METHODS AND TECHNIQUES

Hypothesizing hybrids and parents using character intermediacy, parental distance, and equality

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Summary

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A simple method for objectively screening morphological variation in study sets suspected of containing hybrids is presented. The method applies to a collection of specimens in which two or more species along with some or all of their hybrids are suspected to be represented. The purpose of the method is to hypothesize which specimens might be of hybrid origin, and for each of these specimens to indicate which other two specimens resemble those that might have been its parents. The method is employed in the computer program HYWIN by using two kinds of computationally intense techniques: evaluation of a hybrid optimality score for each triplet generated from three quantitatively defined criteria: hybrid intermediacy (IN), parental distance (PD), and equality (EQ); and simulation of a probability hypothesis to generate measures of the level of statistical certainty. The method can be applied in direct studies of hybrids and their parents, taxonomic treatments, pinpointing of specimens that merit study with other techniques, and screening of data sets for putative hybrids and hybrid species prior to phylogenetic reconstruction.

Introduction

Because of the important role of hybridization in plant speciation and evolution, the study of hybrids has always been an important component of plant systematics research. Recent advances in molecular systematics have provided powerful techniques for discerning hybrids. These techniques are being upgraded and refined and provide access to a large number of molecular markers. These markers enable the determination of both primary and derived hybrids, assessment of levels of gene flow among species, and reconstruction of phylogenetic relationships among hybridizing taxa and their close relatives (Rieseberg & Ellstrand, 1993). Other more recently developed methods based on non-morphological data that are helpful in discerning hybrids are the analysis of secondary compounds by the utilization of the additive pattern of hybrid chemical expression (Harborne & Turner, 1984), seed protein profiles, and allozymes, which allow the determination of the distribution of the products of individual gene loci without the complications of intermediacy and additivity (Gallez & Gottlieb, 1982; Crawford, 1990).

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However, a prerequisite to using any of these methods is recognition of putative hybrids in the wild or in the herbarium, formation of hypotheses about which of the individuals in the population studied are putative parents and which are putative hybrids, and decisions about which individuals to sample for further analyses. These steps involve assessments and analyses of morphological variation. Therefore, a simple but comprehensive method for objectively screening morphological variation in study sets suspected of containing hybrids that would allow generation of more precise hypotheses about hybrid origins would be valuable. These hypotheses would then provide a firm basis for further analyses and also create a starting point for comparisons of morphological data and molecular data. Wilson (1992) also stressed the importance of showing hybridity first before trying to unravel introgression or the structure of a hybrid swarm.

Visual presentations of comparative data, especially scatter diagrams, are among the earliest objective methodologies for the analysis of hybridization and introgression. These early ordination techniques, often including symbols "pictorialized" to show important characters, were perfected by Anderson (1949), who also championed hybrid indices. Two-dimensional plots of pairs of characters can visually show the intermediacy of hybrids compared with their putative parents (e.g. Reznicek & Catling, 1985). Wilson (1992) re-affirmed that pictorialized scatter diagrams properly present evidence for an intuitive interpretation, provided that the intermediacy shown is based on intermediacy in individual character-by-character measurements. An intermediate nearly equal to one putative parent for some measurements and nearly equal to the other putative parent for others may be an ancestor that is intermediate between its descendants, and not a hybrid intermediate.

Goodman (1967) presented an excellent review of classical, statistical approaches. These approaches are based on assumptions that the specimens of each parental species sample a multivariate distribution that is not necessarily the same for each species. These multivariate distributions are described by estimating the variances, covariances, and means for the measurements, using specimens known to belong to particular parental species. These estimated parameters can be used to define and calculate various discriminant functions, early examples of which were described by Anderson (1936), Fisher (1936), and Welch (1939). A discriminant function calculates a number for each specimen using a function that maximizes the distinguishing power of the measurements for that specimen. Specimens with a number typical of a particular parental species are taken to belong to that species. Specimens with numbers between those typical for two parental species may be hypothesized to be hybrids. Multivariate normal probability distributions are hypothesized in order to enable the calculation of the probabilities with which identifications are made correctly by the discriminant functions.

If the species have been correctly delimited, if all the species represented by specimens in the sample have been recognized in advance, if the specimens used to estimate the parameters of the parental species multivariate distribution have been correctly identified, and if the character variation is normally distributed, then classical discriminant function methods should work well to correctly identify specimens that "fall between" the designated parental species. Goodman (1967) presented an example of parental varieties of *Gossypium barbadense* and *G. hirsutum*, with their F_1 and F_2 hybrids and backcrosses, and used this example to compare the accuracy and efficiency of several discriminant functions. With the assumptions met, all the discriminant functions tested worked quite well, though not necessarily better than simple hybrid indices.

There are some weaknesses to discriminant functions. Both or all parental species may not be known. Measurements of specimens of a particular species may not accurately estimate the normal distributions assumed to describe that species. Normal distributions may not adequately describe the species in question, no matter how well the species was sampled.

