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Article

Estimating significances of differences between slopes: A new methodology and software

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Abstract

Determining the significance of slope differences is a common requirement in studies of self-thinning, ontogeny and sexual dimorphism, among others. This has long been carried out testing for the overlap of the bootstrapped 95% confidence intervals of the slopes. However, the numerical random re-sampling with repetition favours the occurrence of re-combinations yielding largely diverging slopes, widening the confidence intervals and thus increasing the chances of overlooking significant differences. To overcome this problem a permutation test simulating the null hypothesis of no differences between slopes is proposed. This new methodology, when applied both to artificial and factual data, showed an enhanced ability to differentiate slopes.

Keywords slope difference; permutation; bootstrap; overlap; randomization tests.

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1 Introduction

Linear regression is the simplest, most common method to systematize the relation between pair-wised variables. It estimates expected values for the dependent variable *y* given fixed values for the independent variable *x*. However, usually causality cannot be assumed and therefore it is incorrect to treat a variable as dependent from another independent one. Even when causality can be assumed, the standard linear regression (i.e, model I linear regression) assumes *x* is measured in the absence of error. When such is not the case, most likely the standard linear regression is inadequate (but see Smith, 2009, and Legendre, 2013). Major axis regression (MA) emerged as an alternative for the oblique simultaneous regression of *x* and *y*. It was fallible when variables were expressed in different units and/or subject to different scales. This was solved with reduced major axis regression (RMA), where the variables are first standardized to zero mean and unit variance before the MA protocol is applied. RMA became the reference methodology as was the case of the self-thinning rule, originally from plant demography and later extended to macro-algae (Creed, 1995; Flores-Moya et al 1997; Scrosati, 1997, 2005; Scrosati and DeWreede, 1997; Creed et al., 1998; Steen and Scrosati, 2004; Rivera and Scrosati, 2008) and animals (Hughes and Griffiths, 1988; Rincón and Lobón-Cerviá, 2002; Keeley, 2003). It states the log densities of individuals and their log biomass are negatively correlated provided the population is crowded enough for competition effects to take place (Yoda et al., 1963; White and Harper, 1970; Weller, 1987; Scrosati, 1997, 2005). In other words self-thinning is the process of concurrent increase in population biomass and decrease in density as some individuals die due to intra-specific competition. A question emerges about determining whether slopes taken from distinct populations are significantly different. These would reflect populations subject to different environments and degrees of competitive stress (Yoda et al., 1963; Lonsdale and Watkinson, 1982; Weller, 1987; Morris, 1996; Scrosati and DeWreede, 1997; Creed et al., 1998; Keeley, 2003; Morris, 2003; Steen and Scrosati, 2004). In biometrics, slopes estimated from RMA (or model II regression) are often used in studies on ontogeny (Schott et al., 2011) and sexual dimorphism (Fairbairn, 1997; Anderson, 2012). Slope differences may be found between species of the same family (Leigh and Shea, 1995) or populations of the same species exhibiting different behaviours (Buffa et al., 2001). Within the same species different degrees of sexual dimorphism may be found in body measurements subject to the independent action of natural selection (Masterson and Hartwig, 1998). The significances of slope differentiation were first determined by parametric tests relying in the normality of data and thus obliging further data manipulation (see Sokal and Rohlf, 1981). More recently slope differentiation became commonly estimated by bootstrapping both populations, determining their slope confidence intervals and their overlap. Similar bootstrap and Jackknife procedures have also been used to estimate significances of metrics taken from PCA (Stauffer et al., 1985; Jackson, 1993; Peres-Neto et al., 2003; Peres-Neto et al. 2005; Zhang, 2011). Yet, with bootstrap there is never the statement or the simulation of a null hypothesis against which the original data may be tested. This is fundamental to the estimation of significances of metrics and can only be obtained from permutation tests, another group of Monte Carlo statistics (also known as randomization tests) developed with that precise objective (Manly, 1991; Dijksterhuis and Heiser, 1995; Anderson and Legendre, 1999; Peres-Neto et al., 2005; Vieira, 2012; Legendre, 2013). As for Bootstrap and Jackknife methods, these were developed with the objective of estimating confidence intervals and not significances (Efron and Tibshirani, 1986; Manly, 1991; Lebart, 2006).

