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Interspecific Variation and Phylogenic Architecture of *Pinus densata* and the Hybrid of *Pinus tabuliformis*×*Pinus Yunnanensis* in the *Pinus densata* Habitat: an Electrical Impedance Spectra Perspective

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ARTICLE INFO

Article history

Received: 21 September 2020

Accepted: 16 October 2020

Published Online: 31 January 2021

Keywords:

Pinus densata

Artificial hybrid

Electrical impedance

Interspecific genetic variation

Homoploid hybrid

ABSTRACT

We evaluated a novel and non-destructive method of the electrical impedance spectroscopy (EIS) to elucidate the genetic and evolutionary relationship of homoploid hybrid conifer of *Pinus densata* (*P.d*) and its parental species *Pinus tabuliformis* (*P.t*) and *Pinus yunnanensis* (*P.y*), as well as the artificial hybrids of the *P.t* and *P.y*. Field common garden tests of 96 trees sampled from 760 seedlings and 480 EIS records of 1,440 needles assessed the interspecific variation of the *P.d*, *P.t*, *P.y* and the artificial hybrids. We found that (1) EIS at different frequencies diverged significantly among germplasms; *P.y* was the highest, *P.t* was the lowest, and their artificial hybrids were within the range of *P.t* and *P.y*; (2) maternal species effect of EIS magnitudes in the hybrids and *P.d* was stronger than the paternal species characteristics; (3) EIS of the artificial hybrid confirmed the mid-parent and partial maternal species characteristics; (4) unified exponential model of EIS for the interspecific and hybrids can be constructed as $|Z|=Af^{-b}$; (5) cluster analysis for species and hybrid combinations in total corroborated with the previous hybrid model of *Pinus densata*. Our non-destructive EIS method complemented the previous finding that *Pinus densata* was originated from *P.t* and *P.y*. We conclude that the impedance would be a viable indicator to investigate the interspecific genetic variations of conifers.

1. Introduction

Pinus densata (*P.d*) is a natural homoploid hybrid conifer originated from two ancestry species (i.e., *Pinus tabuliformis* and *Pinus yunnanensis*), as evidenced by previous genetic

studies of isozyme, cpDNA, and mtDNA^[1]. The natural habitat of *P.d* ecologically diverged from its parental species *Pinus tabuliformis* or *Pinus yunnanensis*. It has the highest habitat range among the Asian *Pinus* species and its habitat ranges from 2,800 to 4,200 above sea level

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(a.s.l.) on the Tibet Plateau, China; *Pinus tabuliformis* distributes in the mountainous, warm and temperate forests in northern China at a lower altitude below 2,200m a.s.l.; the habitat of *Pinus yunnanensis* locates in the southwest of the *Pinus tabuliformis* 'range, from 1,000m to 2,800m a.s.l.^[1-4].

Previous phenotypic studies of the interspecific adaptation and eco-physiology of the three pine species indicate greater fitness and survival rates of *Pinus densata* in stress tolerance such as to lower temperatures^[5], and drought, in the light of greater dry mass production and long-term water-use-efficiency compared to the parental conifers^[2]. Gao, Gao (6) found that *Pinus densata* had a stronger antioxidant process under drought than the parental species, while *Pinusyunnanensis* was drought sensitive.

In ecological genetic studies, Mao and Wang (1) found *Pinus densata* more adapt to high- altitudinal habitat in terms of the niche characteristics, such as temperature seasonality, growing-degree days. Mao, Li (7) studied eight morphometric traits of cone and seeds in *Pinus densata* and the parental species and concluded that *Pinus densata* is more reproductively successful in the natural habitat than the local *Pinus tabuliformis* and *Pinus yunnanensis*. Among the three *Pinus* species, the cross barrier is weak and the fitness differences are determined by local adaptation^[8]. These phenotyping assessments are laborious to compare the interspecific variation, involving field sampling, common garden trials, experimental measurement and destructive evaluation methods.

Biological electrical impedance spectra (EIS) provides an alternative method compared to destructive procedures to monitor physical changes of tissues in trees such as *Pseudotsuga menziesii*^[9] and *Picea glauca*^[10]. Recently, impedance measurements have been frequently applied in root studies including surface area and mycorrhizae^[11-15]. Repo, Laukkanen (16) studied impedance under 40Hz to 340 kHz to assess tree root growth. Then, Repo, Korhonen (13) studied root colonization of *Pinus sylvestris* L. seedlings with mycorrhiza fungi (*Hebeloma sp.* and *Suillus luteus*) under 5Hz-100kHz; and they found 13% to 27% correlated change for the real and imaginary parts under cold vs high-temperature treatment; under mycorrhiza treatment, there are 30%-39% correlation change in the real part, and 28%-38% in the imaginary part of impedance, respectively. Later, Repo, Korhonen (17) used similar 5-100kHz frequency of the voltage to measure the impedance of *Pinus sylvestris* L. under hardiness treatment on roots and developed a new method to categorize root frost injuries by EIS.

Needles are the photosynthetic organs that are easier to collect and measure than other organs such as roots. The

structure and electrical-physiological signatures of needles vary among germplasms or species^[18,19]. EIS is a potential technique to detect such a signature of needles, among germplasms or species, although previous application was frequently in the stem and root studies^[11-13]. Zhang, Li (20) employed EIS to assess the shoot and needles of *Pinus Bugeana* and reported greater correlations with the electrolyte leakage under frost hardiness ($r = -0.8-0.9$).

EIS is also a genotyping tool for crop plants. Kocheva, Georgiev (21) measured leaf impedance under seven Hz to two kHz to assess varietal difference and found low impedance genotypes with higher ion leakage in *cultivar Prelom* variety. In genetic mapping, EIS can build the phenotypic association with the genotypes of quantitative trait locus with statistical models^[22].

In this study, we explored the feasibility of the non-destructive EIS method for assessing the genetic variation among relative species by capturing the needles signature potentially due to evolution and local adaptation. This paper reported the variation and trend of EIS parameters by testing the needles of different germplasms among artificial hybrids of *Pinus tabuliformis*(P.t), and *Pinus yunnanensis* (P.y), as well as the homoploid hybrid,*Pinus densata* (P.d).The basic hypothesis is that EIS signatures of the low or high frequency could reflect the genetic variations between the homoploid hybrid conifer (P.y),and related parental species. Our objectives are(i) to explore the interspecific variation of EIS among P.d, P.t, P.y and the artificial hybrids; (ii) to assess the association between genetic variation and EIS parameters within the hybrid families; (iii) to test the parental effects on the artificial hybrids in terms of the EIS variability. By examined the feasibility of EIS to study the systematic evolution and the adaptation of homoploid hybrid conifers, our study provided a novel technique and strategy for forest genetics and adaptation research.

2. Materials and Methods

2.1 Plant Materials and Study Site

2.1.1 Artificial Interspecific Hybrids, *Pinus tabuliformis* and *Pinus yunnanensis*

We sampled six clones of *Pinus tabuliformis* as the female parents, five *Pinus yunnanensis* as the pollen donors from Kunming, Yunnan, China to conduct the controlled pollination in the seed orchard at Ningcheng, Heilihe, Chifeng City, Inner Mongolia, China. To construct research pedigree, we employed a test cross mating design to obtain 30 hybrid families, within which 13 hybrid families were subjected to the common garden experiment

based upon the seed quality. These 13 families were coded as from 401, 402, to 413 in the experiment and analyses. We focused on both the inter-specific variations and among-family variations of the hybrids.

The *Pinus tabuliformis* germplasms were open-pollinated half-sib families developed from the six female parents that produced the artificial hybrids. The *Pinus yunnanensis* population was developed from the five pollen donors that formed five half-sib families and the donors were used for producing the artificial hybrids. There were 30 *Pinus densata* trees sampled in the stands in Linzhi City, Tibet, China. In the sampled stands, *Pinus densata* is naturally distributed without other conifers stands in the same genus.

2.1.2 Common Garden Field Trial

We constructed common garden experiments in the native range of *P.d* to compare different species and sampled seedlings within the trial for EIS assessment. The common garden field trial locates at the research nursery of the College of Resources and Environment, Tibet College of Agriculture and Animal husbandry, at Bayi County, Linzhi City, Tibet, China (93°25' E, 29°50'W, 2,900m a.s.l.). The study area has a semi-humid monsoon highland climate (mean annual temperature 8.5 °C ; min annual temperature -15.3 °C ; max annual temperature 30.2 °C ; annual precipitation 654.1mm, from April to October; annual sunlight hour 2,022 hours; annual frost-free period >180 days). The nursery was built on the former flood bed. The soil was sandy loam with a pH ranging from six to seven and a depth more than 60cm. The fertility of the soil was from medium to low. The native *Pinus densata* grows normally in the nursery area.