Discriminant functions, principal component ordination, factor analysis, and canonical analysis consider many variables together and are therefore called multivariate methods. These methods include also more recent ordination methods such as reciprocal averaging and multidimensional scaling. Adams (1982) reviewed how these methods have been used in studying hybridization. Results produced by these methods are often presented as two-dimensional scatter plots of specimens from which inferences can be made, much like the two-dimensional scatter plots of Anderson (1949). Wilson (1992) also provided a thorough review of methods in common use for inferring hybridity from morphological intermediacy, and pointed out a fundamental problem: ancestral intermediacy is evidenced by a mixture of the character states of the descendants, but hybrid intermediacy is evidenced by character states that are intermediate between those of the parents. An additional problem with some scatter plot methods are the lingering questions about statistical certainty.

There is another, qualitatively different, class of weaknesses associated with all these multivariate approaches. They were developed before computers capable of performing immense numbers of calculations were developed. These methods had to depend on sophisticated mathematical reasoning and simplifying assumptions in order to reduce calculations to a feasible level. Sometimes this placed systematists in the uncomfortable position of having to argue the differential credibility of interpretations based on mathematical techniques that they did not fully understand and assumptions that were not entirely met. Now, the ubiquity and calculating power of personal computers greatly diminishes the need to reduce the number of calculations. Thus, we no longer need to use simplifying assumptions about multivariate normal distributions nor use complex mathematical arguments in order to reduce calculations to a feasible level. Wilson (1992) used some of this power and freedom in simulations to show how ancestors can be distinguished from hybrids. He simulated intermediates under the hypothesis of phyletic divergence, and other intermediates under the hypothesis of reticulate (hybrid) genesis, and was then able to distinguish hybrids from ancestors by character intermediacy.

Our approach makes explicit the bases for the intuitive interpretation of scatter plots, eliminates the need for *a priori* identification of putative parental species, and avoids *a priori* assumptions about what distributions the measurements might sample. It uses two kinds of computationally intense techniques: one evaluates a hybrid optimality score for every possible combination of three specimens in which one is considered a hybrid of the other two (up to hundreds of thousands of possibilities for a set of hundred specimens); and the other simulates a probability hypothesis to generate measures of the level of statistical certainty.

Wagner (1983) outlined criteria, procedures, and methods that can be employed in the detection of hybrids by using qualitative (discrete) morphological data, and his triplex hybrid detection method – implemented in a computer program devised by Estabrook in 1981 – is the philosophical foundation of our methodology. The concept of character intermediacy appropriate for hybrid detection advocated by Wilson (1992) is consistent with that of Wagner (1983) and was incorporated into the original 1981 program by using character states. This concept is extended in the method we present here to employ continuous measurements. Character intermediacy can apply equally to discrete or continuous data.

However, hybrid intermediacy alone is not always a sufficient indicator of hybrid status, especially if putative parental species had not been identified with certainty in advance. In addition, the need to know the level of statistical certainty had still to be met. Here we present two additional criteria besides hybrid intermediacy for the evaluation and ranking of all the possible hypotheses for hybrids and their parental representatives. We also present a statistical guide to the interpretation of the results based on the null hypotheses that all possible rankings of the typically many thousands of hybrid hypotheses are equally likely.

Rieseberg & Ellstrand (1993) noted that "... the unpredictability of hybrid character expression diminishes the utility of morphological characters for hybrid identification." The advantages they presented of molecular over morphological markers for studies of hybridization are clear. Yet, their sample of 46 papers (most of which present cases of artificially synthesized hybrids) represents only a tiny fraction of the many thousands of known instances of hybridization. Most of these numerous instances of hybridization were presumably insufficiently interesting to warrant publication of detailed studies. Thus, the sample supporting Rieseberg & Ellstrand's conclusions (especially that the presence of extreme characters in hybrids appears to be the rule rather than the exception) is almost certainly biased toward complex and unusual examples that were sufficiently interesting to be studied in detail. Their wholesale rejection of morphological characters for hybrid identification due to the unpredictability of hybrid character expression is unwarranted. Nevertheless, their cautions are justified, and it is possible that adjustments of the weightings of the hybridity criteria in our method may compensate for some of the problems that they noted.

Objectives

The method of analysis of hybrids presented here can be applied towards two primary objectives. First is the direct study of hybrids (including introgressants) for taxonomic treatments, and for the pinpointing of possible populations, taxa, or individuals that may merit further study with other techniques. Second is the scanning of data sets for putative hybrids and hybrid species prior to phylogeny reconstruction or other detailed analyses.

Methodology

Our method is computationally intense in that every combination of three specimens in data sets of up to 100 specimens, in which one is considered the hybrid offspring of the other two, is evaluated and ranked.

The method we describe here applies to a collection of specimens in which we suspect that two or more species along with some or all of their hybrids are represented. The purpose of the method is to hypothesize which specimens might be of hybrid origin, and for each of these specimens to indicate which other two specimens resemble those that might have been its parents. Each specimen is therefore considered a putative hybrid, and every pair of remaining specimens is evaluated as its putative parents.

Quantitative measurements of characters that distinguish the species suspected to be in the study collection are taken from each specimen. These measurements are compiled and tabulated, and then used to evaluate these hypotheses using the computer program described below. For a study collection with N specimens, the number H of hypotheses evaluated is $H = N \times (N-1) \times (N-2) / 2$. This number becomes large as N increases. For example, if $N = 10$ then $H = 360$; if $N = 20$ then $H = 3420$; and if $N = 100$ then $H = 485{,}100$.

