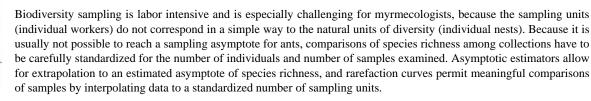# Counting ants (Hymenoptera: Formicidae): biodiversity sampling and statistical analysis for myrmecologists

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**Abstract**

Biodiversity sampling is labor intensive and is especially challenging for myrmecologists, because the sampling units (individual workers) do not correspond in a simple way to the natural units of diversity (individual nests). Because it is usually not possible to reach a sampling asymptote for ants, comparisons of species richness among collections have to be carefully standardized for the number of individuals and number of samples examined. Asymptotic estimators allow for extrapolation to an estimated asymptote of species richness, and rarefaction curves permit meaningful comparisons of samples by interpolating data to a standardized number of sampling units.

Winkler sacks of leaf litter and specialized traps for arboreal ants often yield distinctive sets of species in tropical and subtropical latitudes. These microhabitats are best treated as distinctive assemblages that can be compared with stratified sampling. Within other habitat types, different baiting and sampling methods yield similar, but not identical sets of species, and many authors have advocated using a diversity of methods to gain the greatest coverage of species. However, many of the distinctive species that are sampled by a particular method are rare, and are just as likely to have been found with other sampling methods. The estimated similarity in composition of ants sampled by different methods in the same habitat is probably greater than has been appreciated. Recent published comparisons of sampling efficiency have shown that hand collecting accumulates species more efficiently than the more commonly used pitfall traps or baits. However, if hand-collected samples are to be analyzed quantitatively, hand sampling must be standardized to a constant plot size that is searched for a fixed amount of time, and all nests encountered must be sampled and counted. For comparative studies of ant assemblages across habitats, hand collecting may be superior to either litter sampling or pitfall traps because it can be used in sites that have no leaf litter or are too environmentally sensitive, too rocky, too steep, or contain too much human and domestic animal traffic for pitfall trapping.

Data from hand sampling and grids of pitfall traps lend themselves to slightly different kinds of analyses. Hand sampling probably gives the best estimates of the true frequency of nests of different species in an area, and the data can be used with individual-based rarefaction curves and many standard parametric statistical tests. Data from pitfall-trap grids should usually be treated as sample-based occurrences, and analyzed with sample-based rarefaction and diversity estimators. For the purposes of estimating diversity, myrmecologists should avoid the temptation to analyze the underlying counts of individual workers. Data from replicated grids of pitfall traps can be analyzed with hierarchical occupancy models. These newly developed models provide useful estimates of probabilities of occurrence and probabilities of detection for each species in the assemblage. Biogeographic patterns of ant diversity, such as latitudinal gradients of species richness, are comparable to those seen in other taxa, and the data collected by myrmecologists can contribute in important ways to our general understanding of biodiversity patterns.

**Key words:** Biodiversity sampling, rarefaction, species richness, Winkler sacks, leaf litter, pitfall traps, review.

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## Climbing the species accumulation curve

Biodiversity sampling is a basic activity in community ecology, but it is time-consuming and labor-intensive (LAWTON & al. 1998). Technological advances continue to make genomic and proteomic screening cheaper and faster each year (MAYR & FUERST 2008), but methods of collecting invertebrates in the field have changed little in over a cen-

tury and remain very costly in terms of time and effort. Myrmecologists in particular are very familiar with a diversity of sampling methods, and have struggled to develop specific protocols for efficiently (and thoroughly) sampling ant assemblages (BESTELMEYER & al. 2000).

In this essay, we compare sampling methods used for ants, discuss appropriate statistical analyses of the data, and clarify the kinds of questions we can answer, given the current methods of sampling and analysis. One important goal of sampling is the generation of a complete species list for a location, along with an unbiased estimate of the relative abundance of each species (LONGINO & COLWELL 1997). In theory, this can be done by constructing a species accumulation curve in which the *x*-axis is the number of samples (or individuals) collected, and the *y*-axis is the number of species accumulated. This curve rises very rapidly in the beginning, as most of the common species are detected early in a survey. It continues to rise with more sampling, but at a slower rate, because rare species are encountered infrequently. Finally, with enough effort, the asymptote of the curve is reached, and additional sampling yields no further species (GOTELLI & COLWELL 2001).

