groups, G1-G3 (Table S4, Fig 4b), and we

320 categorized 16 species that were only absent in the 1978-year sites as G4 (Table S4). G2
321 consisted of plants whose MDG values were constantly high at the 2014-, 2011-, and 2003- year
322 sites, and was especially high at the 1978-year sites than that at the other sites (Fig. 4b). G3
323 comprised plants in which the MDG value gradually increased as the establishment years
324 increased. G1 consisted of four species that were not classified into the former two groups and
325 showed higher values of MDG in the 2011- and 2003-year sites than those at other sites. In G4,
326 shrubs excluding *Corylus sieboldiana* had high MDG values in all three establishment years, and
327 perennials were browsed in the 2003- and 2011-year sites but not in the 2014-year sites, and all
328 belonged to an erect growth form.
329
330 DNA barcoding of feces
331   Sequencing of 63 fecal samples yielded 424,114 reads, corresponding to an average of
332 74.7 bp. From the results of DNA barcoding with the P6 loop database, 98 plant taxa (49
333 families) were detected from 401,206 reads (95% of the total sequences) (Table S2). Most of the
334 reads that were not assigned to a specific plant taxon in the P6 loop database (22,908 reads) were
335 short or had a low frequency. The sequence file of the fecal sequences was deposited at the
336 International Nucleotide Sequence Database Collaboration through the DNA Data Bank of Japan
337 (Accession no. DRA011125).
338   Out of 98 plant taxa ~~were~~ detected from the 63 feces samples, 73 plant taxa were detected
339 throughout the four establishment years. In 1978, 2003, 2011, and 2014 year sites, 44, 36, 35,
340 and 36 plant taxa were detected, respectively. We did not detect spatial correlation, as none of
341 the vectors (MEMs) were statistically significant. Diet composition (proportion of reads of plant
342 taxa) differed among the establishment years ($R^2 = 0.13$, $P <0.001$, Fig. 5). This was significant
343 even when feces sampled in November were excluded from the analysis ($R^2 = 0.13$, $P = 0.001$).
344 However, when the site was considered as a block effect, the effect of establishment years was
345 not significant ($R^2 = 0.13$, $P = 0.99$), indicating that the composition of the feces varied among
346 sites. In particular, the 2014-year sites were plotted differently from other establishment year
347 sites (Fig. 5). In the 2014-year sites 26 species were absent or in remarkably low frequency
348 (Table S5). These plants include small plants shorter than 20 cm or rosette plants such as
349 *Hydrocotyle* spp., *Stellaria uliginosa*, *Ajuga* spp., *Oxalis corniculata*, *Lysimachia japonica*, and
350 *Viola vaginata*. Additionally, the number of reads in some shrubs was exceptionally higher in the
351 2014-year sites than that at other sites such as *Stachyurus paraecox*, *Aucuba japonica*, *Rosa*
352 *multiflora*, *Kerria japonica*, and *Euonymus alatus* var. *altus*. Within each category of life forms
353 and plant architectures, there were no significant differences among the establishment years
354 except for trees and shrubs (Table 2, Fig. S2). The number of reads of the trees gradually
355 increased with a greater number of years since establishment, and it was significantly higher in
356 the 1978-year sites than that in the 2014-year sites (Fig. S2a). Conversely, the number of reads of
357 shrubs gradually decreased as the establishment years extended, and it was significantly lower in
358 the 1978-year sites than that in the 2014-year sites.

359   The proportion of reads of plant taxa of the four establishment years combined did not
360   differ either among lifeforms ($R^2 = 0.04$, $P = 0.84$) or among plant architectures ($R^2 = 0.18$, $P = 0.06$). The clustering analysis classified species into three groups G1-G3 (Table S5, Fig. 4c): G1
362   included plants in which the proportion of reads in the 1978-year sites was higher than that in the
363   other year sites, G2 included plants in which the proportion of reads became gradually higher as
364   establishment years became longer, and G3 was species that was not classified into the former
365   two groups and showed a higher proportion of reads in the 2011 year-sites (Fig. 4c).
366
367   Assessment of vulnerability of plant species
368   We integrated the results of the three cluster analyses described above, and 98 plants that
369   were included in either MDG analyses or DNA barcoding in feces were categorized into six
370   groups (Table 3, group A–E). Please see Appendix 3 for detailed description of categorization of
371   the groups.
372