2.1.3 Seedling Preparation and Experimental Design

The common garden field trial was established on a high bed of 5m length from east to west, 1.1m width and 10cm height. A randomized complete block design (RCBD) was employed at the trial. There are 4 blocks, 13 artificial hybrid families, and three pure species as control (*Pinus densata*, *Pinus tabuliformis*, and *Pinus yunnanensis*) with two seedlings within each of the three pure species; for each treatment (13*1 hybrid families + 3*2 families), there are ten seedlings planted per treatment level for the artificial hybrids and ten seedlings for treatment of control species within each block. The row distances and maintenance conditions were consistent for each block. Thus, the complete experiment contained 19 families (i.e., 13+3 treatment levels) x 10 trees per plot x 4 replication,

760 seedlings in total. The common garden experiment and progeny structures were reported in previous studies^[23], but a new set of individual trees were selected from the field trial for this study.

2.2 Impedance Measurement of Needles

Impedance measurement was conducted on the secondary growth needles of age-2 of the seedlings. The sampling process of test trees was as following, from three randomly selected blocks, choose (13+3) treatments (germplasms) in total, and select six trees per treatment. Impedance measurement was carried out for five times per seedling, and in each time, three normally-growing needles were picked. For each germplasms, 90 needles were measured. The total measurements were as 16 germplasms x 6 trees x 5 times x 3 needles = 1,440 needles.

2.3 EIS Measurement Procedures and the Frequency Responses

We connected the impedance analyzer (TH2828S, Changzhou Tonghui Electrical Co. Ltd., Jiangsu, China) with two stainless steel electrodes (the red and black), and calibrated the open and short circuits of the impedance analyzer. Then we sampled two healthy needles from *Pinus tabuliformis*, *Pinus yunnanensis*, *Pinus densata*, and the artificial hybrids at the branches that were at one-third of the tree height aboveground (i.e., ~1mm in diameter and ~1 cm in length, no chlorosis or pathogenic syndromes). The voltage electrodes were clippers and were connected directly to the needles with about one centimeter between each clipper without additional conductive media. The voltage was set to ten mV and the impedance was measured at 53 frequencies between 1Hz to 100MHz to draw the impedance curve. We plotted the EIS trend from one to 80Hz as well as from one kHz to 100kHz. The EIS from one Hz to 80Hz showed more variability comparing to the range from 80Hz to 100kHz and the trajectories were similar in the two spectrum ranges. To address the environmental effects such as the light and temperature, we repeated the measurement for five times at the same location of branches from the morning to the afternoon. We compared the potential polarization effect under low frequency measurement with the higher frequency measurement (Supplementary materials).

We aimed to test the feasibility of impedance parameters for testing species genetic variations. Here we assumed that the physiological responses were negligible and consistent when needles were connected to the current, e.g., the physiological change when the needle cells were ruptured. The frequencies for impedance mea-

surement were between one Hz and 100kHz. We showed both the lower (1-80Hz) and higher frequency results (1k-100kHz). The original status of the needles was consistent during the measurement without cutting the needles off the trees. Nyquist plots of the impedance for different germplasms were constructed to present the interspecific variations. Both the real (Z' , resistance) and imagery part (Z'' , reactance) of impedance were compared in the Nyquistplot among species.

2.4 Statistical Analyses

We took the average impedance of three needles for the interspecific analyses of variance (ANOVA), followed by Tukey's test for multiple comparisons with R software [24]. For interspecific variation, we used the following linear model:

$$y_{ijk} = \mu + S_i + f_{j(i)} + e_{ijk} \quad (1)$$

where y_{ijk} is the sampled impedance reading of individual tree; μ is the grand mean; S_i is the i -th species, which includes *P.t*, *P.d*, *P.y* and the artificial hybrids; $f_{j(i)}$ is the j -th family nested in i -th species; e_{ijk} is the random residual of the k -th tree of ji -th family. For comparing the hybrid families, we applied another linear model as following:

$$y_{jk} = \mu + I_j + e_{jk} \quad (2)$$

where y_{jk} is the sampled impedance reading of each family; μ is the grand mean; I_j is the j -th hybrid family, which includes *P.t*, *P.d*, *P.y*, and the artificial hybrids; e_{jk} is the residual of the EIS average of k -th tree in j -th hybrid family.

We also constructed the unified exponential model of EIS is as following:

$$|Z| = Af^{-B} \quad (3)$$

where A and B are the regression coefficients of species; A is also the amplitude ($A > 0$); B is the exponential coefficient ($0 < B < 1$); $|Z|$ is the impedance magnitude (kOhm); f is the frequency (Hz). The exact specific or phenotypic trait factors to modify A and B are still under studies.

To evaluate the EIS of interspecific kinships, the cluster analyses were conducted based on arithmetic means of EIS within six germplasms of three pine species and family means of each hybrid family. The coefficients of variation were calculated as the standard deviation divided by means of the germplasm- family group. Correlation coefficients 0.6 were the threshold value for hierarchical clustering. Ward's method was used to determine the Euclidean distances of impedance among different germplasms, which is the distance of all clusters to the grand average

of the sample. The distances of impedance and branches of the dendrogram indicated the among-family and inter-specific genetic structure inferred from the EIS. To depict the unified function between EIS and voltage frequencies we used non-linear regression with exponential independent variables (i.e., frequency). We used R software for the statistical analyses of variance components, regression and cluster analyses [24].

3. Results and Discussion

3.1 Among-family Impedance Variation and Impedance of Artificial Hybrids

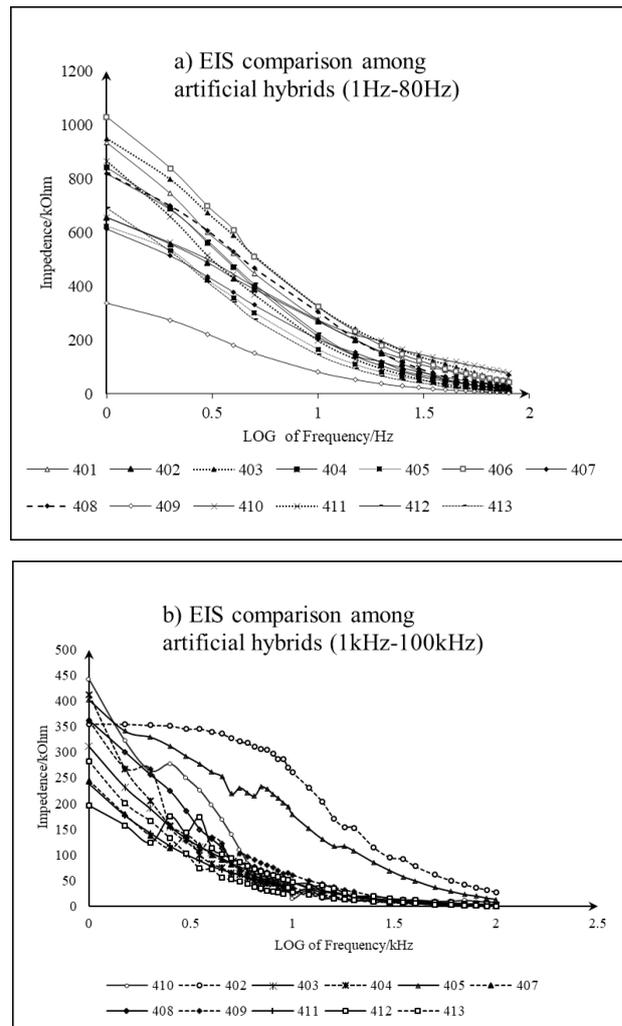


Figure 1. Impedance spectroscopy comparison for artificial hybrids

Note:

The frequency in a) is transformed by logarithm to base 10 ranging from zero to 80 Hz; the frequency in b) ranges from one to 100kHz.

