How cryptic is cryptic diversity? Machine learning approaches to classifying morphological variation in the Pacific Pond Turtle (*Emys marmorata*)

# Introduction

Molecular systematics has repeatedly demonstrated the existence of cryptic species that can only be diagnosed using genetic data (Bickford et al. 2007; Schilck-Steiner et al. 2007; Stuart, Inger, and Voris 2006; Pfenninger and Schwenk 2007; Funk, Caminer, and Ron 2012; Clare 2011). In attempts to streamline the documentation of biodiversity, several methods of species delimitation that rely almost entirely on genetic data have recently been proposed (CITATIONS). Wheres strong caveats on the utility of these methods have been raised (Bauer et al. 2000; Carstens et al. 2013), they are already being used to name species (Leaché and Fujita 2010; Spinks, Thomson, and Bradley Shaffer 2014).

The majority of extant taxa, and almost all extinct taxa, are delimited by morphology alone. This disjunction complicates interpretations of variation and diversity in deep time, as apparent morphological stasis may not reflect the true underlying diversity (G. Hunt 2008; Eldredge and Gould 1972; Gould and Eldredge 1977; Van Bocxlaer and Hunt 2013). Similarly, for many museum specimens of extant taxa (e.g. those preserved in formalin), it is difficult to acquire genetic data to apply to species delimitation methods.

These considerations have sparked interest in whether geometric morphometric analysis can capture similar fine-scale variation that can be used for identifying cryptic species. Most such studies focus on morphometrics to discover differences between taxa that were identified by other means (Polly 2007; Demandt and Bergek 2009; Gaubert et al. 2005; Gündüz et al. 2007; Polly 2003; Zelditch, Swiderski, and Sheets 2004). Additionally, there has been a fair amount of work on automated taxon identification and classification of taxa into groups (Baylac, Villemant, and Simbolotti 2003; Dobigny et al. 2003; van den Brink and Bokma 2011; MacLeod 2007). In cases where genetic data are ambiguous or lacking for many samples, morphometric approaches could help identify cryptic species. This would make the task of identifying and maintaining endangered or conserved groups much easier and could contribute to improved classifications of extinct taxa and populations.

Here, we attempt to address this issue using machine-learning approaches. In particular, we ask whether it is possible to determine which amongst a set of classification hypotheses is best and examine the implications of the results for a recently proposed set of cryptic turtle species.

## Background and system

Machine learning is an extension of known statistical methodology (Hastie, Tibshirani, and Friedman 2009) that emphasizes high predictive accuracy and generality at the expense of the interpretability of individual parameters. The basic statistical mechanics are supplemented by randomization, sorting, and partitioning algorithms and along with the maximization or minimization of summary statistics, in order to best estimate a general model for all data, both sampled and unsampled (Hastie, Tibshirani, and Friedman 2009). Machine learning approaches have found use in medical research, epidemiology, economics and automated image identification such as handwritten zip codes (Hastie, Tibshirani, and Friedman 2009). The two major classes of machine learning methods are unsupervised and supervised learning. Unsupervised learning methods are used with unlabeled data where the underlying structure is estimated and are analogous to clustering and density estimation methods (Kaufman and Rousseeuw 1990). Supervised learning methods are used with labeled data where the final output of data is known and the rules for going from input to output are inferred. These are analogous classification and regression models (Breiman et al. 1984). The application of the alternative approaches used in this study illustrates only a sampling of the various previously derived methods for clustering observations and fitting classification models.

Geometric morphometric approaches to identifying differences in morphological variation between different classes, including cryptic species, mostly have used methods like linear discriminate analysis and canonical variates analysis (Zelditch, Swiderski, and Sheets 2004; Mitteroecker and Bookstein 2011; Polly 2007; Polly 2003; Gündüz et al. 2007; Gaubert et al. 2005; Demandt and Bergek 2009; Sztencel-Jabłonka, Jones, and BogdanowicZ 2009; Mitrovski-Bogdanovic et al. 2013; Francoy et al. 2009). These methods are comparatively straightforward ways of understanding the differences in morphology between classes given their similarity to familiar multivariate approaches like principal components analysis (PCA). They are benefit by producing results that can be easily visualized, which aids in the interpretation and presentation of data and results. Most previous morphometric studies did not assess which amongst a set of alternative classification hypotheses was optimal. For example, studies such as those of Caumul and Polly (2005) and Polly (2007) focused on comparing different aspects of morphology and their fidelity to a classification scheme instead of comparing the fidelity of one aspect of morphology to multiple classification schemes. In this context, the study of Cardini et al. (2009), is noteworthy because they compared morphological variation in marmots at the population, regional, and species level and determined the fidelity of shape to divisions at each of these levels.