The study collection should include specimens suspected of having hybrid origin, as well as specimens that represent the variation of the species to which the putative parents belong. This will ensure that the hypotheses of particular interest are included. The order of the specimens in the data set does not affect the results. Although the program will accept sets of measurements for up to 100 specimens, the amount to be included in the analysis does not need to be that large in most instances. When variation is redundantly represented, the number of plausible hypotheses proliferates, and the results become cluttered with essentially identical hypotheses. The characters used for this procedure should be continuous quantitative, discrete quantitative, or qualitative characters that can be coded numerically with proper justification.

The number of characters measured will depend on the nature of the investigation. If the parent species differ for the characters being measured and the variation is heritable, then increasing the number of characters measured can increase the accuracy of the results without cluttering the outcome. However, each additional character is expected to improve accuracy proportionally less. If one is aware of the species present, ideal quantitative characters should each differentiate among them with minimal overlap.

Inclusion of heritable variation may reduce accuracy if the parent species do not differ significantly for the character measured. Including such characters would simply place another random element into the evaluation of hypotheses. If researchers can plausibly determine which specimens represent pure species, then analysis of variance may identify which characters measured do not differ significantly among the species. Such characters should be removed from the data set. If there is uncertainty about species limits, the use of as many characters as possible showing differentiation within the study collection is recommended.

Evaluating hypotheses. – A triplet, consisting of three specimens of which the first is hypothesized to be a hybrid and the other two are hypothesized to resemble its parents, is evaluated with three criteria that serve as indicators of hybridity: (1) a hybrid should have for each measurement a value that is between the values of its two parents; (2) its parents should be sufficiently different in their measurements to belong to different species; and (3) a hybrid should not resemble one parent much more closely than the other. These three criteria are defined quantitatively as hybrid intermediacy (IN), parental distance (PD), and equality (EQ), and the program reports them as numerical values.

Hybrid intermediacy (IN) for a triplet is determined by considering each measurement of a specimen in turn. If the specimen designated as a putative hybrid has a value exactly midway between the two values of its putative parents, then this is considered the best evidence in support of the hypothesis that the hybrid in this triplet is a product of the hybridization between the taxa to which the other two specimens belong. Consequently, this measurement is given a value of 1 for that triplet. At the other extreme, if the hybrid value exceeds the larger, or is exceeded by the smaller of the two parent values by more than half the difference in the parent values, then this is considered strong evidence against the hypothesis, and the measurement is given a value of -1 for that triplet. Measurements that have the same value for the hybrid and one parent are given a value of zero. Measurements with other values are given a value between -1 and $+1$ as determined by linear interpolation. An intermediacy value for that triplet is calculated by adding up the values for all the measurements and dividing by the number of measurements.

To state this symbolically, denote with $m\ (i, j)$ the value of the j th of r measurements of specimen i. The triplet $(h, p1, p2)$ in which with no loss of generality $p2$ has a higher value for measurement j than does $p1$, would have half the difference between the parental values given by

$$\mathrm{a} = (m\ (p2,j) - m\ (p1,j))\ /\ 2$$

and would assign to variable j the value $\mathrm{I}\ (j,\ (h, p1, p2)) =$

$$\begin{array}{lll}
\mathrm{I}\ (j,\ (h, p1, p2)) = -1 & \text{if} & m\ (h, j) - m\ (p2, j) \geq a \\
\mathrm{I}\ (j,\ (h, p1, p2)) = -1 & \text{if} & m\ (p1, j) - m\ (h, j) \geq a \\
\mathrm{I}\ (j,\ (h, p1, p2)) = (m\ (h, j) - m\ (p1, j))\ /\ a & \text{if} & m\ (p1, j) - a < m\ (h, j) < m\ (p1, j) + a \\
\mathrm{I}\ (j,\ (h, p1, p2)) = (m\ (p2, j) - m\ (h, j))\ /\ a & \text{if} & m\ (p2, j) - a < m\ (h, j) < m\ (p2, j) + a
\end{array}$$

This triplet would have an intermediacy score

$$\mathrm{IN}\ (h, p1, p2) = (\sum_{j=1}^{r}\ \mathrm{I}\ (j,\ (h, p1, p2)))\ /\ r$$

Parental distance (PD) is calculated here by rescaling each measurement to have a range from zero to one in order that all measurements be weighted more nearly equally in the determination of difference between two specimens. This is done by subtracting the minimum value actually assigned to any specimen under study from the value assigned to each specimen. Then, each of these new values is divided by the maximum new value. The specimen with this maximum value will now have the value one, while the specimen with the minimum value will have the value zero. The other specimens will have intermediate values relative to each.