The artificial hybrids of *Pinus tabuliformis* and *Pinus yunnanensis* showed a decreasing impedance when the

frequencies of the external alternating electric field rise (Figure 1). The impedance values of all hybrids peaked at one Hz. When the frequency increased, the dropping speed of each hybrid varied. The gradient of impedance reached its maximum (400-1,500 kOhm) when the frequency changed from one Hz to five Hz. At 10Hz of the field frequency, the impedance curve inflected; and the impedance was only 30% of the initial value at one Hz. Then the declining speed of the impedance curve reduced and reached to 10% of the initial value at 50Hz.

We found a discernable genotypic variation of impedance between hybrid individuals when the voltage reached 30Hz. And the differences of impedance between hybrids kept stable when the frequency was greater than 30Hz. When the frequency was lower than 30Hz, the interspecific variation dropped, in which Hybrids 409 and 406 expressed of the lowest and the highest impedances, respectively. The interspecific variation was negligible as the frequency was lower than five Hz. To magnify the interspecific variation in the low frequencies, we took the log-transform for frequency (Figure 1). Figure 2 and Figure 3 showed impedance variation among hybrids and species in terms of the aggregated means of the bands of frequencies.

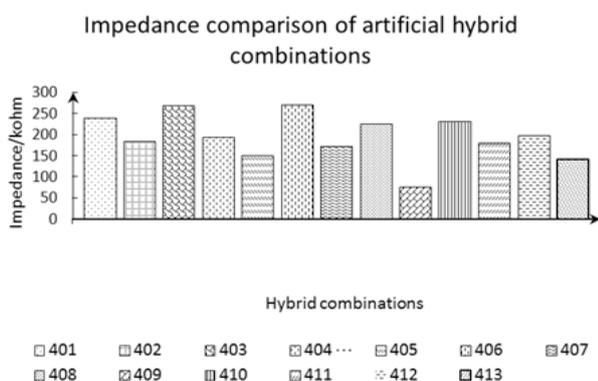


Figure 2. Impedance comparison of artificial hybrid combinations

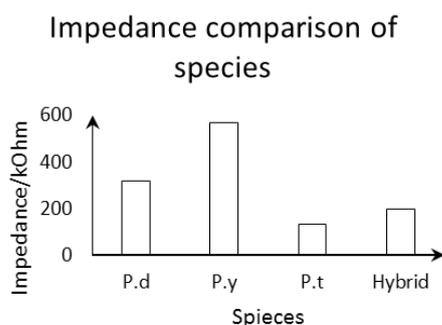


Figure 3. Impedance comparison of species (artificial hybrid)

3.2 Impedance Means and Variances of Hybrids

We did not find significant differences of impedance among hybrids in the analyses of variance (Table 1). The ratio of variance component among hybrids was 5.2%, while the ratio was 10.1% among individuals of each hybrid family. There was greater variation within the hybrid families than among the hybrids.

Table 1. Impedance variance analysis for artificial hybrids

	DoF	Impedance		Significance
		Mean Square	Variance components/ %	
Among the combinations	12	7875.9	5.2	ns
Among the individuals	39	4679.1	10.1	ns
Error	215	7133.8	84.7	

Notes:

** $P < 0.001$; * $P < 0.05$; ns $P > 0.05$.

Table 2. Artificial hybrids mean and standard deviation of electrical impedance

ID	Impedance \pm SD	Impedance similarity
401	84.13 \pm 98.98 ^{ab}	<i>P. t-like</i>
402	63.22 \pm 70.67 ^{ab}	<i>P. t-like</i>
403	78.00 \pm 44.24 ^a	<i>intermediate</i>
404	63.72 \pm 69.57 ^{ab}	<i>P. t-like</i>
405	83.67 \pm 74.27 ^{ab}	<i>intermediate</i>
406	95.98 \pm 94.39 ^{ab}	<i>P. t-like</i>
407	106.39 \pm 88.58 ^a	<i>intermediate</i>
408	77.30 \pm 95.45 ^{ab}	<i>P. t-like</i>
409	64.86 \pm 84.76 ^b	<i>P. t-like</i>
410	55.81 \pm 53.86 ^{ab}	<i>P. t-like</i>
411	92.57 \pm 72.56 ^a	<i>intermediate</i>
412	113.03 \pm 115.64 ^a	<i>intermediate</i>
413	54.21 \pm 55.50 ^{ab}	<i>P. t-like</i>

Note: *P. t-like* means the mean impedance of the hybrids is not significantly different from that *P.t*; *Intermediate* means the impedance value of the hybrid is significantly different but within the range of the parental species the *P.t* and *P.y*. The different letter signs "a" and "b" indicates significant differences ($P < 0.05$) of average impedance between hybrids based Tukey test adjusted for multiple comparisons.

We calculated the average impedance with three repeated measurements at a fixed frequency. The impedance of eight hybrids was similar as that of *Pinus tabuliformis* and five hybrids showed impedance between that of *Pinus tabuliformis* and *Pinus yunnanensis*; no artificial hybrids showed similar impedances as *Pinus yunnanensis*.

3.3 Interspecific Variation of EIS

Impedances of the hybrids was lower than *P.d*, although the Hybrid 404 at one Hz was higher than *P.d* (Figure 1). Among four hybrids, the impedance of *P.y* was the highest, followed by that of *P.d*. And the impedance of Hybrids was within the impedance range of the parental

species (*P.y* and *P.t*), while the impedance of the Hybrids 405, 409, and 403 was lower than that of *P.t*. and *P.y*. The impedance curves became leveled off after 20 - 40 Hz (Figure 4). *P.d* and hybrids' curves were close to *P.t*. The variation among germplasms groups decreased with increasing frequency. However, the coefficient of variation among species groups increase by frequency and leveled off after 60Hz except for the EIS of hybrids (Figure 5).

Besides the impedance pattern of the frequency from one Hz to 80 Hz, from one kHz to 100 kHz, we tested the impedance of all germplasms. The curve trend and shape obtained were similar to the previous low-frequency EIS. We found the trajectory of impedance responses from one

Table 3. Impedancemean and variance comparison for artificial hybrid, parental species, and *Pinus densata*

Frequency	Mean±standard deviation				Variance components ratio/%	
	<i>P.t</i>	AH	<i>P.d</i>	<i>P.y</i>	Among species	Residual
1 Hz	690.8±265.4	754.1±324.8	1,067±388.8	1,450±418.8	32.2**	67.9
2	549.2±244.5	621.9±283.2	844.5±273.4	1,195±380.4	29.8**	70.2
3	434.8±218.7	517.6±253.5	695.3±218.2	1,017.8±344	29.1**	70.9
4	356.4±200.0	443.7±232.3	603.4±203.7	911.1±324.9	29.8**	70.2
5	300.2±185.4	383.3±213.5	532.9±197.7	835.9±314.5	31.3**	68.7
10	158.3±119.8	232.4±163.1	367.8±177.6	652.3±293.1	37.7**	62.3
15	103.3±87.3	163.8±133.4	293.4±165.6	562.4±269.3	42.6**	57.2
20	74.4±66.2	127.4±115.5	245.5±160.9	506.3±252.3	45.2**	54.8
25	51.2±52.7	101.0±97.5	213.5±156.2	466.0±238.7	48.9**	51.1
30	43.9±40.1	84.2±87.3	190.6±151.4	434.1±228.2	48.9**	51.1
35	35.8±32.7	71.9±80.1	171.9±148.4	407.3±219.0	51.0**	49.0
40	26.9±29.4	61.6±73.9	157.4±146.1	385.6±213.8	51.6**	48.4
45	24.6±22.8	55.1±70.6	147.9±143.0	365.5±207.9	48.3**	51.7
50	22.2±20.5	48.9±65.9	139.6±140.8	347.4±203.0	51.1**	48.9
55	19.4±18.6	43.7±62.1	132.1±137.3	331.5±197.5	51.2**	48.8
60	17.2±16.0	39.4±58.7	125.0±133.5	316.4±190.2	51.3**	48.7
65	15.7±14.0	35.4±56.2	119.1±131.4	304.1±184.0	51.5**	48.5
70	13.9±11.6	32.2±53.3	128.4±127.1	287.2±181.3	51.0**	49.0
75	12.2±9.8	30.6±50.9	123.1±125.7	276.7±177.8	50.55**	49.5
80	11.1±9.0	26.9±47.8	117.6±123.5	265.4±174.8	50.45**	49.6

Note:

*, $P < 0.05$; **, $P < 0.01$; *P.d*, *Pinus densata*; *P.t*, *Pinus tabuliformis*; *P.y*, *Pinus yunnanensis*; AH, a hybrid of *Pinus tabuliformis* × *Pinus yunnanensis*.