Matlab (Mathworks) based software was developed to allow for the use of randomization tests to estimate the significance of the difference between two slopes. Both bootstrap and permutation based methods are available. These were tested and their results compared. We used artificially generated bivariate datasets and two empirical datasets: one of the frond and biomass densities of the seaweed *Laminaria digitata* (Huds.) Lamouroux and the other on the biometrics of the lobster *Nephrops norvegicus* (Linnaeus, 1758). As calculus may turn out to be computationally intensive, it is also provided a parallel processing version of the software. However, this requires the 'parallel processing toolbox' only available in Matlab R2011a and later releases.

2 Methods

2.1 Slope estimation

The bivariate data sets had m observations divided between g groups. The slope was estimated for each group using RMA and compared to every other slope. The RMA is the geometric mean of the two regression coefficients, that is, of y regressed upon x and vice versa. However, the slope of x regressed on y must be inversed ($1/b_{xy}$) due to the axes transposition. The RMA becomes then in its simplest form $|b|=s_y/s_x$, where s_x and s_y are the standard deviations of x and y, respectively. The slope sign is that of the Pearson correlation coefficient r.

2.2 The permutation tests

Permutations tests with PCA permuted variables independently breaking their correlations (Dijksterhuis and Heiser, 1995; Peres-Neto et al., 2005; Zhang, 2011; Vieira, 2012). However, these tests were developed to estimate the significances of the PCA itself, which implied a null hypothesis of no correlations between variables. Permutations tests have been applied to multiple regression to estimate the significance of the correlation of Y with the X_n predictor variables (Zhang, 2011). It requires keeping the correlations within independent variables while breaking their correlations with the dependent variable (Ferrarini, 2011). In the present case the permutations redistributed the observations randomly through the full dataset keeping the correlations between the two variables, i.e: it was a redistribution of the original (x, y) pairs as it was only intended to test the differences between groups and not the correlation between the two variables. The original difference between two slopes was estimated for each possible combination of two groups. The original dataset was randomly permuted along its full length. This procedure simulated the null hypothesis that all groups came from the same population. The slopes were re-estimated as well as the slope differences. When the random permutations procedure was repeated n times the probability density functions (pdf) for the slope differences among groups under the null hypothesis were obtained. The n was usually around 10000 iterations. In order to estimate their significances, the original slope differences were compared to their pdf under the null hypothesis. It was found the permutation method could not be applied to the original x and y variables. Randomly permuting elements between two populations with distinct centroids produces a systematic bias in slope estimation. This was illustrated comparing the self-thinning of kelp in replicate 1 of 2000 plants/m^2 and replicate 4 of 5186 plants/m^2 densities (Appendix A). The solution was standardizing the variables to each population's mean, thus forcing the centroids to coincide in the origin of the coordinate system (Appendix B).

2.3 The bootstrap tests

Each group was bootstrapped and its new slope estimated. This means all observations were taken out from their groups and then re-sampled back with repetition into their original groups. When this was done n times probability density functions (pdf) of the slope values for the groups were obtained. However, these pdf did not correspond to any null hypothesis but rather to estimates of the error around the original values. The n was usually around 10000 iterations. The significances of the differences between groups were given by the overlap of their confidence intervals. Two slopes were significantly different at α level only if their 1-α confidence intervals did not overlap (Sokal and Rohlf, 1981).