Symbolically, the distance between two specimens $p1$ and $p2$ for some rescaled measurement j is $|\ m'\ (p1,j)\ -\ m'\ (p2,j)\ |$, i.e., the absolute value of the difference between the value assigned by j to $p1$ and the value assigned by j to $p2$. The overall difference between two specimens $p1$ and $p2$ is simply the average over all the rescaled measurements of these individual differences. If there are r measurements, this is written:

$$\mathrm{d}\ (p1, p2) = (\sum_{j=1}^{r}\ |\ m'\ (p1,j) - m'\ (p2,\ j)\ |)\ /\ r$$

The distance between the parents of a hypothesized hybrid triplet is simply the difference between the hypothesized parents:

$$\mathrm{PD}\,(h, p1, p2) = d\,(p1, p2)$$

A hybrid would be expected to have intermediate character values that are sometimes closer to one parent and sometimes closer to the other parent. This would result in it being nearly equidistant from each other parent. Of two specimens of the same species, one near the limit of variation toward a neighbouring species would have a high IN score if a member of that neighbouring species was considered as the other putative parent, but its intermediate character values would usually be closer to its conspecifics, resulting in unequal distances to the putative parents.

Equality (EQ) of the distances between a putative hybrid and its putative parents in a triplet is calculated by using the same formula used for parental distance to calculate the distance between the putative hybrid and each putative parent. The distance between the hybrid and the second parent is then subtracted from the distance between the hybrid and the first parent, and the remainder is divided by the distance between the parents (PD). This is defined symbolically as:

$$\mathrm{EQ}\,(h, p1, p2) = d\,(h, p1) - d\,(h, p2)\,/\,d\,(p1, p2)$$

The value for EQ is constrained to vary from -1 to $+1$. When the hybrid is identical to the first parent then $d(h, p1) = 0$ and $d(h, p2) = d(p1, p2)$ so that $\mathrm{EQ} = -1$. When the hybrid is equidistant from each parent, then $d(h, p1) = d(h, p2)$ so $\mathrm{EQ} = 0$. When the hybrid is equal to the second parent then $d\,(h, p2) = 0$ and $d\,(h, p1) = d\,(p1, p2)$ so that $\mathrm{EQ} = 1$. Thus, EQ also shows to which parent the "hybrid" is closer. Unlike the other criteria, EQ provides strongest evidence for hybrid origin when its values are near zero.

Each of the IN, PD, and EQ values provides some indication about possible hybrid status. As an indicator of character intermediacy, IN may be considered the most important. However, as we have argued, without some contribution from PD and EQ, which are based on overall difference, IN could make misleading suggestions. Thus, we calculate a hybrid score (HS), which is the weighted sum of these three measures.

The hybrid score of the hypothesis $(h, p1, p2)$ can now be explicitly defined as:

$$\mathrm{HS}\,(h, p1, p2) = w1 * \mathrm{IN} + w2 * \mathrm{PD} + w3 * |\,1 - \mathrm{EQ}\,|$$

in which $w1$, $w2$, and $w3$ are weights specified by the user. Weights for each of the three criteria can range from 0 for no weight to 1 for maximum weight. HS, produced by summing the three weighted criteria, is a ranking device used to sort out from the large numbers of potential hybrid hypotheses those that are most plausible.

Intermediacy on a character-by-character basis is an established protocol for determining putative hybridity (Gottlieb, 1972; Wagner, 1983; Wilson, 1992), but heterosis, introgression, and sometimes also negative heterosis can confound the situation (Murrell, 1994). The computer program provides the user with a default weight of one for each criterion, as well as an alternative choice to assign a different weight to each criterion. Weighting the three criteria can be both an empowerment, enabling flexibility to meet different needs, as well as a source of anxiety, requiring the user to be responsible for determining these weights. The default weights should function adequately in many circumstances, at least for a "first pass" evaluation of the data. However, by allowing weighting of the degree of intermediacy, interparental distance, and inequality, this method provides flexibility in dealing with problems, such as heterosis and introgression, if they are suspected. Researchers in different situations may therefore prefer to weight these three criteria differently. For example, in

Table 1. Results of the HYWIN Analysis of the *Carex* data set. The top 50 of the 163 highest ranking triplets of putative hybrids and respective putative parents at 0.5 probability. Letters preceding the collection numbers: C = *Carex ×caesariensis;* P = *C. pellita;* T = *C. trichocarpa;* S = *C. striata.* Rank = Rank of first time as hybrid; IN = intermediacy score; EQ = equality score; PD = parental distance score; NP = distance to the nearest parent. When the EQ value is positive, the putative hybrid is closer to putative parent 2; when it is negative, the putative hybrid is closer to putative parent 1. The weights assigned for the ranking criterion: *w*I = 1.0, *w*E = 1.0, *w*P = 1.0.