Hz to 100Hz, and less variability occurred from 80Hz to one MHz. In the frequency ranging from one kHz to 100 kHz, the hybrids showed similar trajectories as that in the low frequency.

3.4 Variance Comparison of Impedance

The interspecific variations of impedance were significant at different frequencies (Figure 2). Impedance was an effective indicator to differentiate germplasms due to the physiological responses and activity under the electromagnetic fields. The variance component among species reached to 29% at one Hz to 30Hz; and the variance ratio between species exceeded 50% when the frequency was greater than 35 Hz.

We found reduced impedance when the frequency of electromagnetic field increases (Table 3). The mean impedance of the hybrid and *P.d* between the parental species (Table 3), aligned with the trend in Figure 3. The standard deviation (SD) of impedance followed the same trend as the impedance means among species and hybrids. *P.y* also peaked in the standard deviation of impedance; *P.t* showed the lowest impedance standard deviation, while the hybrid was within the range of *P.t* and *P.y*. The standard deviation of *P.d* was lower than that of the artificial hybrid except at one Hz. Table 3 showed high standard deviations equivalent to individual genotypic differences; thus, the impedance can differentiate intraspecific genetic variation of needles.

Figure 6 depicted the means of each species over the gradient of frequency from zero to 100 Hz and showed the interspecific variation of species. In Figure 5, the positive correlation existed between the standard deviation (SD) and voltage frequency in the hybrid families. However, the SD of *P.y* decreased when the frequency increased. Within the frequency of one Hz to 25Hz the SD curves of *P.d*, *P.y* paralleled from one to ten Hz. From 20- to 50Hz, SD of *P.d* was between *P.t* and *P.y*. From 45 to 85Hz, *P.d* followed the similar trend as *P.t*. Above 45Hz, the EIS of hybrids diverged from *P.y*. Except for the SD of impedance from one to ten Hz, SD of *P.d*, and the hybrid showed more tendencies of *P.t* than *P.y*. The maternal species characteristic was discernible.

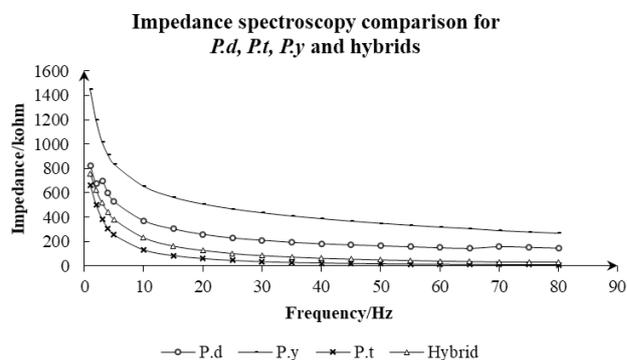


Figure 4. Impedance spectroscopy comparison for *Pinus densata* (*P.d*), *Pinus tabuliformis* (*P.t*), *Pinus yunnanensis* (*P.y*), and artificial hybrids

Coefficients of variation of EIS in four species groups

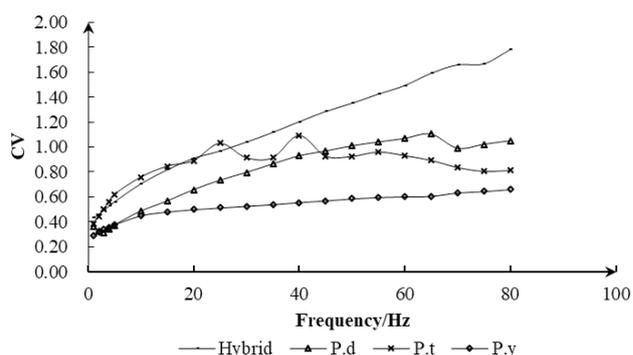


Figure 5. Comparison of the coefficient of variation (CV) of EIS for four species groups

3.5 Interspecific Impedance Models and Cluster Analyses

Per the linear regression of EIS curves of *P.d*, *P.t*, *P.y* and the hybrids in Figure 1 and 6, there was an exponential relationship between the impedance and voltage frequencies. The curves of each species were following exponential patterns with R^2 greater than 0.9.

The cluster analyses of EIS among *P.d*, *P.t*, and *P.y* and the hybrids showed that six clusters with the threshold value as 0.4 (Figure 7). There were eight hybrids categorized in the *P.d* cluster indicated the similarity of impedance within the cluster. Hybrids 405 and 413 were clustered with *P.t*; *P.y* and three hybrids were not classified in the same group of *P.d* or *P.t*. *P.d* shared similarity with hybrids because the impedance was closer related than to *P.t* or *P.y*.

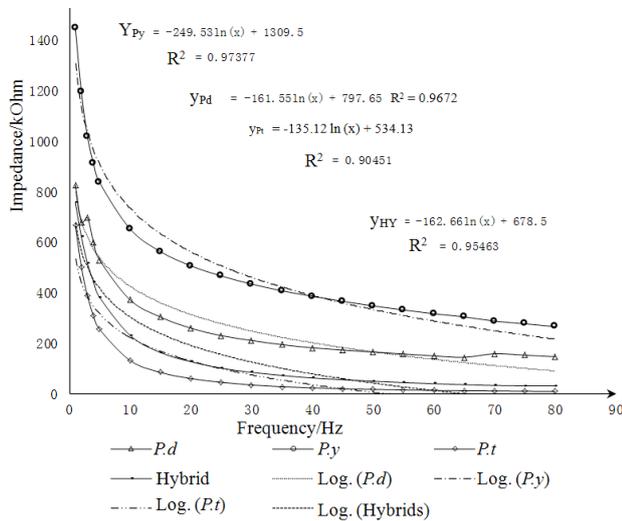


Figure 6. Impedance model of *P.d*, *P. t*, *P.y*, and the hybrids

Note:

Y_{Py} is the predicted impedance of *P.y*, the R^2 is 0.97377 which is a good fit; Y_{Pd} is the predicted impedance of *P.d* ($R^2 = 0.9672$); Y_{Pt} is the predicted impedance of *P.t* ($R^2 = 0.9045$); Y_{HY} is the impedance of the artificial hybrids ($R^2 = 0.9546$).

3.6 Impedance Complex plane

In the complex plane of impedance in the alternating circuits (AC), the real part, resistance (Z'), and the imaginary part (reactance, Z'') were both presented. The Nyquist graph [25] depicted both the magnitude of impedance $|Z|$ and the phase difference between the voltage and current in the

polar coordinate system (Figure 8).

The artificial hybrid was in the third quadrant; *P.d* entered the fourth quadrant in the high-frequency band, while the rest located in the third quadrant (the entering point is at 12kHz-13kHz). The impedance of *P.t* fell in the fourth quadrant and entered the third quadrant near the eight Hz. The impedance of *P.y* located on the third and the fourth quadrants and the intersection was at 8.5 to nine kHz that bent at 60Hz and formed a closing curve.

The artificial hybrids and *P.d* shared the similar curve, while the curve of *P.d* shifted towards the left. This shift of *P.d* could be the result of genetic variation of needles due to natural selection and adaptation, which needed further studies. The curves of *P.d* and the hybrids tended to be a result of 180 degrees rotation, of that of *P.t* along the Z'' axis. Besides, the curve of hybrids was closer to the shape of the *P.t* curve. The curves of *P.d*, hybrids and *P.t* showed slower bending at the high-frequency regions, comparing to the steeper bending trends of *P.y* at the high frequency. *P.d* showed similar arcs as *P.y* in the low-frequency area, while *P.d* curve was similar as *P.t* at the high-frequency area.