2.4 The data

An artificial data set was created specifically to determine the probability of type 2 error (false negatives), which occurs due to bias from the sampling and bias from the numerical method. It implied comparing two factually distinct slopes from two distinct populations, each with 1000 (x, y) pairs. Variable x was randomly distributed from 1 to 5. Variable $y_1 = a + bx + er_1$, variable $y_2 = a + (b+\Delta b)x + er_2$, $a = 10$, $b = 1$, $\Delta b = 0.5$ and er_i was randomly selected from a normal distribution with zero mean and s_y standard deviation. One sample sized n was randomly selected from each population, their slopes estimated, the randomization methods applied as described in sections 2.2 and 2.3, and decided whether to consider them different (true positive) or equal (false negative). By repeating this procedure 1000 times it was obtained an estimate of the probability of type 2 error. This probability was estimated for samples sized 4 to 48 with standard deviations from 0.1 to 1. Another artificial data set was created specifically to determine the probability of detecting false differences between slopes i.e., false positives or type 1 error. The procedure was similar to the one above in all except the two samples were taken from a single homogeneous population (x, y_1). Therefore, any differences obtained corresponded to false positives.

Macrostages of *Laminaria digitata* were cultivated on 10×15cm plates with initial densities of 10, 20, 30, 40 and 80 individuals per plate corresponding to 650, 1334, 2000, 2668 and 5186 individual per m^2. Each density had four replicates and experiments were carried in culture tanks at Port Erin Marine Laboratory, Isle of Man. The density and stand biomass in each plate was accessed for 11 sampling times at approximately 19 day intervals. Details of methods and results were presented in Creed et al (1998). Here were used replicates 1, 2 and 3 of density 2000 ind/m^2 and replicates 1, 2, 3 and 4 of density 5186 ind/m^2, and only the first eight sampling times.

Males of the lobster *Nephrops norvegicus* were sampled at seven sites from the south coast of Portugal (Algarve) in the Atlantic and across the Mediterranean in the Alboran Sea, the Catalan Sea, the Ligurian Sea, the Tyrrhenian Sea, the Adriatic Sea and the Gulf of Euboikos (Castro et al., 1998). The relation between the carapace standard length and carapace width were used to compare these populations.

3 Results

The probability of type 1 error was generally around 0.04 to 0.05 for the permutation method and 0.03 for the bootstrap method (*not shown*). The permutation method was more robust against type 2 error than the bootstrap method (Fig. 1) identifying real differences between slopes with higher data scatter. However, with samples sized four or smaller both methods were incapable of identifying true differences. In order to study this particular case two artificial populations of four elements were created where y was a linear combination of x (r = 1). One population had a slope of 1 whereas the other had a slope of -1. The permutation method did not consider these as significantly different (p=0.5107). Two new artificial populations of five elements were created where y was no longer a linear combination of x. Still, x and y were conspicuously correlated (r_1 = 0.8865 and r_2 = -0.8982) and slopes were conspicuously different (Fig. 2). Neither method was able to differentiate slopes. The permutation method gave p = 0.3233 whereas the bootstrap method entirely overlapped the 95% confidence intervals due to the occurrence of odd combinations when resampling very small sample sizes. Resampling only units 2, 3 and 4 (starting counting from the left) from population 1 yielded slopes more negative than the slopes of population 2. Resampling only units 2 and 3 from population 2 yielded slopes more positive than the slopes of population 1.

The kelp self-thinning time series of every replicate, plotted in Fig. 3, started in the bottom right and evolved to the top left. Replicates 1, 2 and 3 of density 2000 plants/m^2 had the steepest self-thinning slopes, replicates 1, 2 and 3 of density 5186 plants/m^2 had the flattest self-thinning slopes and replicate 4 of density 5186 plants/m^2 had an intermediate self-thinning slope. According to both methods all replicates of 2000 plants/m^2 and replicate 4 of 5186 plants/m^2 made a homogeneous group while replicates 1, 2 and 3 of 5186 plants/m^2 made another homogeneous group (Table 1). Slopes tended to be significantly different between these two groups. The permutation method showed difficulty differentiating replicate 3 of 2000 plants/m^2 from replicate 1 of 5186 plants/m^2 (p=0.0521). The bootstrap method overlapped the 95% confidence intervals of replicate 3 of 2000 plants/m^2 with both replicates 1 and 3 of 5186 plants/m^2. This was because for the lower density time series it was sufficiently easy to obtain particular combinations of points yielding flatter slopes while for the higher density time series it was sufficiently easy to obtain particular combinations of points yielding stepper slopes.