Discussion

374   The results of the browsing damage and diet analysis using DNA barcoding indicate~~d~~ that
375   ~~the~~ deer selection may vary among deer establishment years. The categorization based on the
376   three cluster analyses showed that 16 species (group A) were browsed at a greater rate from the
377   initial stage of the deer establishment and showed lower coverage in sites with more than 10
378   years since deer establishment. The 16 plant species belonged to various life forms and plant
379   architectures~~,~~ indicating that these indices might not always be effective in predicting plant
380   vulnerability to browsing. Moreover, ~~the results that~~ 11 of the 16 species were detected both in
381   the ~~Browsing~~ browsing damage survey and DNA barcoding of feces, suggest~~ed~~ing that deer
382   exhibited a high selection for these species. Conversely, it was revealed that 14 species (group C)
383   were not as highly browsed from the initial stage of deer establishment as group A, but showed
384   lower coverage in sites with more than 10 years since deer establishment. On the other hand, 11
385   species (groups E and F) were selected but displayed negligible ~~to~~ browsing effects as their
386   coverage did not differ among deer establishment years. These results are consistent with those
387   reported by Akashi et al. (2015), who stated that although ~~the browsed level~~the level of browse
388   was the same, ~~the~~ recovery differed among different species in juvenile trees. This may be
389   because ~~the~~ tolerance to browsing differs among species. Noteworthy, these categorizations of
390   plant vulnerability based on ~~damage~~ degree of damage and coverage in the summer among deer
391   establishment years might differ in other seasons as studies have reported seasonal changes in
392   deer diet both in medium and severely damaged forests (Takatsuki, 1986, Nakahama et al.,
393   2020). Takatsuki (1986) studied the deer diet at Mt. Goyo in Japan, which is located in the region
394   of our study, and showed that *Sasa* spp. was the most dominant plant species in~~of~~ the deer diet
395   and its proportion increased, especially in the winter and early spring. Further study of seasonal
396   changes in deer diet is necessary to better understand vulnerability to browsing among plant
397   species.

Vegetation coverage and number of species was lower only in the 1978-year sites. These results are consistent with the reports of previous studies showing declines in species richness in other forest in Japan after more than 20 years of browsing (Fujiki & Takayanagi, 2008, Takatsuki, 2009a, Tamura et al., 2011). The coverage of perennials and vines was only low in the 1978-year sites compared to other establishment year sites. However, the coverage of shrubs was lower in 2011-year sites than in 2014-year sites, suggesting that shrubs are more vulnerable to browsing and cover may decline in the early stage of deer establishment. In the plant architecture category, while coverage did not differ among establishment years ~~in~~ for erect, rosette plants, the coverage of tussock plants was considerably lower in the 1978-year sites but prostrate plants showed lower coverage in the 2003-year sites. Previous studies reported that long lived perennials and~~,~~ erect plants are likely to be more affected by browsing than annuals~~,~~ and rosette plants (Daiz et al., 2007, Tamura, 2011), and dwarf shrubs are less abundant toward the high end of the herbivory gradient than juvenile trees (Hegland & Rydgren, 2016). Our results partly support these reports; however, the large variation we observed suggests that the response to ~~the~~ deer establishment year differs at the plant species level within each category.