3.7 Electrical Impedance Evidence of the P.d Hybrid Evolution

Pinus densata has been demonstrated as the ancient homoploid hybrid of *Pinus tabuliformis* and *Pinus yunnanensis*. The EIS revealed the interspecific variability of

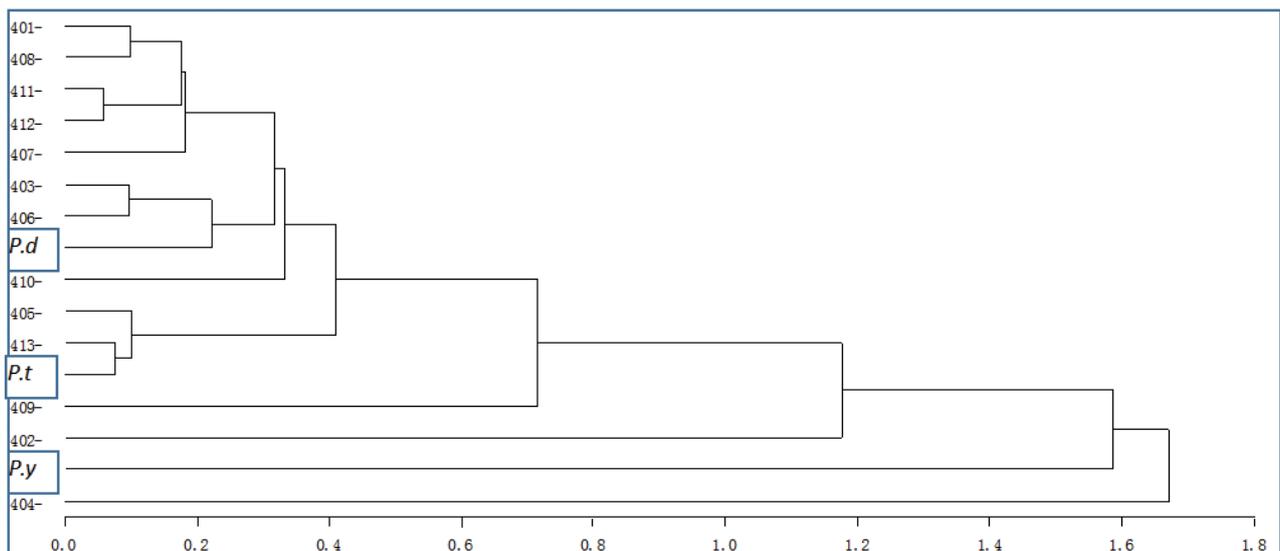


Figure 7. The hybrid combination with *P.d* and parent species impedance clustering dendrogram (1Hz~80Hz). The x-axis showed the threshold values to form different clusters based on the distance of impedance

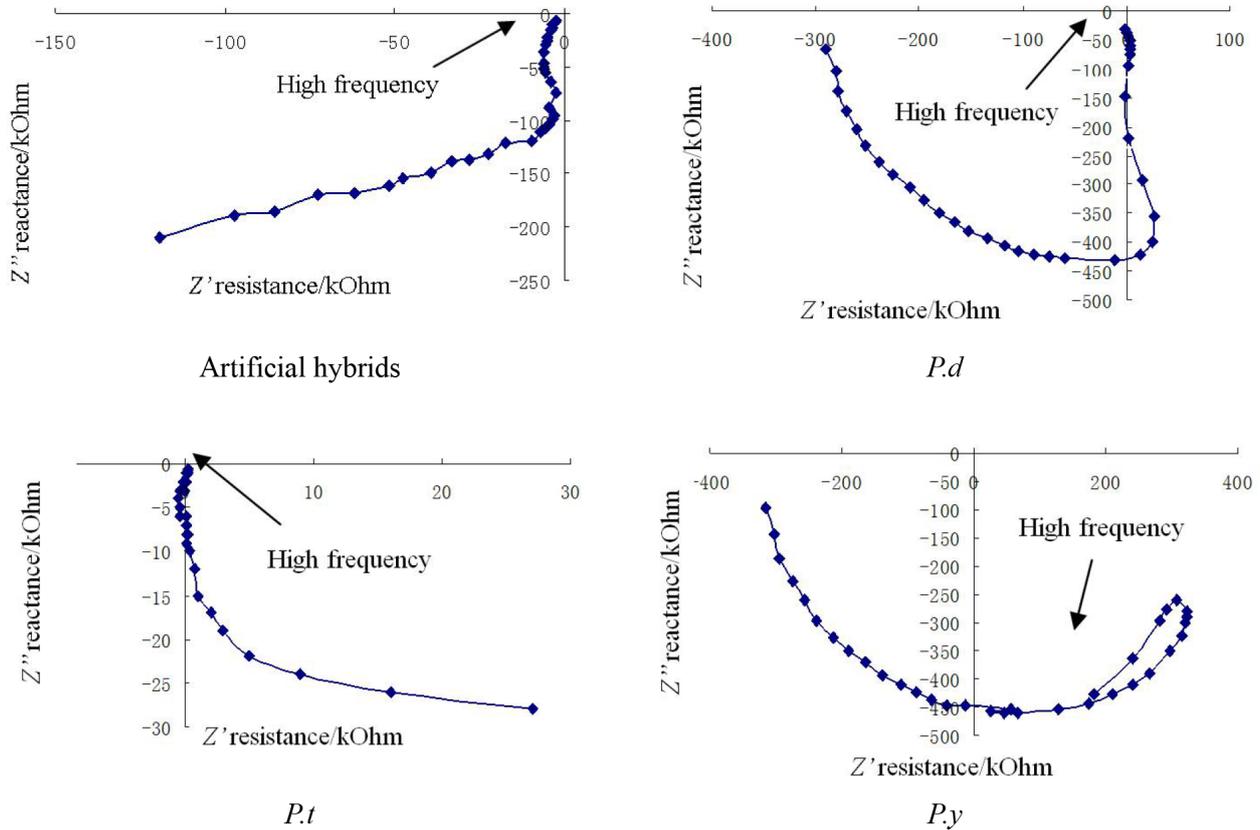


Figure 8. Impedance complex plane for artificial hybrids, *Pinus densata* (*P.d*), *Pinus tabuliformis* (*P.t*) and *Pinus yunnanensis* (*P.y*)

needles that complemented the evolution and physiological relationship between three pure coniferous species. The Nyquist plot showed contrasting trajectories of impedance indicating the specific variability of needles; and the trend of the interspecific genetic variation is aligned with the previous hybridization and evolution findings of *P.d*, *P.t*, and *P.y*^[1,23]. When comparing the germplasms with references of both paternal *P.y* and maternal parent of *P.t* for the hybrids, maternal factor was stronger than the paternal factor for the hybrids. The responses of impedance under different frequencies showed interspecific variations of needles might link to the hybridization history (e.g., Figure 1, Table 2).

P.d adapts to the plateau climate of lower temperature and intense ultraviolet radiation, though the needles of *P.d* and *P.t*, *P.y* are specialized in terms of adapting to the cold and ultraviolet radiation conditions^[7,23]. The nature selection gradient changed the functional and genetic features of needles of the three species^[23], though adaptive optimality is more evident in other tree species such as *Pinus contorta* Dougl^[26] and more studies on the biochemistry of

needles is needed.

Contrasting to *P.d*, the parental species *P.y*, and *P.t* are not locally-adapted as *P.d* in Tibetan Plateau. Local adaptation is demonstrated on the morphological and ecological characteristics of the needles, including needle length, the mean number of stomata, mean stomatal density, mesophyll vascular bundle area ratio, mesophyll/resin canal area ratio, mesophyll/(resin canals and vascular bundles) area ratio, vascular bundle/resin canal area ratio etc^[23].

Interestingly, the artificial hybrids showed a higher coefficient of variation in impedance (Figure 6) after 40Hz, indicating the hybrids have greater genetic variability in the needle structure comparing to *P.d*, *P.y*, and *P.t*. The potential heterosis of impedance magnitude occurred among the Hybrids 405, 409, and 403.

The contrast of polar molecules in the needles tissues can be detected by EIS signatures, such as proline. Non-polar amino acid exists in plants, though their structures contain polar molecules. For an instance, proline has the polar structure as a nonpolar amino acid that can be found freely in plants. Higher concentration of proline was

caused by stress such as drought preconditioning in species such as *Lolium perenne* L.^[27], so testing the proline content can evaluate physiological parameters for selecting the drought-resistant varieties. Thus, proline content is a physiological indicator for stress in frost or drought-resistance of plants^[28]. Here EIS could detect the signature in normally-grow needles among the studied species. However, the exact relationship of proline concentration in needles between the three coniferous and artificial hybrids needs to be tested in the future studies.

3.8 EIS Reveals Interspecific Variation and Parental Species Effects

The signature of our impedance profiles could describe the interspecific genetic difference of these conifers. *P.y* expressed the highest impedance while *P.t* showed the lowest; impedance of *P.d* and the artificial hybrids were within the range of the two parental species, which agrees with the kinship relations of samples. Xing et al. (2015) reported significant differences in thirteen needle traits between artificial hybrids and *P.y*, the male parent, while the needle characteristics are similar to that of *P.t*(the female parent) and *P.d*. Based on the similarity of needles of *P.d* and artificial hybrids^[23], the parental trend is reflected by impedance profile and the cluster analyses.