Unfortunately, we almost never reach that asymptotic endpoint, particularly for ants and other invertebrates. In one famous example, new species records (and in some cases undescribed species) continue to be found at the La Selva Biological Station in Costa Rica, even after 30 consecutive years of arthropod sampling (LONGINO & al. 2002). John Longino (pers. comm.) humorously speculates that this accumulation will never end, and he will eventually record all Neotropical ant species at La Selva! A temperate zone example is provided by ESPADALER & LÓPEZ-SORIA (1991). Over a 5-year period, these authors sampled a small urban garden (400 m$^2$) in Barcelona, Spain, and accumulated records of 23 nesting species, 7 hypogaeic or parasitic species that probably nested, and an additional 10 species from nearby. This high level of ant diversity, including many rare species, suggests that ant species lists for most parts of the world may be very incomplete.

Even in subtropical and temperate locations, the additional sampling effort necessary to collect all of the undetected species in an area is often 1 to 10 times greater than the original sampling effort (CHAO & al. 2009). Consequently, ecologists must extrapolate the species accumulation curve to account for incomplete sampling. *Extrapolation* means extending the species accumulation curve and using the existing data to estimate what the asymptote would be if sampling were to continue indefinitely. To date, the most useful indices used to extrapolate the species accumulation curve have been a class of non-parametric asymptotic estimators (CHAO 1984). These indices use information on the frequency of rare species – those represented by only one ("uniques") or two ("duplicates") incidences in the collection – to yield a minimum estimate (and a confidence interval) for the asymptote (COLWELL & CODDINGTON 1994). These indices can be extended to use information on the full range of incidence classes in the data set (CHAO & LEE 1992, COLWELL & CODDINGTON 1994). The choice of an optimal index depends in part on species evenness and sampling intensity (BROSE & al. 2003).

In contrast to extrapolating species richness for a single assemblage, comparison of species richness of two or more assemblages requires an adjustment for differences in the number of samples or individuals collected from the different assemblages. This adjustment must be done by *interpolation*. By taking random subsets of samples or individuals, the species accumulation curve can be extended back towards the origin. This approach – known as rarefaction (SANDERS 1968) – allows investigators to compare species richness of two or more collections at comparable levels of abundance or sampling effort. Both asymptotic estimators and rarefaction are now standard statistical tools for the estimation and comparison of biodiversity (COLWELL & CODDINGTON 1994, GOTELLI & COLWELL 2001), and free, user-friendly software (CHAO & SHEN 2003, COLWELL 2009, GOTELLI & ENTSMINGER 2009) is available that performs these calculations.

## Seeing the forest for the leaves

It is one thing to discuss reliable statistical methods for estimating biodiversity from small samples. It is another thing entirely to deal with such methods in the context of real field data. Many obstacles arise when we attempt to apply rarefaction and asymptotic estimators to ant diversity data as they are typically collected. For example, a standard comparison of diversity in two forests might rely on counts of individual ants from 25 pitfall traps in each forest. These counts certainly can be used to calculate asymptotic estimators and to fit rarefaction curves. However, there is a fundamental problem with this method of analysis: It assumes that the individuals in a collection are randomly and independently sampled, like snacking on a small handful of jelly beans drawn from a large, well-mixed candy jar (LONGINO & al. 2002, GOTELLI & COLWELL 2010). But that is certainly not the case for pitfall catches. When we find a single pitfall trap that is stuffed with hundreds of workers of a single species, it is most likely the case that a single nest entrance is present nearby that has supplied all of the workers. The raw count of the number of workers may dramatically overestimate the abundance of colonies of some species. This inflation is a problem even if between-species differences may average themselves out over a large number of pitfall traps. Conventional statistical methods (such as contingency-table analysis) that treat each individual as an independent observation will have artificially inflated degrees of freedom, and the resulting P-values will not be reliable.

In some ecological contexts, ant abundance in pitfalls may still be a useful measure, especially if the investigator is trying to estimate ant biomass, or perhaps the collective ecosystem services (such as seed dispersal or soil turnover) that are contributed by a species. But for the estimation of biodiversity, the small-scale patchiness introduced by treating individual workers as independent observations can cause problems.

For many ecological questions, the individual workers sampled in pitfall traps do not represent the true units of ant biodiversity. Instead, we should be sampling independent colonies. It is as though we wanted to survey tree diversity in a forest, but we could not count individual trees directly. Instead, we could only sample leaves from the forest floor, which are analogous to workers caught in traps. The frequency of individual workers in pitfall traps may be related to the density of nests, although there seem to be few published empirical data demonstrating the strength of this relationship (SCHLICK-STEINER & al. 2006).

ELLISON & al. (2007) censused ants in 18 plots of a 10 ha tract of mature red-oak forest in the northeastern USA with both replicated transects of 10 pitfall traps per plot and hand searching for nest entrances, allowing for a direct comparison of the two methods. For the 28 species detected by hand searching, total pitfall catches were indeed a highly significant predictor of nest density per species (Fig. 1; $r^2 = 0.58$; $P = 8 \times 10^{-7}$). The data points for the three most common species (*Aphaenogaster rudis* species complex [139 workers, 40 nests], *Formica neogagates* [103 workers, 27 nests], and *Myrmica punctiventris* [57 workers, nine nests]) fall nicely near the fitted regression line. However, as in most assemblages, the remaining 25 species collected were uncommon or rare (< 20 workers total in pitfall catches), and the significant correlation disappears once the top three species are removed from the analysis ($r^2 = 0.09$; $P = 0.08$).