The weak positive effect of coverage on the MDG of each species suggests that deer are likely to feed on the more dominant plants as their foraging availability is known to depend on plant density (Boulanger et al. 2009), and plant apparency (Furedi, 2004). Although reduced apparency can delay ungulates detecting ~~the~~ a plant, a study has shown that wallabies tend to browse more on lower biomass plants due to visual cue of younger plants or new growth in plant~~s~~ (Stutz et al. 2017). Thus, plant apparency may not always correspond to the level of herbivory ~~level~~. Unlike the vegetation coverage, plant life form and plant architecture did not explain the pattern of deer food selection among establishment years, as shown by the large variance of MDG within each establishment year. The nutritional value and chemical content of ~~the~~ plants determine the forage selection of deer (Champagne et al. 2020). Thus, ~~the~~ plant resistance based on phytochemicals, which may vary among plant life forms and plant architectures, is likely to be more important than plant apparency for ~~the~~ food selection ~~of~~by deer. However, many of the shrubs that were classified as G2 and G4 (browsed in 2003, 2011, and 2014 but absent in 1978-year sites), and the perennials classified as G2 and G4 were ~~all~~ erect in growth form. Diet analysis using DNA barcoding also showed that shrub species were detected at a much higher frequency in ~~the~~ feces from the 2014-year sites than the other sites. These results suggest that shrubs and erect herbs may be more heavily browsed than other plants, and some may disappear in forests where browsing occurred for more than 20 years. Conversely, the average proportion of rosette plant taxa detected in the DNA barcoding was 2.27 in the 1978-year sites, while it was 0.93, 0.53, 0.92 in 2003-, 2011-, and 2014-year sites, respectively (Table S5). This may reflect the deer forage on rosette plants more in forests where browsing occurred for more than 20 years. This may be explained by the nearby presence of other more attractive species in the initial deer established sites where the rich plant community remained, but not in the severely browsed sites (Boulanger et al., 2009).

437   The diet analysis using DNA barcoding provided high-resolution identification of food

438   plants and detected 28 plant taxa that were included in the analysis of browsing damage. Thus,

439   DNA barcoding is a useful method ~~to~~ for detecting a wide array of plant species within the

440   feeding range of deer. However, 21 plant taxa included in the browsing damage analysis were

441   not detected by the DNA barcoding. This discrepancy may be due to insufficient sampling of

442   feces or due to issues related to low PCR amplification in some species, which is a future

443   challenge ~~of~~ for the use of this method (Nichols et al. 2016).

444   The species composition of the understory vegetation varied with deer establishment

445   year. These results indicated that although the sum of cover and number of species did not

446   change until decades of browsing had occurred, the species composition can change across a

447   much shorter time scale. However, we acknowledge the limitation of our study as we did not

448   account for the difference in the plant community among sites before deer establishment.

449   Because there is a longitudinal cline in ~~the~~ deer establishment years, we could not separate the

450   effect of deer establishment year~~s~~ from ~~the~~ abiotic factors such as ~~the~~ snow depth (~~higher~~greater

451   snow depth in Japan seaside than in Pacific seaside) on the difference in vegetation among sites

452   (Takatsuki 2009b). The DNA barcoding of feces indicated that the diet of deer largely differed

453   among sites and we could not exclude the possibility that ~~the~~ deer diet is more affected by site

454   specific factors. It is noteworthy that, however, that the plant community differed even when we

455   limited our data analysis to Pacific seaside sites (R^2=0.12, P=0.004), indicating that the

456   difference in the vegetation between Pacific seaside and Japan seaside ~~reflecting~~ resulting from

457   snow depth difference is less likely to explain the plant community difference~~s~~ observed in our

458   sites. In addition, we cannot deny the possibility that the deer food selection had been influenced

459   by the initial composition of the plant community, which may had led to different trajectories in

460   deer impacts on the ecosystem as deer food choices are known to be influenced by the plant

461   species composition (Champagne et al. 2018). Considering ~~the~~ data ~~of~~from the plant community

462   in ~~the~~ sites without deer invasion and/~~-~~or monitoring the temporal changes of the plant

463   community in the recent established sites are essential in future studies.

464   Previous studies have suggested the ~~concept~~ use of indicator species, which is an

465   approach ~~of~~that monitor~~s~~ing ~~the~~ plant size or browsing damage of a specific species ~~that are~~

466   selected by ungulates, to predict ungulate abundance (Augustine & deCalesta, 2003; Mysterud et

467   al., 2010; Akashi et al., 2015; Iijima & Nagaike, 2015; Inatomi et al., 2017). Waller et al., (2017)

468   have also shown that twig ages provide a direct indicator of browsing on regenerating trees with

469   lower sampling variance, greater sensitivity, and are reliable indicator of deer impacts and

470   habitat conditions. These studies have shown that these indicators may be effective in gaining

471   information not only when the ungulate population is increasing, but also when it ~~after~~ decreas~~es~~