The EIS under higher frequency (i.e., >40 Hz) indicates more discernable inter-and-intra-specific genetic variation (Figure 5). The inter-specific variation is greater than the within-specific variation. Hybrids showed less significant differentiation in impedance, which indicated the limitation of EIS for interspecific studies in pines, though EIS is feasible for interspecific studies.

3.9 Voltage Frequency

The impedance varies greater when the frequency is more than 100Hz. Here we assume the physiological and structure response of needles during the electric stimulus does not modify inter- and intra-specific genetic variation of sampled trees, although the fewer errors of the measurement relies on the controlled and consistent experiment condition settings. More studies are necessary to dissect the effects of physiological and structure response variation under low and high frequency electric stimulus. EIS of the kHz scale could indicate intracellular variation among individual samples based on previous studies^[17,21]. Here, we could not exclude the distortions of EI at low frequency (1-80 Hz) due to the potential electrode resistance and extracellular resistance.

We found the frequency below 100 Hz is capable of

testing needle genotypic variation compared to previous studies focusing on the kHz frequencies^[17,21]. The unified regression model has good fitting in low frequency spectrum according to R^2 . High frequency at kHz scales show similar trends with moderate to low variations comparing to EI in the low frequency (1-80Hz) in our case (Figure 1 and Supplementary materials). The EIs are linked between the low and high frequency (Supplementary materials).

Potential fractal effects may occur though more studies are necessary. The discrepancy between the low and high frequency also occur in some hybrids (e.g., Hybrid 402). Other than that, EI of hybrids low frequency can still differentiate the genotypic groups.

4. Conclusions

The AC electrical impedance parameters of normal growth needles were measured at a range of frequencies among 1, 440 needles of 96 trees sampled in 760 seedlings in the habitat of *Pinus densata* in Tibet plateau. We depicted the profile of impedance and constructed the unified regression model, the descent ranking of EIS is *P.y*, *P.d*, hybrids, and *P.t*. Low voltage frequency (<100 Hz) is capable of demonstrating species variations, while the EIS of high frequency (80Hz-100kHz) showed overlapping trajectories with less interspecific variation. Within the hybrids, family-variation is less than the within-family variation of EIS. The mid-parent and partial maternal species effects of EIS is demonstrated in the hybrid families.

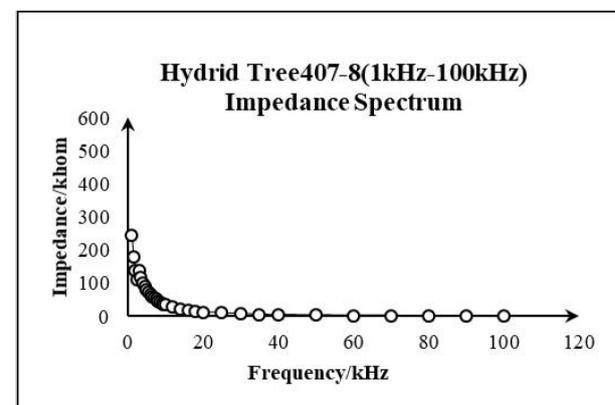
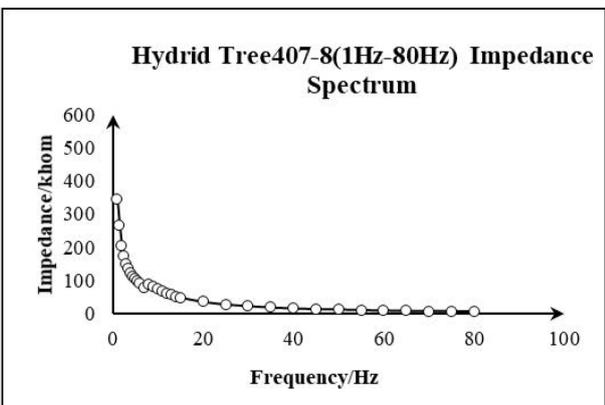
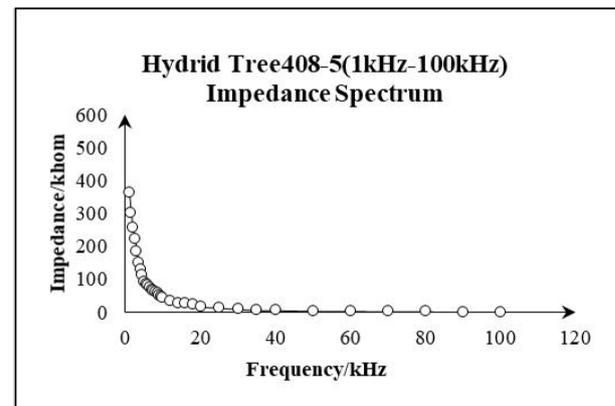
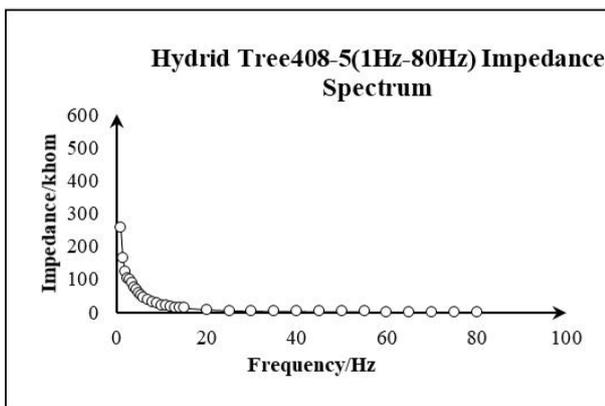
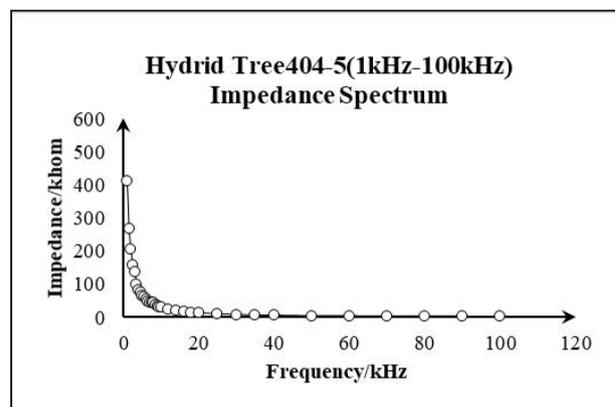
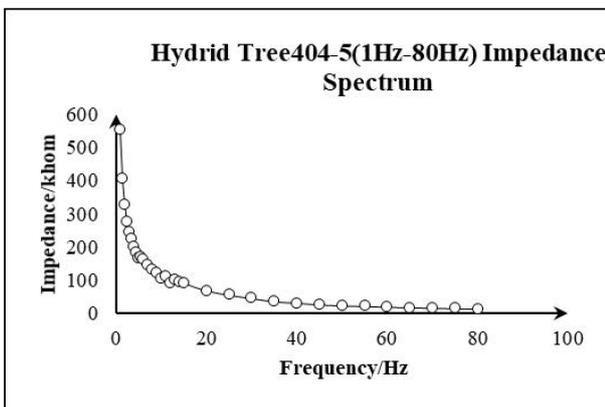
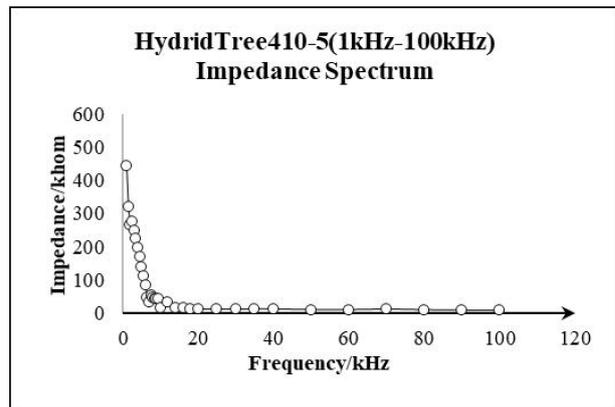
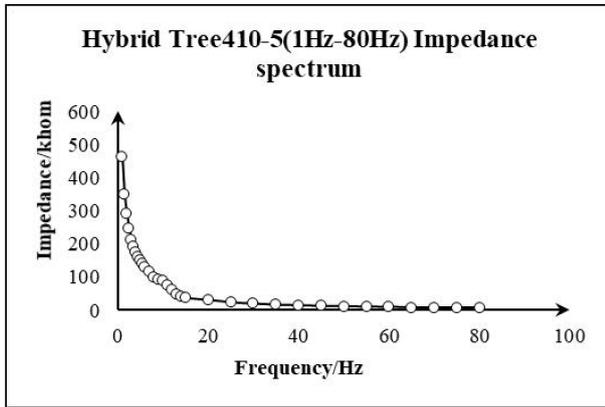
Our non-destructive EIS method is feasible to analyze the genetic relationship between homoploid hybrids, parental conifer, and the artificial hybrids. The EIS pattern not only complemented the previous findings of *P.d* hybrid history, but provided a novel tool for evaluating interspecific variations of conifers due to adaptation differentiation in a non-destructive way.