Here, we used multiple machine learning methods, both unsupervised and supervised, in order to compare different classification hypotheses. These methods provide different and unique advantages for understanding how to classify taxa, and with what accuracy. While machine learning methods such as neural networks have been applied to studying shape variation (Baylac, Villemant, and Simbolotti 2003; Dobigny et al. 2003; van den Brink and Bokma 2011; MacLeod 2007), including in the context of automated taxon identification and classification of groups, the number of cases remains limited. In the current study, we not only consider pure classification accuracy but also use a statistic of classification strength that reflects the rate at which taxa are both accurately and inaccurately classified.

## *Emys marmorata*

We analyzed the problem of whether there are distinct subspecies or cryptic species exist within the western pond turtle, *Emys marmorata* (formerly *Clemmys marmorata*; see (Feldman and Parham 2002)). *E. marmorata* is distributed from northern Washington State, USA to Baja California, Mexico. Traditionally, *E. marmorata* was classified into two named subspecies: the northern *E. marmorata marmorata*, the southern *E. marmorata pallida* (Seeliger 1945), while recognizing a central Californian intergrade zone between these subspecies. *Emys marmorata marmorata* is differentiated from *E. marmorata pallida* by the presence of a pair of triangular inguinal scales and darker neck markings. It should be noted that the triangular inguinal plates can sometimes be present in *E. marmorata pallida* though they are considerably smaller.(Seeliger 1945) did not formally include the Baja California populations of *E. marmorata* in either taxon, implying the existence of a third distinct but unnamed subspecies.

Previous work on morphological variation in *E. marmorata* has focused primarily on differentiation between populations over a portion of the species’ total range (Lubcke and Wilson 2007; Germano and Bury 2009; Germano and Rathbun 2008; Bury, Germano, and Bury 2010); comparatively few studies have included specimens from across the entire range (Holland 1992). Most of these studies considered how local biotic and abiotic factors may contribute to differences in carapace length and found that size can vary greatly between different populations (Lubcke and Wilson 2007; Germano and Bury 2009; Germano and Rathbun 2008). There also has been interest in size-based sexual dimorphism in *E. marmorata* (Lubcke and Wilson 2007; Germano and Bury 2009; Holland 1992), with males being on average larger than females based on total carapace length and other linear measurements. However, the quality of size as a classifier of sex can vary greatly between populations (Holland 1992), because of the amount of size differences among populations (Lubcke and Wilson 2007; Germano and Bury 2009). However, the effect of sexual dimorphism on shape, *sensu* David G. Kendall (1977), has not been assessed (Holland 1992; Lubcke and Wilson 2007; Germano and Rathbun 2008).

Of particular importance in the context of cryptic diversity in *E. marmorata* is the morphometric analysis of carapace shape carried out by Holland (1992), who compared populations of *E. marmorata* from three areas of its range. This study concluded that geographic distance was a poor indicator of mophological differentiation, and instead geographic features such as breaks between different drainage basis are probably more important barriers to reproduction. Additionally, (Holland 1992) suggested that morphological differences were more observable as the magnitude of barriers and distance increased, but the variation required many variables to adequately capture, implying only very subtle morphological differentiation between putatively distinct populations. That study concluded that *E. marmorata* is best classified as three distinct species: a northern species, souther species, and a Columbia Basin species. This classification is similar to that of Seeliger (1945), except elevated to the species level and without recognition of a distinct Baja species.