472   ~~after~~ ing due to management. The categorization of ~~the vulnerability of~~ plant species

473   vulnerability to deer browsing ~~from~~ identified by multiple aspects ~~obtained from the~~of the

474   present study ~~would~~ provide~~s~~ insight into the selection of ~~the~~ indicator species. Selecting

475   appropriate indicator species would allow evaluation ~~on~~ of~~the~~ browsing impact ~~of~~before and

476   after ~~the~~ management. Our ~~study~~ results strongly emphasize the ~~that we~~ need for ~~more~~

studies~~more studies~~ of ~~on the~~ temporal changes in plant abundance and food selection ~~by~~ ~~of~~ deer at ~~a~~the local scale, which would allow ~~for~~ more effective conservation of forest vegetation. This would also allow ~~us to~~better ~~e~~evaluat~~eion of~~ the validity of ~~the use of the~~using a chronosequence of deer establishment years ~~for predicting~~to predict changes in plant community composition caused by deer browsing.

Conclusions

By combining multiple approaches to investigate deer diet and vegetation, we categorized 98 plant taxa into six groups that reflect ~~the~~ vulnerability to deer browsing. In future studies, a clear separation between the effects of deer browsing and other environmental factors is necessary for a precise prediction ~~in~~of forest plant community composition. The differ~~enting~~ responses to browsing among plant species inferred from this study could be a first step in predicting the short- and long-term responses of forest plant communities to deer browsing.

Acknowledgements

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Figure 1

Geographic location of study sites in northern Japan and the 5 km meshed area categorized into four sika deer establishment years.