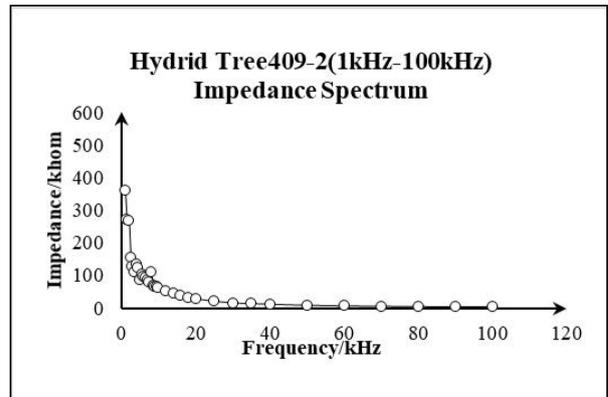
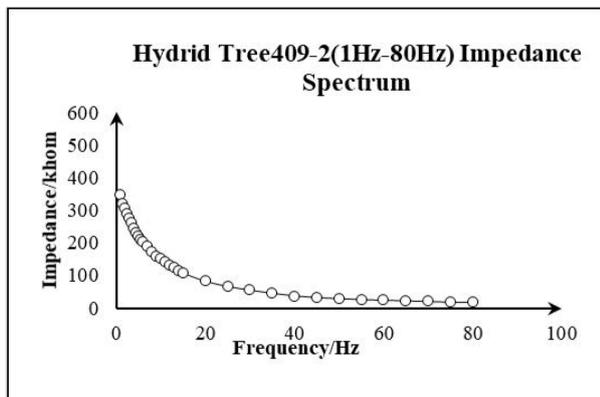
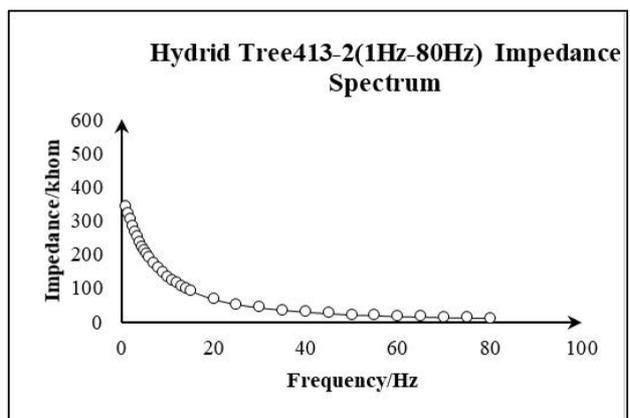
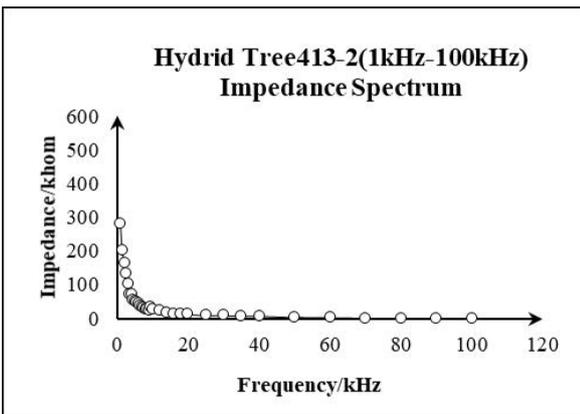
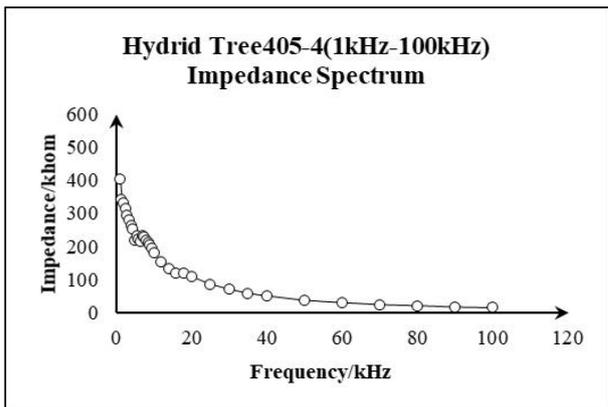
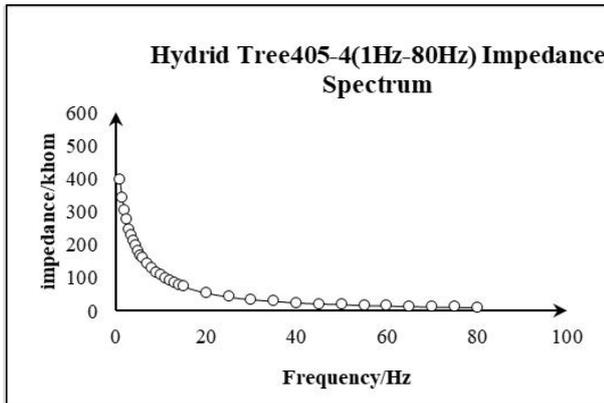
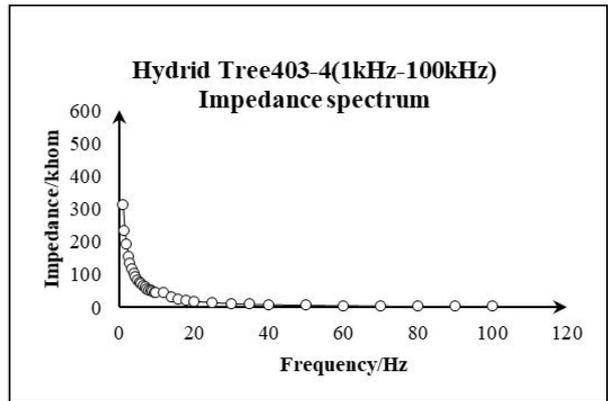
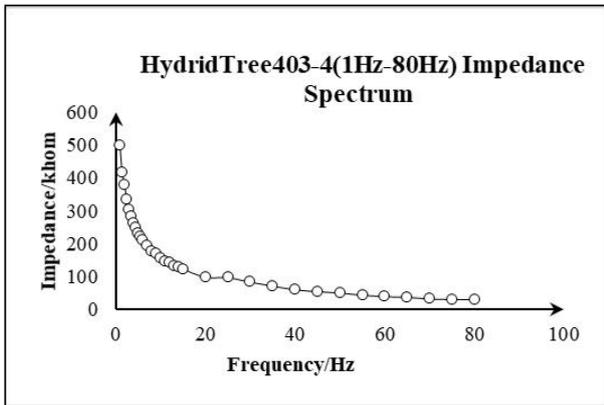
Acknowledgement

The authors gratefully acknowledge Drs. Wang Xiaoru and Mao Jianfeng's help on the materials and experiment. We thank Drs. Francis Yeh and Wenqing Zhang's comments on the abstract, materials and methods. This research was funded by National Natural Research Fund 31070591, "Adaptation research of *Pinus densata*, *Pinus tabuliformis*, *Pinus yunnanensis* and the hybrids at high elevation habitats", P. R. China.

Author's Contribution: F-X M lead the study and finished the writing; X-Y C and Y L conceived the ideas and edited the manuscript.