The biometrics data was divided into seven groups with 45 to 50 observations each and slopes b_{PO}=0.422, b_{CA}=0.433, b_{LI}=0.399, b_{TY}=0.446, b_{AD}=0.573, b_{GR}=0.372 and b_{AL}=0.355. The bootstrap method only recognized the bigger slope as significantly different from the two smallest slopes whereas the permutations method exhibited a better capability of differentiating between slopes from both extremes (Table 1).

Table 1 Tests to the differences between slopes for the *Laminaria digitata* self-thinning data and the *Nephrops norvegicus* biometry data. (D3) 2000 plants/m^2, (D5) 5186 plants/m^2, (R) replicate. (TY) Tyrrhenian sea, (AD) Adriatic sea, (GE) Gulf of Euboikos, (LI) Ligurian sea, (CA) Catalan sea, (PO) Portugal and (AL) Alboran sea. For the Bootstrap method it is given the overlapped proportion of the 95% confidence intervals. Only when it is 0 (in bold) the slopes are significantly different. For the permutations method is given the significance of the differences with $p<0.05$ in bold and p close to 0.05 in grey bold.

	Bootstrap						**Permutations**					
Laminaria digitata data												
	D3 R2	D3 R3	D5 R4	D5 R1	D5 R2	D5 R3	D3 R2	D3 R3	D5 R4	D5 R1	D5 R2	D5 R3
D3 R1	1	0.492	0.391	**0**	**0**	**0**	0.493	0.249	0.212	**0.008**	**0.007**	**0.005**
D3 R2		0.871	1	**0**	**0**	**0**		0.706	0.597	**0.041**	**0.027**	**0.025**
D3 R3			0.901	0.071	**0**	0.010			0.842	**0.052**	**0.039**	**0.043**
D5 R4				**0**	**0**	**0**				**0.015**	**0.012**	**0.009**
D5 R1					0.707	1					0.413	0.757
D5 R2						0.714						0.584
Nephrops norvegicus data												
	AD	GE	LI	CA	PO	AL	AD	GE	LI	CA	PO	AL
TY	0.516	0.290	0.758	0.904	0.885	0.084	.0165	**0.059**	0.458	0.835	0.669	**0.007**
AD		**0**	0.212	0.412	0.356	**0**		**0.0004**	0.136	0.134	0.119	**0**
GE			1	0.516	0.426	0.836			0.483	0.130	0.139	0.629
LI				0.862	1	0.656				0.599	0.626	0.28
CA					1	0.288					0.846	**0.029**
PO						0.146						**0.054**

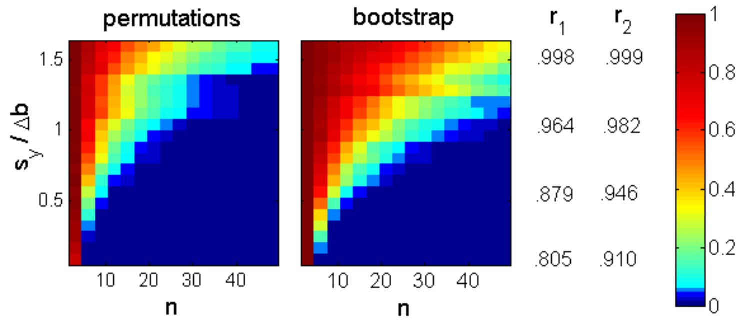

Fig. 1 Type 2 error probability tested on artificial data. (s_y) standard error of y, ($\Delta b = b_2 - b_1$) slope difference, (n) sample size and (r_x) regression coefficient for population x.