Putative Hybrid	Putative Parent 1	Putative Parent 2	Rank	IN	EQ	PD	NP
C26083	P2266	T1982	1	0.659	0.033	0.401	0.194
C26083	P5352	T1982	2	0.585	−0.016	0.382	0.188
C26083	P1840	T1982	3	0.534	−0.035	0.375	0.181
C23212	P12843	T13434	4	0.456	0.042	0.421	0.212
C23212	P2492	T43952	5	0.507	0.026	0.343	0.167
C23212	P2266	T13434	6	0.461	0.081	0.439	0.212
C26083	P2266	T13434	7	0.461	−0.092	0.439	0.207
C23212	P6090	T43952	8	0.530	−0.042	0.321	0.153
C26083	P1928	T43952	9	0.452	−0.057	0.409	0.202
C23212	P6818	T43952	10	0.521	0.091	0.368	0.167
C23212	P5013	T13434	11	0.398	−0.003	0.401	0.211
C23212	P12843	T43952	12	0.554	0.161	0.398	0.167
C23212	P2266	T6689	13	0.403	−0.130	0.514	0.250
C23212	P5352	T13434	14	0.402	0.023	0.408	0.212
C23212	P21594	T43952	15	0.515	−0.055	0.317	0.150
C26083	P12843	T43952	16	0.494	−0.117	0.398	0.179
C26083	P6818	T43952	17	0.621	−0.215	0.368	0.146
C23212	P5013	T43952	18	0.504	0.117	0.378	0.167
C23212	P5352	T43952	19	0.510	0.142	0.390	0.167
C26083	P2266	T6803	20	0.399	−0.023	0.380	0.207
C26083	P2266	T43952	21	0.384	−0.045	0.417	0.207
C23212	P2266	T6847	22	0.372	−0.028	0.409	0.250
C26083	P5013	T1982	23	0.455	−0.066	0.359	0.170
C23212	P6818	T13434	24	0.380	−0.029	0.390	0.201
C23212	P1928	T6847	25	0.365	−0.044	0.410	0.242
C26083	P12843	T6807	26	0.470	−0.122	0.383	0.179
C26083	P12843	T13434	27	0.474	−0.164	0.421	0.179
C23212	P1840	T13434	28	0.333	0.007	0.401	0.212
C23212	P2266	T43952	29	0.508	0.198	0.417	0.167
C23212	P1840	T43952	30	0.468	0.126	0.382	0.167
C23212	P12843	T6847	31	0.366	−0.067	0.417	0.231
C26083	P2266	T6826	32	0.471	−0.228	0.469	0.207
C26083	P1840	T43952	33	0.441	−0.112	0.382	0.181
C23212	P12843	T6807	34	0.364	−0.041	0.383	0.231
C23212	P6853	T43952	35	0.459	−0.067	0.313	0.146
C23212	P5352	T6847	36	0.394	−0.086	0.390	0.223
C23212	P2266	T6807	37	0.343	−0.002	0.352	0.250
C26083	P1928	T1982	38	0.347	0.020	0.363	0.194
C26083	P433	T1982	39	0.343	−0.020	0.365	0.186
C26083	P2266	T6813	40	0.425	−0.179	0.437	0.207
C26083	P6818	T6807	41	0.556	−0.221	0.347	0.146
C23212	P1928	T43952	42	0.448	0.184	0.409	0.167
C26083	P5013	T43952	43	0.434	−0.143	0.378	0.170
C26083	P1840	T6826	44	0.504	−0.291	0.454	0.181
C23212	P5352	T1982	45	0.359	0.077	0.382	0.191
S6964	T6847	SNJ	46	0.337	0.087	0.414	0.242
C23212	P1840	T6847	47	0.380	−0.102	0.383	0.215
C23212	P5352	T6813	48	0.407	−0.153	0.406	0.223
C23212	P1072	T43952	49	0.341	−0.000	0.319	0.167
C23212	P5352	T6803	50	0.317	−0.016	0.357	0.223

a situation where one putative parent is a polyploid and one is a diploid, EQ may be given less weight, as putative hybrids might be expected to resemble more closely the parent contributing most genetic material. If heterosis or negative heterosis is suspected, then IN should be given less weight, as hybrid measurements conceivably could fall beyond the ranges of their parents. Weighting PD more may be appropriate when dealing with highly variable species or cases of introgression. Otherwise triplets composed of three members of the same species may be ranked high, since they can have high intermediacy values although they will have low parental distance values.

This weighting of properties of characters is quite different from the individual weighting of each character proposed by Hatheway (1962). In the triplex hybrid detection method (Wagner, 1983), the user weights judgements on hybrid intermediacy character by character by giving index values to intermediate states, and evaluates all possible three-way taxon comparisons (triplets). However, neither parental distance nor equality is used.

To understand the role of PD and EQ, notice that intermediacy can occur among three very similar specimens belonging to the same species. The hypothesis that the middle specimen is a hybrid of the other two would be irrelevant for most studies because all three specimens belong to the same species. To ensure that such hypotheses are not ranked highly, PD must be given some weight. If hypothesized parents were quite different but the hypothesized hybrid is very much closer to one than to the other, it may belong to the same species as its closest "parent". To ensure that such hypotheses are not highly ranked, EQ must be given some weight. If specimens of several possible parental species are present in the study, hypotheses with distant putative parents and a putative hybrid equidistant from them could be very wrong if the putative hybrid is not intermediate according to the criteria of Wagner (1983) and Wilson (1992). Intermediacy in the form of equality of distances between three specimens of a triplet (EQ) does not necessarily indicate hybrid intermediacy. Giving significant weighting to IN will help ensure that intermediates are putative hybrid intermediates.

How many hypotheses should be considered? – Two basic questions can be examined in a study of hybrids employing this method. Are there putative hybrids in the data set, and what are the parents of suspected hybrids?