A partial solution to this problem is to avoid using counts of individual workers in the analysis. Instead, the analysis can be based on the number of *samples* (or the number of occurrences; LONGINO & al. 2002) in which a species was represented (regardless of its abundance). Both rarefaction and asymptotic estimators have been developed for use with sample-based data (GOTELLI & COLWELL 2010). The issue here is that the samples need to be sufficiently separated in time or space so they can be treated as independent observations. However, the distance of separation that is required may well vary between species: Pitfall traps separated by 2 m may be effectively independent for a tiny *Temnothorax* species nesting in a single acorn, but not independent for a large-bodied, relatively wide-ranging *Camponotus* species. Because we are unlikely to know either the effective sampling range of a particular pitfall trap or the foraging ranges and traveling distances of all the species in an assemblage, we can only use our intuition to arrive at a proper spacing of sampling units. Nevertheless, the use of sample-based methods at least avoids the most egregious problems that arise from treating individual workers as if they were independent observations.

## Diverse sampling methods for diverse ants

Although one goal of collecting can be to develop a comprehensive species list for a single site ("strict inventory"), sampling also is often done with an eye toward comparative analyses of different habitats or geographic regions (LONGINO & COLWELL 1997). The wide variety of methods that are used for collecting ants raises additional complications when we want to compare results across habitats or sites. For comparing assemblages across habitats or sites, it is more useful to use a single, standardized method that can be used effectively in many different habitats, even if it is not the optimal method to use in any particular habitat (STEINER & al. 2005). For example, many myrmecologists favor Winkler sacks of leaf litter (FISHER 1999, IVANOV & al. 2010), but of course this sampling method can only be used in forests and shrubland, and cannot be used to estimate ant diversity in grassland, desert, tundra, wetland, and agricultural habitats, which collectively comprise the majority of the earth's surface.

The challenge is that different methods differ widely in their costs and efficiency, no single method will provide a comprehensive sampling, and each method has its own par-
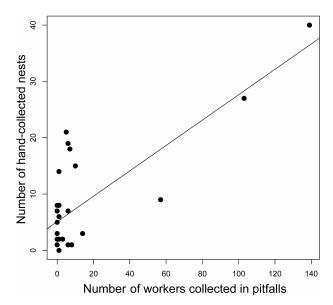


Fig. 1: Relationship between total pitfall catch and measured nest density for 28 species of ground-dwelling ants in a northeastern US forest. The solid line is the fitted least-squares regression ($r^2 = 0.58$; $P = 8 \times 10^{-7}$). Data from ELLISON & al. (2007).

ticular biases and oddities. These sampling biases have been appreciated for some time. For example, pitfall traps and baits do not catch a "random" sample of ants in a particular location. Instead, small pitfall traps appear to be biased against large-bodied species (ABENSPERG-TRAUN & STEVEN 1995), and the number and composition of ants collected in pitfall traps is even affected by whether the trap contains water or ethylene glycol (CALIXTO & al. 2007). Species composition from Winkler sacks of leaf litter can be affected by the amount of moisture present in the soil (DELSINNE & al. 2008), and resource baits (either protein or carbohydrate) may be biased in favor of behaviorally dominant species that sequester and defend these food resources. The composition of ants that visit baits can also change diurnally (TAVARES & al. 2008).

However, in one interesting application, a popsicle stick dipped in peanut butter was given to every child on the big island of Hawaii (which has no native ant species), and the children had to report back what they found. This cheap (but child-labor-intensive) method yielded 26 species of ants (two of which had never been recorded before), characterized the distribution of the invasive Argentine ant, *Linepithema humile*, in Kona and Hilo, and revealed an isolated population of the tramp species *Wasmannia auropunctata* that was successfully eradicated (ANTWATCH 2010). Although all sampling methods have their biases, large numbers of standardized samples collected over a substantial geographic area are bound to yield useful data, as this peanut-butter assay demonstrates.

Comparative studies have repeatedly found that, when different sampling methods are used in the same habitat, each of them will contain a few unique species that are not represented in any other sampling method (LONGINO & COLWELL 1997). For this reason, a common recommendation is a structured inventory in which a diversity of methods is simultaneously used to yield the most species-rich inventory (BESTELMEYER & al. 2000, KING & PORTER 2005,

GROC & al. 2007, LOPES & VASCONCELOS 2008). For example the ALL (Ants of Leaf Litter) protocol is a set of standardized methods using Winkler sacks and pitfall traps for sampling ground-dwelling ants (AGOSTI & ALONSO 2000). Using multiple methods to sample ants at a single location of course has the important downside of increasing time, labor, and expense.