Geographic location of study sites in Japan and (b) in the northern Tohoku region indicated by ~~numeric~~ numbers and the 5 km meshed area categorized into four establishment years of sika deer according to the Japanese Ministry of the Environment: 1) deer established before 1978 (1978 year site), 2) deer established during 1979-2003 (2003 year site), 3) deer established during 2004-2011 (2011 year site), 4) deer established during 2012-2014 (2014 year site), and 5) not established. The site numbers correspond to the description in Table S1. Areas with no mesh indicate areas with no records of deer establishment. Although sites 18-22 are located in the unestablished meshed area, deer were observed ~~in~~ all sites, and thus, these sites were categorized as 4). ~~The~~ photos ~~of~~ the typical forest floor of each establishment year ~~are shown~~.
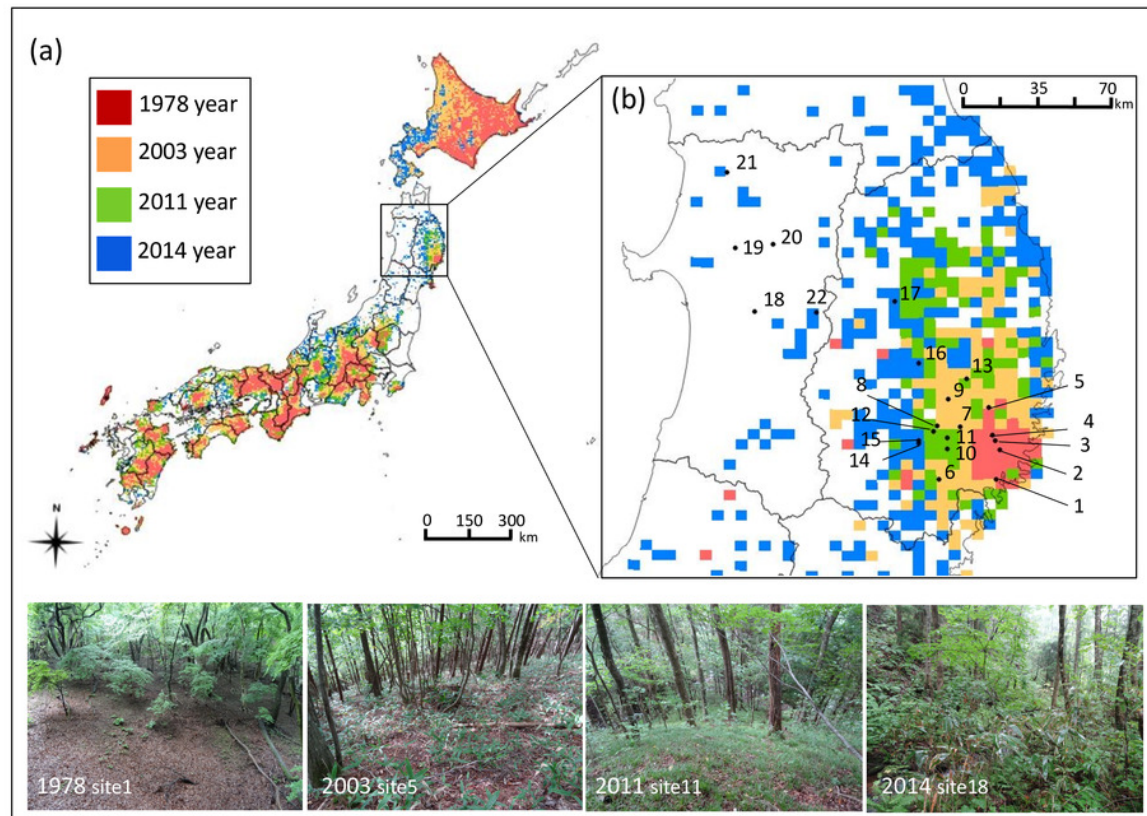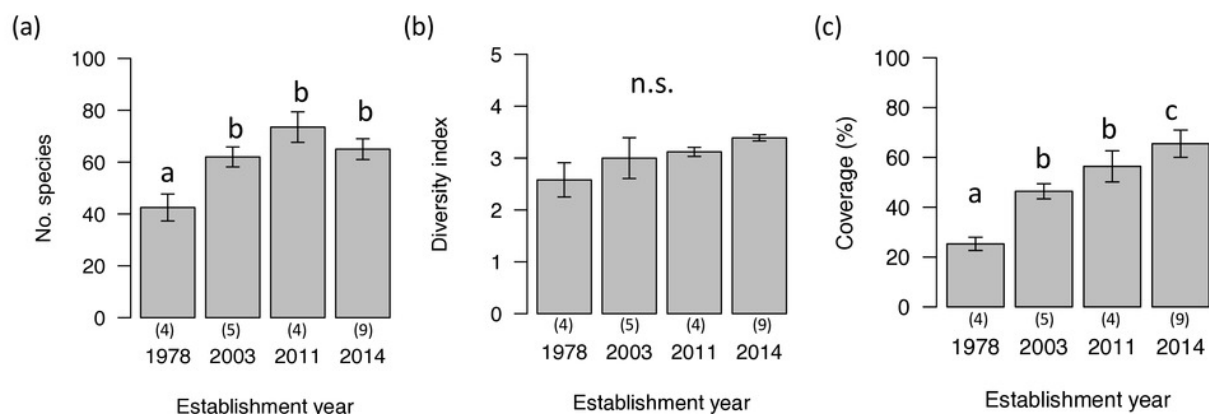
Figure 2

Plant community indices (mean ± SE) of the four establishment years of sika deer.

(a) Number of plant species per site, (b) Shannon-Wiener diversity index per site, (c) sum of vegetation coverage per plot. Different letters indicate significant pairwise difference after post hoc adjustment among establishment years. n.s. indicate no significance difference ($P>0.05$). The number of sites per establishment years are provided by the number in parenthesis above each year. For coverage, 20 plots are included per site.

Figure 3

Differences in plant community including 273 species ~~of~~ 22 sites with four establishment years of deer using non-metric multidimensional scaling (NMDS).

Different symbols indicate sites with different establishment of sika deer. Analyses were based on ~~the~~ Bray–Curtis dissimilarity in the ~~plant~~ coverage of each species. Stress values indicate the extent to which the two disagree is reflected in a stress coefficient, where stress ~~tends to~~ zero when the rank orders reach perfect agreement. Stress is a statistic of goodness of fit, and it is a function of non-linear monotone transformation of observed dissimilarities and ordination distances (Oksanen 2015).
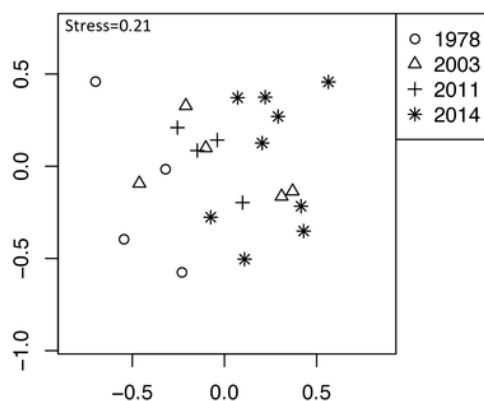
Figure 4

Plant vulnerability groups defined with K-means analyses.