If the primary purpose of the study is to recognize which specimens warrant further scrutiny to test their hybrid status, examine the top ranked one or very few hypotheses with additional tests, such as those outlined by Wagner (1983). If they remain plausible, remove these probable hybrids from the data set, and repeat the analysis. Continue to examine the top ranked hypotheses, remove hybrids, and reanalyse until there are no longer hypotheses that are plausible.

If the primary purpose of the study is to help determine which of several species may have been the parents of specimens whose hybrid nature is fairly clear, then the failure of certain specimens to participate in the top ranked hypotheses becomes significant. One approach to determining how many of the top ranked hypotheses to consider is to ask what we would expect if one ranking of the hypotheses was as likely as any other (instead of being based on measurements of specimens). For a given number of specimens, we generate, with equal probability among the possible rankings, 10,000 of them. For each ranking, we count down from the top ranked

Table 2. The HYWIN summary output of the 163 highest ranking triplets at 0.5 probability of the *Carex* data set. Letters preceding the collection numbers: C = *Carex* ×*caesariensis*; P = *C. pellita*; T = *C. trichocarpa*; S = *C. striata*.

Collection number	Number of times ranked as a hybrid	Rank of first time as a hybrid	Number of times as a parent	Rank of first time as a parent
C23212	71	4	2	110
C26083	54	1	1	113
S6964	12	46	3	51
P6685	7	78	1	137
T6803	4	88	8	20
T1982	2	65	18	1
T6847	2	51	19	22
S4205	2	135	10	70
P21594	1	110	3	15
T6826	1	109	16	32
T6807	1	158	9	26
S26174	1	156	1	129
T6824	1	98	9	79
T13434	1	144	29	4
P2492	1	132	8	5
P12843	0	–	12	4
P1928	0	–	10	9
P6818	0	–	6	10
P6853	0	–	1	35
P2101	0	–	8	55
T43952	0	–	24	5
T6813	0	–	15	40
T6689	0	–	9	13
P2266	0	–	21	1
P5013	0	–	13	11
P6090	0	–	6	8
P1840	0	–	16	3
P433	0	–	5	39
P1072	0	–	1	49
P5352	0	–	16	2
SNJ	0	–	6	46
S3225	0	–	0	–
S44496	0	–	1	156
S8934	0	–	0	–
S2145	0	–	0	–
S3061	0	–	3	115
S9004	0	–	1	155
SBoston	0	–	6	74
S6976	0	–	3	98
S6977	0	–	2	140

hypothesis until each specimen has appeared at least once as a putative hybrid. These 10,000 counts are placed into a table and sorted from the highest to the lowest count. The count in place 5001 represents the number of top ranked hypotheses we would need to examine for a 0.5 probability of encountering every specimen hypothesized at least once in the role of hybrid, if the rankings were completely arbitrary. By the same reasoning, the count in place 501 is the number of top ranked hypotheses that would make this probability 0.95. For example, with 10 specimens, the top 26 hypotheses would contain all 10 specimens in the role of hybrid at least once with probability 0.5 if the rankings were arbitrary. The top 47 would bring that probability up to 0.95. For 20 specimens, the top 67 hypotheses give a probability of 0.5 of including all 20 in the role of hybrid, and the top 115 bring that probability to 0.95. In the case where most specimens are hybrids, the meaningful contribution of this approach would be the recognition of parents by failure to hypothesize them as hybrids in any high scoring. The 0.95 threshold would give a number of triplets, within which failure to hypothesize specimens as hybrids would be given a specific meaning of significance.

Although these numbers of top ranked hypotheses are still arbitrary, this approach provides a probabilistic basis for limiting the number of hypotheses, rather than listing thousands. Once a number of hypotheses has been specified by the user, the number of hypotheses in which each specimen occurs as hybrid, the rank of the highest ranked hypothesis, and the identity of specimens that never occur as hybrid, are counted and reported in a summary table (see Tables 2 and 4 for examples).

The computer program

Our method is implemented in the computer program HYWIN (*hy*pothesizing h*y*brids and parents using *w*eighted *in*termediacy) that is written in PASCAL, and runs on IBM and compatible personal computers under DOS. A copy of the program, a sample data set, and a user's guide can be obtained from the World Wide Webb (http://www-personal.umich.edu.~gfred/).

The output from the program consists of five tables. The first table is a matrix of the original input data. The second (optional) table is a matrix of the standardized data. The third (optional) table is a symmetric matrix of distances. The fourth table reports all the requested top ranked triplets of hybrids and their putative parents along with listings of the IN, EQ, PD, and HS scores for each triplet. In addition, the distance from the putative hybrid to its nearest putative parent, NP (see the calculation of EQ under Methodology), is given for each triplet. The fifth table provides a summary for each specimen consisting of the number of times the specimen is being suggested as a hybrid (when applicable) within the number of top ranked hypotheses for the selected probability, the highest rank of a triplet in which a specimen is suggested as a hybrid, the number of times the specimen appears in the role of a parent, and the highest rank of a triplet (when applicable) in which the specimen is suggested as one of the putative parents.

Examples

In order to evaluate our approach and to test the program, we used data sets from two published studies.