More recently, the phylogeography of *E. marmorata* and the possibility of cryptic diversity was investigated using molecular data (Spinks and Shaffer 2005; Spinks, Thomson, and Shaffer 2010; Spinks, Thomson, and Bradley Shaffer 2014). Based on mitochondrial DNA, Spinks and Shaffer (2005) recognized four subclades within *E. marmorata*, a northern clade, a San Joaquin Valley clade, a Santa Barbara clade, and a southern clade. Analyses with nuclear DNA (Spinks, Thomson, and Shaffer 2010) with single-nucleotide polymorphism (SNP) data suggest a primarily north–south division in *E. marmorata*, although the dataset differs in the location of this break point. These studies discussed the potential taxonomic implications of their results, with Spinks, Thomson, and Bradley Shaffer (2014) going so far as to strongly advocate for the recognition of at least two species (*E. marmorata* and *E. pallida*), and a possible third based on populations in Baja California. However, they did not discuss in detail the morphological characters that would help to diagnose these species beyond those specified by Seeliger (1945). Given that these characters are somewhat variable within the proposed species, and that Holland (1992) described shell shape variation that might be consistent with this taxonomy, a geometric morphometric analysis of shell shape might provide a reliable way to diagnose groups (whether species or subspecies) within *E. marmorata*.

In this study, we attempt to estimate the best classification scheme of *E. marmorata* based on variation in plastral (ventral shell) shape in order to determine whether this character is consistent with any of the past divisions based on other morphological features or molecular data. We use the plastron …

Because of unclear geographic boundaries between subgroups of *E. marmorata*, we compare multiple hypotheses of morphologically– and molecularly–based classification. We hypothesize that if morphological variation corresponds to class assignment, then it should be possible to determine the best classification hypothesis of *E. marmorata* from amongst multiple candidate hypotheses. However, if morphological variation does not correspond to any of the standing hypothesis, then supervised learning model generalization performance will be poor.

# Materials and Methods

## Specimens, sampling, morphometrics

We collected landmark-based morphometric data from 354 adult *E. marmorata* museum specimens. These specimens are a subset of those included in Angielczyk and Sheets (2007), Angielczyk, Feldman, and Miller (2011), and Angielczyk and Feldman (2013) and represents adult individuals. We chose to focus on adults because significant changes in plastron shape occur over the course of ontogeny in *E. mamorata* and other emydines (Angielczyk and Feldman 2013).

We assigned a classification to each specimen for the different binning schemes based on geographic occurrence data recorded in museum collection archives. When precise latitude and longitude information were not available we estimated them from locality information. Because the specimens sampled to obtain the genetic data used to define the subclades were not available for study, all specimen classifications were based solely on the geographic information, not explicit assignment in previous studies. Because the exact barriers between different biogeographic regions are unknown and unclear, we represented each hypothesis with two different schemes; we compared a total of six different schemes. The schemes differed based on where geographic boundaries were assigned. This changes how certain individuals were assigned two one of the groups within in hypothesis such as which of the three morphologically defined groups, which of the four mitochondrially defined groups, and so on. Following previous work on plastron variation (Angielczyk and Sheets 2007; Angielczyk, Feldman, and Miller 2011; Angielczyk and Feldman 2013), we used TpsDig 2.04 (Rohlf 2005) to digitize 19 landmarks (Fig. [fig:plastra]). Seventeen of the landmarks are at the endpoints or intersection of the keratinous plastral scutes that cover the platron. Twelve of the landmarks were symmetrical across the axis of symmetry and, in order to prevent issues surrounding degrees of freedom and other similar concerns (Klingenberg, Barluenga, and Meyer 2002), we reflected these landmarks across the axis of symmetry (i.e. midline) prior to analysis and used the average position of each symmetrical pair. In cases where damage or incompleteness prevented symmetric landmarks from being determined, we used only the single member of the pair. We conducted all subsequent analyses on the resulting “half” plastra. We superimposed the plastral landmark configurations using generalized Procrustes analysis (Dryden and Mardia 1998), after which, we calculated the principal components (PC) of shape using the shapes package for R ([CSL BIBLIOGRAPHIC DATA ERROR: reference "2013" not found.]; [CSL BIBLIOGRAPHIC DATA ERROR: reference "Dryden2013" not found.]).