(a) plant coverage, (b) MDG (mean damage grade) of ~~feeding traces~~, and (c) DNA barcoding of feces. Plants categorized in the same group show similar patterns of variation in each category among deer establishment years. Values indicate mean ± SE of each group among sika deer establishment years.
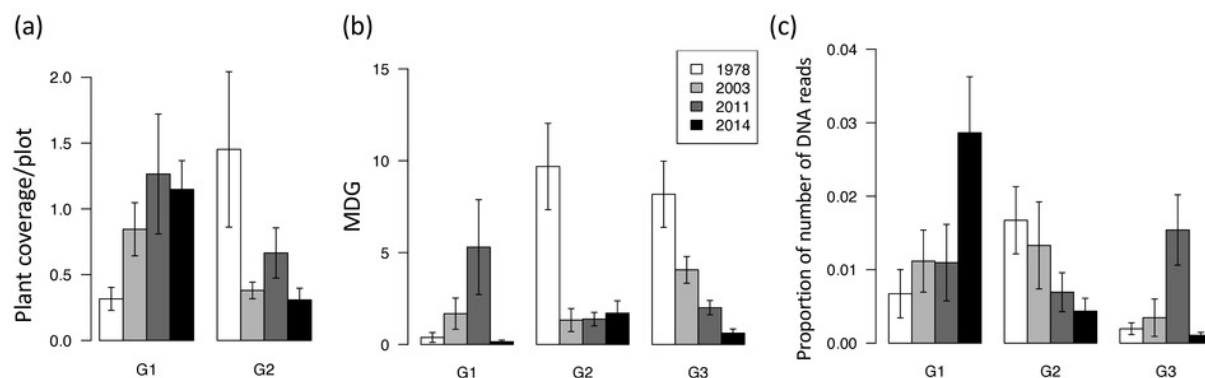
Figure 5

Differences in sika deer diet determined by fecal analysis including 63 feces sampled in 16 sites using NMDS.

Analyses were based on the Bray–Curtis dissimilarity in the read rates of individual samples.

See Fig. 3 for explanation of stress values.
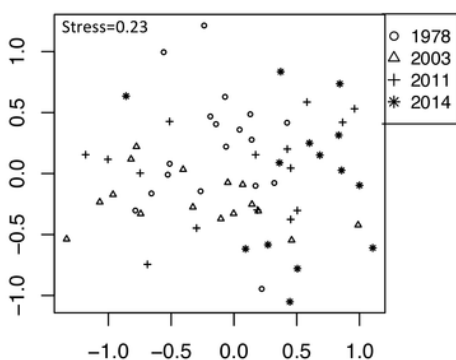
Table 1(on next page)

Results of the GLMM explaining the effect of deer establishment years on plant coverage of each categories.

The significance of the variables ~~were~~ determined using a likelihood ratio test, compared to the chi-square distribution. Bold letters indicate categories which differed among sika deer establishment years (P<0.05). Sample size is 440 plots.

1

		Establishment year (d.f. = 3)	
Life form		χ^2	P
	Annual/biannual	0.96	0.81
	Perennial	**14.01**	**0.003**
	Vine	**20.08**	**<0.001**
	Tree	5.60	0.13
	Shrub	**28.21**	**<0.001**
Plant architecture	**Prostrate**	**10.32**	**0.016**
	Errect	3.56	0.31
	Rosset	2.68	0.44
	Rosset (seasonal)	6.15	0.10
	Tussock	**13.65**	**0.003**

2

Table 2(on next page)

Results of the GLMM explaining the effect of deer establishment years on proportion of number of DNA reads in deer feces of each plant taxa of each categories.