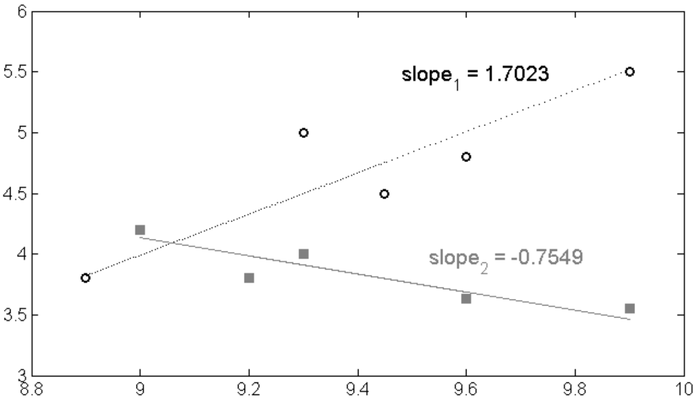

Fig. 2 Artificially generated populations of 5 elements.

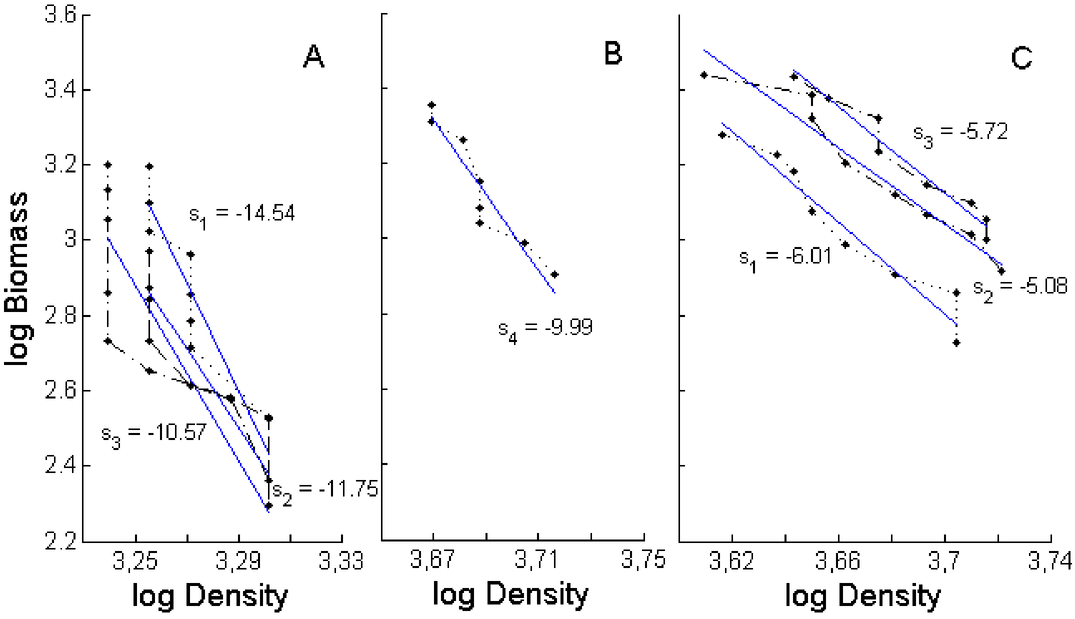

Fig. 3 Self-thinning of *Laminaria digitata* cultures with densities of (A) 2000 plants/m^2 replicates 1, 2 and 3, (B) 5186 plants/m^2 replicate 4 and (C) 5186 plants/m^2 replicates 1, 2 and 3. (s) slopes.