Example 1: *Carex ×caesariensis.* – Reznicek & Catling (1985) studied the identity of *Carex ×caesariensis,* a putative hybrid with two competing parentage hypotheses: *C. trichocarpa* × *C. striata (C. walteriana)* and *C. trichocarpa* × *C. pellita (C. lanuginosa).* In this example, two-dimensional scatter diagrams of morphological characters, though suggesting a parentage of *C. trichocarpa* × *C. pellita,* were inconclusive. However, a discriminant function analysis that employed 11 quantitative characters on data from two collections of this putative hybrid (both from the same clone), 10 specimens of *C. trichocarpa,* 13 specimens of *C. striata,* and 15 specimens of *C. pellita,* demonstrated that *C.* ×*caesariensis* was unambiguously a hybrid of *C. trichocarpa* and *C. pellita.* This hybrid, treated as a species by several authors, is notable because it is intersectional. Thus, the two parents are so distantly related that if the hybrid was included in a phylogenetic analysis, it would distort a cladogram (McDade, 1992).

Applying the methodology presented in this paper to this *Carex* data set very clearly eliminated the hypothesis that *C. striata* is one of the parents of *C.* ×*caesariensis,* and confirmed the results of the earlier discriminant function analysis. The HYWIN analysis of the *Carex* data set was conducted by using the default weights ($wI = 1$, $wE = 1$, and $wP = 1$) for the ranking criterion. The 0.5 probability option noted above was used, as we were only interested in the top-ranked few hypotheses. For ease of data retrieval, the specimens were labelled in the matrix of the input data set with the first letter of the specific epithet followed by collection number or an abbreviation for locality when a collection number was unavailable.

A portion of the results of this analysis is presented in Table 1, and summarized by frequency counts in Table 2. The count for the 0.5 probability option for the 40 specimens examined was the first 163 highest ranking triplets. Within this group, the two specimens of *Carex* ×*caesariensis* (C23212 and C26083) in the data set appeared as hybrids of *C. pellita* (P) and *C. trichocarpa* (T) through the top 45 triplets. Of the top 163 triplets, this combination appeared 114 times (66 for one specimen and 48 for the other) while the triplet of *C.* ×*caesariensis* hypothesized as a hybrid of *C. trichocarpa* × *C. striata,* appeared only 10 times and far down in the rankings; the first appearance of this triplet was in the 70th position from the top. The greatest frequency for any of the other specimens appearing as a putative hybrid was 12, and all other hybrid triplets appeared only a total of 36 times, again far down in the rankings. Eliminating the two specimens of *C.* ×*caesariensis* from the data set, and running the analysis again to try to detect other potential hybrids produced no plausible candidates for hybrids. All three species *(C. trichocarpa, C. striata,* and *C. pellita)* appeared as suggested "hybrids" even among the top five triplets, and the greatest frequency for any single specimen was 31 appearances, scattered through the ranks with no discernible pattern. Thus, applying this method using the default weighting, distinguished unambiguously and easily between the two rather similar competing hypotheses without the statistical assumptions and mathematical complexity inherent in methods like discriminant function analysis.

Example 2: Introgression among "stemless white" *Viola.* – A more complex example, a highly variable population of "stemless white" *Viola,* was also examined using this method. This example was first presented by Anderson (1954) and also by Hatheway (1962), who actually included the data set in a paper introducing an intricate, weighted hybrid index method based on canonical analysis. Anderson concluded that introgression was responsible for the variation in a population of *V. macloskeyi (V. pallens)* and that the other parent might have been either *V. blanda* var. *blanda* or *V. blanda* var. *palustriformis (V. incognita).* He did not comment on the identities of individual specimens. Using his weighted hybrid index, Hatheway concluded that two specimens (No. 1 and 2) were "... close to *Viola macloskeyi*" and two others (No. 3 and 18) were "... probably closely related to the coarser of the

Table 3. Results of the HYWIN Analysis of the "stemless white" *Viola* data set (data from Hathaway, 1962).The top 50 of the 149 highest ranking triplets of putative hybrids and respective putative parents at 0.95 probability. Headings as in Table 1. The weights assigned for the ranking criterion: *w*I = 0.1, *w*E = 0.1, *w*P = 1.0.