## Machine learning analyses

### Unsupervised learning

In order to preserve the relationship between all landmark configurations in shape space, we measured the dissimilarity between observations using Kendall’s Riemannian shape distance or (D.G. Kendall 1984; Dryden and Mardia 1998). We chose this metric because shape space, or the set of all possible shape configurations following Procrustes superimposition, is a Riemannian manifold and thus non-Euclidean (Dryden and Mardia 1998). varies between 0 and when there is no reflection invariance, which should not be a concern in the case of the half plastral landmark configurations used in the study.

We divisively clustered the shape dissimilarity matrix using partitioning around mediods clustering (PAM), a method similar to *k*-means clustering except that instead of minimizing the sum of squared Euclidean distances between observations and centroids, the sum of squared dissimilarities between observations and mediods is minimized (Kaufman and Rousseeuw 1990). Because the optimal number of clusters of shape configurations in the study was unknown, being possibly three, four, or some other value, we estimated clustering solutions in which the number of clusters varied between one and eight. We compared clustering solutions using the gap statistic, which is a measure of goodness of clustering (Tibshirani, Walther, and Hastie 2001).

We conducted this analysis using the cluster package for R ([CSL BIBLIOGRAPHIC DATA ERROR: reference "Maechler2013" not found.]).

### Supervised learning

We used three different supervised learning, or classification, approaches: linear discriminate analysis, multinomial logistic regression, and random forests. Linear discriminate analysis, also known as canonical variate analysis, is commonly used in studies of geometric morphometric data (Zelditch, Swiderski, and Sheets 2004; Mitteroecker and Bookstein 2011). The other two methods, however, are not. In all cases, the optimal number of PCs used as predictors was chosen via maximum within-sample AUC value, explained below.

Linear discriminate analysis (LDA) attempts to find a linear combination of predictors that best model two or more classes. LDA is very similar to PCA except that instead of finding the linear combination of features that maximize the amount of explained variance in the data, LDA maximizes the differences between classes. The results of this analysis produces a transformation matrix by which the original features can be transformed to reflect the best discrimination between the classes. In this study, we applied LDA to the eigenscores from a subset of the total number of PCs, ranging from two to 6 in increasing order of complexity. In total, this produced nine different LDA scaling matrices.

Multinomial logistic regression is an extension of logistic regression, where instead of a binary response there are three or more response classes (Venables and Ripley 2002). Similar to the odds ratios calculated from the coefficients of a logistic regression, the relative risk of a classification can be determined from the coefficients of the model.

Random forest models are an extension of classification and regression trees (CART) (Breiman et al. 1984; Breiman 2001). The goal of CARTs is to use a series of different features (i.e. predictors) to estimate the class of an observation. In top-down induction of decision trees for each member of a given set of predictor variables, attribute value tests are used to estimate the differences between classes. This process, called recursive partitioning, is then repeated on each subset. The recursion continues until the resulting observations all share the same class or no more meaningful partitions are possible. The resulting model is a tree structure by which observations are classified at each intersection via the estimated cutoff points from the attribute tests made during model fitting. In a random forest model, many CARTs are built from a random subsample of both the features and the observations (specimens). This process is then repeated many times and the parameters of the final model are chosen as the mode of estimates from the distribution of CARTs (Breiman 2001). In addition to classifying the observations, this procedure allows for the features to be ranked in order of importance. This is a generally useful property for studies in which the goal is to describe and model the differences between classes and the relative importance of different predictors.

In this analysis, we used 1000 subtrees to estimate the random forest model parameters. We estimated the best set of predictors necessary for each classification scheme was estimated using a recursive feature selection algorithm, and we chose the optimal number of PCs to include based on the AUC of the model. Following the backwards selection algorithm implemented in caret ([CSL BIBLIOGRAPHIC DATA ERROR: reference "KuhnMAN2013" not found.]), the maximum number of features were included in the initial model, their importance ranked, and the AUC of the model calculated. The lowest ranked feature was then removed, and the AUC of the model recalculated. This was repeated until only one feature, remained. Because PCs were kept in order of importance and not in relation to the amount of variance each PC described, these means that the PCs are not included in the order of ascending eigenvalue.