The significance of the variables were determined using a likelihood ratio test, compared to the chi-square distribution.Bold letters indicate categories which differed among sika deer establishment years (P<0.05). Sample size is 63 feces.

1

		Establishment year (d.f. = 1)	
Life form		χ^2	P
	Annual/biannual	2.01	0.08
	Perennial	1.40	0.23
	Vine	0.00	0.99
	Tree	**6.59**	**0.01**
	Shrub	**4.86**	**0.03**
Plant architecture	Prostrate	0.48	0.49
	Erect	0.31	0.58
	Rosset	0.003	0.95
	Rosset (seasonal)	0.32	0.56
	Tussock	2.34	0.13

2

Table 3(on next page)

98 plant species categorized into 6 groups differing in ~~its~~ vulnerability to sika deer browsing.

Plant species in bold letters indicate species that were detected in both ~~feeding traces~~ and DNA barcoding of feces. Plant species with underline letters indicate plants that were present except in 1978 year site. A: plant species browsed in initial establishment years and started to decrease its coverage after 10 years of deer establishment; B: plant species browsed in initial establishment year but the coverage difference among deer establishment was unknown; C: plant species which suffered low browsing level in initial establishment years (less than ten years) but high browsing in the latter years, and started to decrease ~~its~~ coverage after 10 years of deer establishment ; D: plant species which suffered browsing in the latter years but their coverage difference among deer establishment was unknown.

1

Category	Family	Species	Life form	Plant architecture
A	Actinidiaceae	***Actinidia arguta***	vine	
A	Asteraceae	***Ainsliaea apiculata***	perennial	errect
A	Asteraceae	***Eupatorium makinoi***	perennial	errect
A	Asteraceae	*Petasites japonicus*	perennial	rosset(seasonal)
A	Fabaceae	*Hylodesmum podocarpum*	perennial	errect
A	Fabaceae	*Wisteria floribunda*	vine	
A	Fagaceae	*Quercus crispula*	tree	
A	Fagaceae	*Quercus serrata*	tree	
A	Lamiaceae	***Callicarpa japonica***	shrub	
A	Lauraceae	***Lindera umbellata var. membranacea***	shrub	
A	Rosaceae	***Kerria japonica***	shrub	
A	Rosaceae	***Rubus crataegifolius***	shrub	
A	Sapindaceae	***Acer amoenum***	tree	
A	Sapindaceae	***Acer pictum***	tree	
A	Sapindaceae	***Acer sieboldianum***	tree	
A	Staphyleaceae	***Staphylea bumalda***	shrub	
B	Adoxaceae	*Viburnum furcatum*	shrub	
B	Anacardiaceae	*Toxicodendron trichocarpum*	shrub	
B	Asteraceae	***Rudbeckia laciniata***	perennial	rosset(seasonal)
B	Betulaceae	*Corylus sieboldiana*	shrub	
B	Celastraceae	*Celastrus orbiculatus*	shrub	
B	Colchicaceae	*Disporum smilacinum*	perennial	errect
B	Ericaceae	*Rhododendron kaempferi*	shrub	
B	Garryaceae	*Aucuba japonica var. borealis*	shrub	
B	Hydrangeaceae	*Heteromalla paniculata*	shrub	
B	Lythraceae	*Lythrum anceps*	perennial	
B	Moraceae	*Morus australis*	shrub	
B	Pinaceae	*Larix kaempferi*	tree	