Putative Hybrid	Putative Parent 1	Putative Parent 2	Rank	IN	EQ	PD	NP
V11	V2	V3	1	0.276	−0.006	0.606	0.367
V20	V2	V3	2	0.294	−0.028	0.606	0.397
V9	V2	V3	3	0.339	−0.137	0.606	0.320
V10	V1	V3	4	0.324	0.099	0.603	0.362
V24	V2	V3	5	0.315	−0.132	0.606	0.282
V11	V1	V3	6	0.249	0.053	0.603	0.372
V4	V2	V3	7	0.279	0.110	0.606	0.329
V7	V1	V3	8	0.230	−0.045	0.603	0.319
V20	V1	V3	9	0.208	−0.028	0.603	0.397
V21	V2	V3	10	0.328	−0.182	0.606	0.260
V7	V2	V3	11	0.259	−0.122	0.606	0.273
V7	V23	V1	12	0.338	0.026	0.588	0.319
V25	V2	V3	13	0.507	−0.382	0.606	0.187
V16	V2	V3	14	0.242	−0.134	0.606	0.292
V9	V1	V3	15	0.307	−0.186	0.603	0.290
V24	V1	V3	16	0.258	−0.148	0.603	0.273
V21	V1	V3	17	0.292	−0.183	0.603	0.259
V4	V1	V3	18	0.222	0.116	0.603	0.329
V16	V1	V3	19	0.311	−0.206	0.603	0.251
V25	V23	V1	20	0.521	0.262	0.588	0.217
V10	V2	V3	21	0.208	0.136	0.606	0.362
V4	V1	V18	22	0.319	0.142	0.595	0.312
V13	V2	V3	23	0.315	−0.266	0.606	0.229
V21	V23	V1	24	0.373	0.151	0.588	0.259
V25	V1	V3	25	0.379	−0.317	0.603	0.217
V24	V23	V1	26	0.320	0.116	0.588	0.273
V4	V2	V18	27	0.325	0.135	0.589	0.312
V9	V23	V1	28	0.348	0.158	0.588	0.290
V19	V1	V3	29	0.290	−0.266	0.603	0.262
V12	V2	V3	30	0.253	0.254	0.606	0.282
V5	V2	V3	31	0.191	0.194	0.606	0.277
V6	V1	V3	32	0.247	0.249	0.603	0.317
V12	V1	V3	33	0.243	0.246	0.603	0.282
V15	V2	V3	34	0.219	0.258	0.606	0.292
V4	V23	V1	35	0.250	−0.111	0.588	0.333
V6	V2	V3	36	0.233	0.278	0.606	0.317
V20	V23	V1	37	0.129	−0.001	0.588	0.397
V11	V2	V18	38	0.330	0.247	0.589	0.222
V20	V1	V18	39	0.032	−0.008	0.595	0.397
V24	V1	V18	40	0.136	−0.126	0.595	0.273
V5	V1	V3	41	0.166	0.245	0.603	0.277
V19	V2	V3	42	0.118	−0.225	0.606	0.287
V16	V23	V1	43	0.256	0.185	0.588	0.251
V21	V1	V18	44	0.129	−0.161	0.595	0.259
V15	V1	V3	45	0.137	0.258	0.603	0.292
V20	V2	V18	46	0.027	−0.008	0.589	0.397
V11	V1	V18	47	0.262	0.302	0.595	0.222
V17	V1	V3	48	0.258	−0.385	0.603	0.214
V25	V2	V18	49	0.380	−0.364	0.589	0.187
V24	V2	V18	50	0.122	−0.109	0.589	0.282

Table 4. The HYWIN summary output of the 149 highest ranking triplets at 0.95 probability of the "stemless white" *Viola* data set.

Collection number	Number of times ranked as a hybrid	Rank of first time as a hybrid	Number of times as a parent	Rank of first time as a parent
V16	10	14	0	–
V21	10	10	0	–
V9	10	3	0	–
V25	9	13	0	–
V24	9	5	0	–
V20	8	2	0	–
V4	8	7	0	–
V7	8	8	0	–
V5	7	31	0	–
V19	7	29	2	144
V13	7	23	0	–
V17	7	48	0	–
V11	6	1	0	–
V15	6	34	2	138
V8	5	52	1	140
V14	5	60	11	70
V6	5	32	14	62
V22	5	74	4	141
V12	5	30	0	–
V10	5	4	0	–
V23	4	80	29	12
V18	3	68	44	22
V1	0	–	82	4
V2	0	–	65	1
V3	0	–	44	1

parental species – either *Viola blanda* or *V. incognita*". Hatheway also presented an alternative interpretation that "plants 3 and 18 may not be specimens of the coarser parent but instead backcrosses to it."

The *Viola* data set consisted of 10 characters measured or scored on 25 specimens. This example represented a case of putative introgression and a search for putative parental specimens. Since the putative parents will not be expected among the highest ranking hybrid hypotheses, we used the 0.95 probability option to be comprehensive. We wanted very stringent criteria for "parenthood", such that specimens imbedded in differing positions near the middle of the putative introgressed swarm would not be reciprocally regarded as "parents" of nearby specimens. Thus we set the weighting for PD at 10 times than that of EQ and IN ($wI = 0.1$, $wE = 0.1$, and $wP = 1.0$). Specimens having the lowest scores as hybrids (the best "parents") would have participated the least often as hybrids and the most often as parents among the trials tabulated. A portion of the results of this analysis is presented in Table 3 and summarized by frequency counts in Table 4.

Examination of the bottom-most four specimens of Table 4 (V18, V1, V2, and V3; specimen numbers are those in Hatheway 1962 prefaced by "V") discloses that they are the same four specimens as were thought by Hatheway to most closely resemble the putative parents. Three of these four never participated as a hybrid in the trials tabulated, and V18 participated as a hybrid only 3 times. On the other hand, these four specimens participated as parents in the trials far more often than any other specimens. Applying our method using the default weights produced somewhat similar results, but the eight bottom-most specimens never participated as hybrids; the four noted above (V1, V2, V3, and V18) plus V8, V14, V22, and V23. These additional four specimens were near one end of the scattered diagram in Hatheway (1962), but were considered introgressants by both Hatheway and Anderson (1954). However, this method is also unable to distinguish between Hatheway's two alternative hypotheses for specimens V3 and V18.

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