In classification studies, such as this one, a common metric of performance is area under the receiver operating characteristic curve (AUC). AUC is an estimate of the relationship between the false positive and true positive rates, as opposed to just the true positive rate (accuracy). This relationship is especially useful in cases where misclassification needs to be minimized just as much as accurate classification, as in this study. AUC ranges between 0.5 and 1, with 0.5 indicating classification no better than random and 1 indicating perfect classification (Hastie, Tibshirani, and Friedman 2009).

The standard AUC calculation is defined for binary classifications, however in this application there are multiple categories. The alternative calculation that we used follows an all-against-one strategy where the individual AUC values for each class versus all others are averaged to produce a multiclass AUC (Hand and Till 2001). To estimate confidence intervals on the out-of-sample AUC values, we performed a nonparametric bootstrap in which the true and estimated classifications were resampled with replacement. This was done 1000 times.

The ultimate measure of model fit is accurately predicting the values of unobserved samples (Hastie, Tibshirani, and Friedman 2009; Kuhn and Johnson 2013). Within-sample performance is inherently biased upwards, so model evaluation requires overcoming this bias. With very large sample sizes, as in this study, part of the sample can be used as the “training set” and the remainder acts as the “testing set.” The former is used for fitting the model where as the later is used for measuring model performance, and this process is called model generalization. In this analysis, we used 75% of samples as the training set while the remaining 25% were used as the testing set.

It is common for some out of sample observations to be misclassified. This misclassification may be due to the model not accurately representing shape variance, systematic differences between the training and test sets, or systematic differences between the accurately and inaccurately classified samples. Testing and training sets are determined completely at random within each class and with respect to shape. Results were not effected by changes in testing or training set assignment.

To determine if there were systematic differences between the correctly and incorrectly classified samples, we used a permutation test to estimate if the dissimilarity between the correctly and incorrectly classified individuals were significantly different from random. The group labels were permuted 1000 times and the distance between the new centroids was calculated. The number of permutations less than the empirical difference divided by 1000 gives a *p*-value for the test. Significant results indicate that correctly and incorrectly classified specimens are systematically different. This was done only for classes where there were 10 or more observations.

# Results

## Unsupervised learning

Comparison of gap statistic values from PAM clustering show that the optimal, minimal number of clusters is most likely one (Fig. [fig:gap]). There is some ambiguity in choice because, although it is not statistically different from a solution with only one group, the solution with two groupings does have the greatest mean gap statistic. However, there is no geographical signal in the results of this clustering solution (Fig. [fig:gapmap]). Because of this, we assert that this means that there is no means of naturally partitioning plastron shape into distinct subgroups with out reference to external information.

## Supervised learning

AUC–based model selection revealed some important patterns of variation and congruence between the classification schemes and the actual data. Generally, the best performing models tended to include as many PCs as possible [fig:sel]). Note that the best random forest models were determined via recursive feature selection, so PCs were not included in order of percent variance explained. That almost all LDA and multinomial logistic regression models were as complex as possible indicates that the differences between the different groups within each classification scheme are very small.

As part of fitting a random forest model, a ranking of variable importance also is determined. Interestingly, the order of variable importance is not the same as the order of the PCs ([fig:varimp]). This means that the variance describing the differences between the classes does not align with the major axes of variance (i.e. the PCs). This result would be the case if variation between classes was extremely fine grained and not a part of the principal form or function of the plastron, which makes sense given that the plastron is involved in both protection and hydrodynamics and not necessarily mate choice (Germano and Bury 2009; Holland 1992; Lubcke and Wilson 2007; Rivera 2008). Moreover, this result is congruous with the results from the AUC–based model selection for the multinomial logistic regression and LDA models.

Observed AUC values for all of the optimal models are not exceptionally high ([fig:sel]). In most cases the different proposed classification schemes are generally poor descriptors of the observed variation. It appears that the data set is overwhelmed by noise, making any accurate classifications difficult at best. This observation is cemented with the generalizations of the models to the testing data set ([fig:genhist]).