B	Plantaginaceae	*Plantago asiatica*	perennial	rosset
B	Polygonaceae	*Persicaria thunbergii*	annual/biennial	errect
B	Rhamnaceae	*Berchemia racemosa*	vine	
B	Rosaceae	*Neillia incisa*	shrub	
B	Rosaceae	***Padus grayana***	tree	
B	Rosaceae	*Rosa multiflora*	shrub	
B	Saxifragaceae	*Rodgersia podophylla*	perennial	errect
B	Stachyuraceae	*Stachyurus praecox*	shrub	
B	Urticaceae	***Boehmeria silvestrii***	perennial	prostrate
C	Adoxaceae	***Viburnum dilatatum***	shrub	
C	Araliaceae	*Aralia cordata*	perennial	errect
C	Araliaceae	*Kalopanax septemlobus*	tree	
C	Asteraceae	***Aster microcephalus***	perennial	rosset(seasonal)
C	Asteraceae	*Parasenecio farfarifolius var. bulbifer*	perennial	errect
C	Hydrangeaceae	***Hortensia cuspidata***	shrub	
C	Hydrangeaceae	*Schizophragma hydrangeoides*	vine	
C	Lamiaceae	*Clinopodium sp.*	perennial	prostrate
C	Oleaceae	***Fraxinus sieboldiana***	tree	
C	Poaceae	*Sasa sp.*	perennial	tussock
C	Rosaceae	***Rubus palmatus***	shrub	
C	Saxifragaceae	***Astilbe thunbergii***	perennial	errect
C	Ulmaceae	*Zelkova serrata*	tree	
C	Urticaceae	*Elatostema involucratum*	perennial	prostrate
D	Adoxaceae	*Viburnum opulus var. sargentii*	shrub	
D	Anacardiaceae	*Rhus javanica*	shrub	
D	Aquifoliaceae	*Ilex macropoda*	tree	
D	Asteraceae	*Artemisia indica*	perennial	errect
D	Asteraceae	***Aster savatieri***	perennial	errect
D	Caprifoliaceae	*Abelia spathulata*	shrub	

D	Caprifoliaceae	*Weigela hortensis*	shrub	
D	Caryophyllaceae	*Stellaria uliginosa var. undulata*	perennial	
D	Celastraceae	*Celastrus orbiculatus*	vine	
D	Celastraceae	*Euonymus alatus var. altus f. striatus*	shrub	
D	Clethraceae	*Clethra barbinervis*	tree	
D	Commelinaceae	*Commelina communis*	annual/biennial	prostrate
D	Cornaceae	*Cornus controversa*	tree	
D	Cupressaceae	*Chamaecyparis obtusa*	tree	
D	Fabaceae	*Amphicarpaea edgeworthii*	vine	
D	Fabaceae	*Lespedeza bicolor*	shrub	
D	Fagaceae	*Castanea crenata*	tree	
D	Juglandaceae	*Juglans mandshurica*	tree	
D	Lamiaceae	*Ajuga sp.*	perennial	
D	Oxalidaceae	*Oxalis corniculata*	perennial	prostrate
D	Polygonaceae	*Persicaria muricata*	perennial	
D	Primulaceae	***Lysimachia fortunei***	perennial	errect
D	Primulaceae	*Lysimachia japonica*	perennial	
D	Rhamnaceae	*Hovenia trichocarpa*	tree	
D	Rosaceae	*Aria alnifolia*	tree	
D	Rosaceae	*Cerasus sp.*	tree	
D	Rosaceae	*Potentilla centigrana*	perennial	
D	Rosaceae	*Rubus mesogaeus*	shrub	
D	Rosaceae	*Sorbus commixta*	tree	
D	Sapindaceae	*Aesculus turbinata*	tree	
D	Schisandraceae	*Schisandra repanda*	vine	
D	Ulmaceae	*Ulmus davidiana*	tree	
D	Ulmaceae	*Ulmus laciniata*	tree	
D	Violaceae	*Viola vaginata*	perennial	rosset
D	Vitaceae	*Ampelopsis glandulosa*	vine	

D	Vitaceae	*Vitis flexuosa*	vine	
E	Sapindaceae	***Acer rufinerve***	tree	
E	Liliaceae	*Tricyrtis affinis*	perennial	errect
F	Apiaceae	*Torilis japonica*	annual/biennial	errect
F	Asteraceae	*Solidago virgaurea*	perennial	rosset(seasonal)
F	Betulaceae	***Carpinus cordata***	tree	
F	Betulaceae	*Carpinus laxiflora*	tree	
F	Polygonaceae	*Persicaria debilis*	annual/biennial	errect
F	Polygonaceae	***Persicaria filiformis***	perennial	errect
F	Urticaceae	*Laportea bulbifera*	perennial	prostrate
F	Urticaceae	***Laportea cuspidata***	perennial	prostrate
	Chloranthaceae	*Chloranthus serratus*	perennial	errect

2