Supplementary Materials





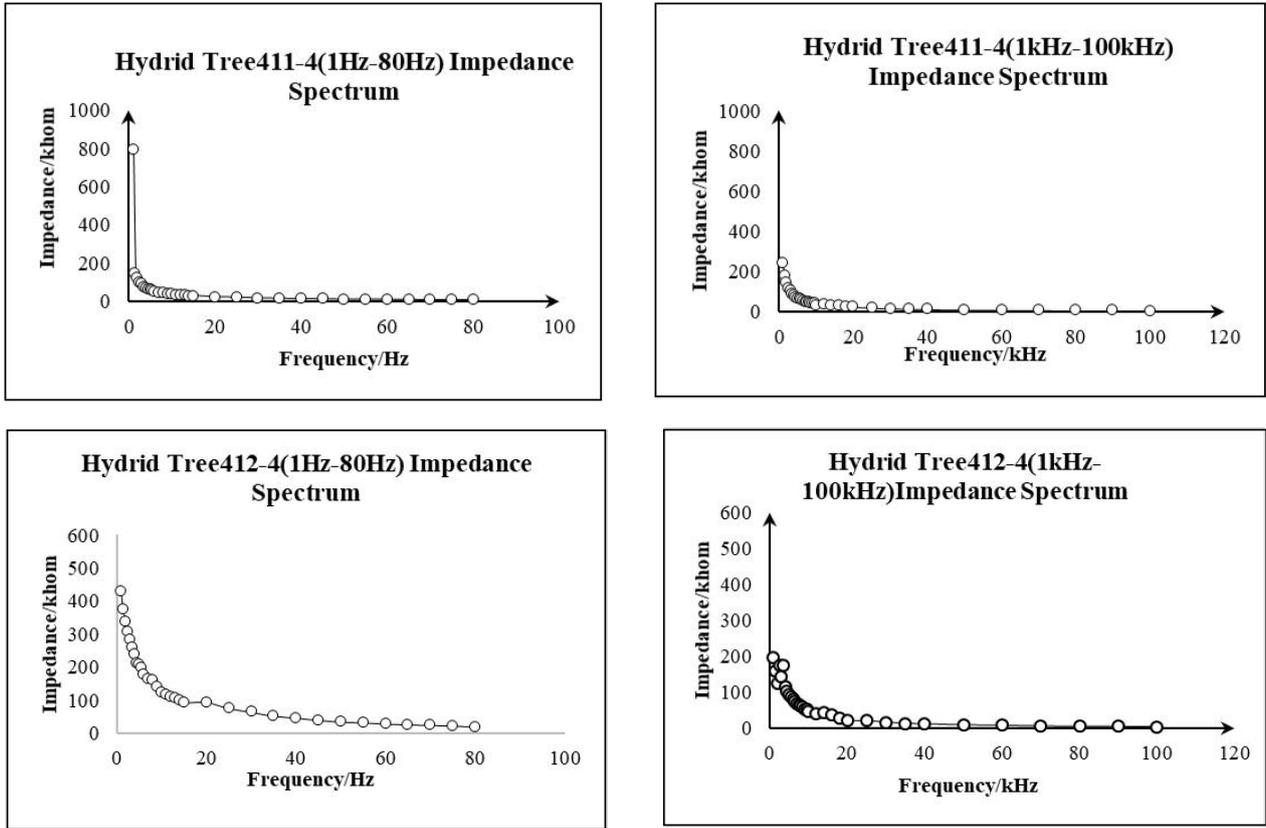
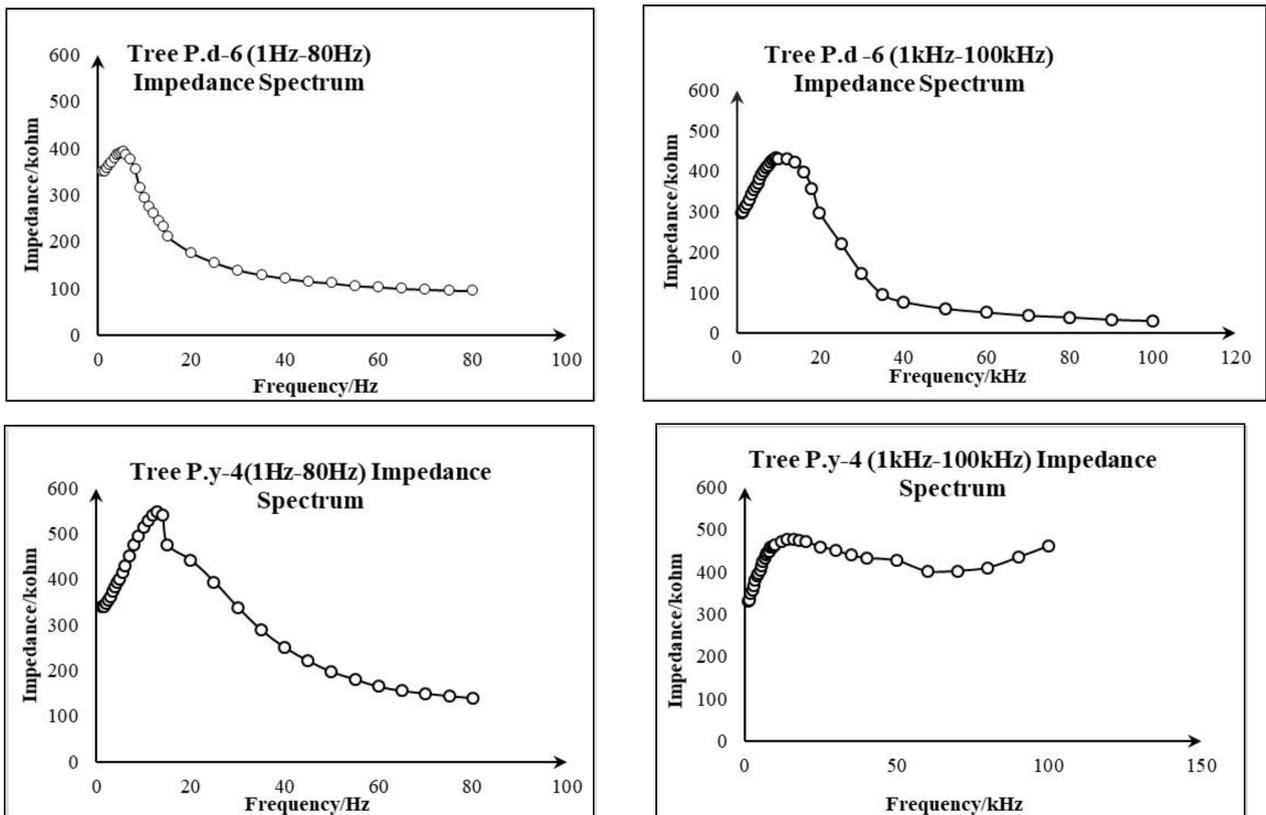


Figure S1. Impedance of hybrids under two spectrums of frequencies, where each curve is the average EI of one sampled tree



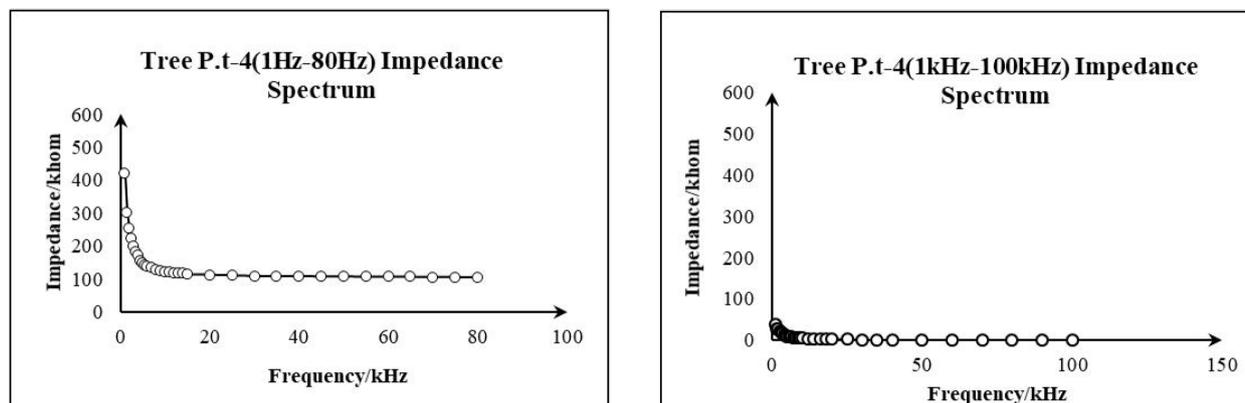


Figure S2. Impedance of *Pinus densata*, *P.yunnanensis* and *P.tabuliformis* at different spectrums of frequencies

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