Mean AUC values for the model generalizations, in most cases, are approximately equal to the observed AUC values from the training data set (Table [tab:comp]). The cases in which the AUC from the generalizations is less than the observed, indicate poor model fit and a poor classification scheme. AUC values from model generalization, or estimating testing data set membership, does not indicate a clear “best” classification scheme (Fig. [fig:genhist]). Although the scheme with two species has the greatest AUC point estimate for each modeling approach, this scheme is not significantly greater than any other except in some limited cases (e.g. LDA, Table [tab:gentests]). Differences in mean shape between correctly and incorrectly classified observations from test set frequently were statistically significant, though there are exceptions. Again, this test was to determine if the mean shapes were statistically different or not. The frequency of these results, however, is important because it means that the different models are poor predictors of class membership. This may be because differences in plastron shape do not align with the any of the hypothesized classification schemes.

# Discussion

The results of this study indicate that there is no clear grouping of *E. marmorata* based on plastron shape.

The unsupervised learning results indicate only a single group of observations being optimal is congruous with the results from the generalizations of the supervised learning models. The classification schemes used in the supervised learning models correspond, loosely, to unsupervised learning solutions with multiple groups. Because unsupervised learning solutions with multiple groups are poor descriptors of the observed variation, it is important to see this generally supported by the supervised learning results.

The results from fitting the various supervised learning models to each of the classification scheme generally shows that no one scheme is “best.” Possible explanations include that the genetic differentiation is not associated with plastral change and/or that local selective pressures (e.g. from hydrological regime) ocerwhelm morphological differentiation. Another possibility (explored below) is that the classification schemes themselves do not represent significant evolutionary lineages.

Both the low AUC values () and the significant difference between the correctly and incorrectly classified observations support the conclusion that none of the hypothesized classification schemes are good descriptors of the observed plastral variation.

## Is there more than one species of Pacific Pond Turtle?

The lack of morphological support for the distinctiveness of *E. pallida* does not, on its own, preclude the recognition of this taxon. However, this apparent lack of congruence does prompt a reexamination of the methods and concepts that led to that taxonomic revision. In other words, before we can assess the significance of the non-diagnosablity, it is essential to evaluate the methods and concepts that led to taxonomic revision. Spinks, Thomson, and Bradley Shaffer (2014) elevated *E. pallida* based on a Bayesian species delimitation analysis of SNP data using BPP (Yang and Rannala 2010). However, Spinks, Thomson, and Bradley Shaffer (2014) did not heed the caveats about species delimitation methods raised by Carstens et al. (2013). In addition to specifically addressing the shortcomings of validation methods such as BPP that rely on guide trees and so “should be interpreted with caution,” Carstens et al. (2013) also strongly emphasize that “Inferences regarding species boundaries based on genetic data alone are likely inadequate, and species delimitation should be conducted with consideration of the life history, geographical distribution, morphology and behaviour (where applicable) of the focal system…” These caveats evoke the development of the Unified Species Concept (Dayrat 2005; De Queiroz 2007) and Integrative Taxonomy CITATIONS (Padial 2010), and other pluralist approaches to species delimitation. None of these considerations were brought to bear on the *E. marmorata* system until now, and in doing so we find the proposal that *E. pallida* is a distinct species to be lacking in several aspects. For one, the natural history and geographical distribution of E. marmorata make the recognition of this taxon implausible. The data from Spinks, Thomson, and Bradley Shaffer (2014) show extensive introgression and admixture in Central California, which makes sense because there are no significant barriers to gene flow in this region. Combined with the well-demonstrated ability for testudinoid turtles, including emydids and even *Emys*, to hybridize (e.g. Buskirk, Parham, and Feldman (2005; Spinks and Shaffer 2009; Parham et al. 2013)) it is hard to imagine how *E. marmorata* and *E. pallida* could maintain their integrity. Because the geography, natural history, and demonstrated genetic admixture of *E. marmorata* conflict with the recognition of *E. pallida*, it is likely that the inability to classify the morphological data by proposed species is because *E. pallida* is not a real species. We agree with Carstens et al. (2013) that “the inferences drawn from species delimitation studies should be conservative, for in most contexts it is better to fail to delimit species than it is to falsely delimit entities that do not represent actual evolutionary lineages.” Although we do not consider “*E. pallida*” to be a valid species, we do recognize that the genetic analysis of Spinks, Thomson, and Bradley Shaffer (2014) are extremely powerful and useful for delineating an Evolutionary Significant Unit or Distinct Population Segment that should be included in conservation management plans.

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