4 Discussion

Permutation tests have already been proposed to estimate the significance of slopes (Legendre, 2013) and partial regression coefficients (Anderson and Legendre, 1999) but not of differences between slopes. The latter has been determined from overlap of their bootstrapped slope probability distributions (Creed, 1995; Scrosati and DeWreede, 1997; Creed et al., 1998; Steen and Scrosati, 2004), following Sokal and Rohlf (1981). Sokal and Rohlf (1981; Chapter 14, section 8 box 14.9) present an algorithm to estimate significances of differences between slopes. They state (point 4) that “a pair of regression coefficients, b_i and b_j, is declared significantly different at the experimentwise error rate α if and only if their difference equals or exceeds the critical difference”. This is precisely the base of the permutations methodology proposed in the current work. Sokal and Rohlf (1981) propose three alternatives to estimate the critical difference. They require a quantity taken from the Student’s t-distribution with further data manipulation, the degrees of freedom and the standard error of the slopes for each population under the restriction these follow a normal distribution. Less data manipulation and assumptions would be desirable. Presently, the critical difference is estimated directly from many runs of permutation tests. In the 1980s powerful computers were not easily available and randomization methods were at their early stages. Consequently, in their following point 5, Sokal and Rohlf (1981) suggest the use of Gabriel's (1978) approximate method to avoid extensive recomputation of the critical difference when many statistical populations are present. Thus, two slopes are significantly different at α level if and only if their 1-α confidence intervals do not overlap. However, Gabriel (1978) is just an approximate method, developed for differences between means and not slopes and restricted to samples of equal sized. Furthermore, the confidence intervals must be extracted from a theoretical distribution of the slope derived from the Student's *t* of known sample size and standard error, and not from its bootstrapped empirical distribution. The approximation could be justifiable when computational abilities were week. But this is no longer the case. The last chapter in Sokal and Rohlf (1981) about "miscellaneous methods" presents randomization tests. At that time these were such a novelty that they were only considered divided into (i) *exact randomization* (ex: Jackknife) when it is feasible to calculate all possible outcomes and (ii) *sampled randomization test* (ex: bootstrap) when the number of possible outcomes is overwhelming. Their example of a *sampled randomization test* was actually a "primordial" permutation test applied to a data set of 25 ticks with scutum length measurements. Its variability supposedly reflected general genetic variability. The ticks were subject to a temperature shock and only 9 survived. The hypothesis was that variability of the scutum length was smaller in the surviving group as only a restricted portion of the genetic spectrum would survive. So, in their case variance was tested whereas here we tested slope. To compare both variances Sokal and Rohlf (1981) estimated their ratio whereas in the present case we compared the difference. Sokal and Rohlf (1981) argued their ratio did not follow the *F*-distribution as the two variances were not obtained from independent, normally distributed samples. So, they proposed a randomization test where the number of possible ways to select 9 ticks out of 25 is given by Newton's binomial (over 2 million). This corresponds to the permutation test design where both the surviving 9 and the dying 16 are randomly selected from the null hypothesis that all belong to the same homogeneous group of 25 ticks.

The present work demonstrated both bootstrap and permutations methods are robust to detecting false differences between slopes (i.e., false positives or Type 1 error) but unable to detect true differences (i.e., false negatives or Type 2 error) with large data scatter or very small samples. It was demonstrated with very small sample sizes (≤5) both methods are too vulnerable to the occurrence of odd combinations. Unfortunately, relatively small sample size is often the case of studies about self-thinning (Ellison, 1989; Rincón and Lóbon-Cerviá, 2002; Keeley, 2003) including those where a null hypothesis was accepted (Weiner and Whigham, 1988; Morris, 1996; Arenas and Fernández, 2000; Steen and Scrosati, 2004; Scrosati and DeWreede, 1997) raising the possibility of false negatives. With bigger samples the implications of choosing either method was well illustrated with the *Laminaria digitata* self-thinning data (Creed et al., 1998) and the *Nephrops norvegicus* biometry data (Castro et al., 1998). The present work demonstrated the bootstrap method had greater difficulties than the permutation method in detecting true differences. Even with samples sized 50 the bootstrap method still delivers results widely divergent from the permutation method, with a tendency to underestimate significances of honestly different slopes. Therefore, we recommend the permutation method to differentiate slopes.

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