However, species that show up in only a single kind of trap are often rare, and rare species present major statistical challenges (DIXON & al. 2005). There is no reason to assume that the unique species captured with one particular sampling method (e.g., pitfall traps) would not have been also captured with another sampling method (e.g., hand collecting). Three identical grids of pitfall traps each might yield a small set of "unique" species represented only once, just as we would see in a comparison of three single grids of pitfall traps, tuna fish baits, and cookie baits.

## Comparing the similarity of ant species sampled by different methods

How similar should the lists of ant species (and their abundances) derived from different collecting methods be before we conclude that these methods are sampling the same fauna? Two recent studies approached this question quantitatively. KING & PORTER (2005) used the familiar Jaccard index to compare the pair-wise similarity of ants collected with pitfall traps, baits, hand sampling, and Winkler sacks from central Florida USA uplands. Jaccard's index is calculated as $J = a / (a + b + c)$, where $a$ is the number of species in common to both collections and $b$ and $c$ are the number of species uniquely represented in each of the collections. Jaccard's index ranges from $J = 0.0$ (no shared species in two collections) to $J = 1.0$ (identical composition of two collections). KING & PORTER (2005: tab. 3) calculated similarity indices for the different methods ranging from 0.38 to 0.60. In a study of ant diversity of the Great Smoky Mountains (southeastern USA), LESSARD & al. (2007: tab. 3) also compared species lists from Winkler sacks, pitfall traps, and Malaise traps, and calculated similarities from 0.39 to 0.70. Both studies concluded that at least some of the different sampling methods revealed differences in species composition that would not have been found using only a single method.

However, the Jaccard index itself inherently underestimates the similarity between two collecting methods. Just as species richness counts underestimate the true number of species, the Jaccard index understimates the true similarity between two collecting methods because it does not account for shared, but undetected, species that are sampled by both collection methods. CHAO & al. (2005) modified the Jaccard index to account for shared, undetected species and derived a confidence interval that can be used to test the null hypothesis that differences in composition between two samples are no greater than would be expected by chance. Using this method, ELLISON & al. (2007) compared the composition of species lists derived from hand sampling, pitfall trapping, tuna baits, cookie baits, and leaf litter sifting in a northern deciduous forest and found that only assemblages collected at cookie baits and by hand sampling were statistically distinct. Other pair-wise comparisons (e.g., between different types of baits, litter, or pitfall sampling) were not statistically distinct, although ELLISON & al. (2007) still recommended both hand sampling and leaf-litter sampling for complete coverage. More comparative studies of this sort are needed, but it may be that – once we account for incomplete sampling – the assemblages accumulated by different sampling methods are more similar than our intuition suggests.

Important exceptions to this generalization are the sampling of leaf-litter, arboreal, and hypogeal ants in tropical forests; very distinctive ant faunas are found in these microhabitats, which are poorly sampled by pitfall traps, baits, or hand collecting. Winkler sacks (FISHER 1999) and other specialized methods (KASPARI 2000, BERGHOFF & al. 2003, REIS & al. 2008, OLIVEIRA-SANTOS & al. 2009) are needed to effectively sample ants from these microhabitats. But they should be treated and recognized as distinct microhabitats in accounting for ant diversity in comparative studies.

Outside of the tropics and subtropics, there is little evidence to suggest that there are distinct arboreal or leaf-litter ant assemblages. For example, Ponerinae (sensu lato) are cryptic and can be found in leaf-litter samples, but in New England, of ~ 125 ant species (ELLISON & al. in press), only six ponerine species have ever been recorded (*Ponera pennsylvanica*, *Hypoponera punctatissima* (Ponerinae sensu stricto); *Proceratium silaceum*, *P. crassicorne*, *P. pergandei* (Proceratiinae); *Amblyopone pallipes* (Amblyoponinae)). All six of these species are routinely collected in hand samples, and *H. punctatissima* is an exotic that is only found in buildings, again by hand sampling.

## Standardized hand sampling

One effective way to choose among different sampling units is to compare their efficiency at accumulating species. Comparative studies of this sort (e.g., ANDERSEN 1991, FISHER 1999, BESTELMEYER & al. 2000, DELABIE & al. 2000, WANG & al. 2001, MARTELLI & al. 2004) have demonstrated that sampling efficiency usually ranks as Winkler sacks or leaf litter traps > pitfall traps > bait traps. Even in temperate forests, Winkler sacks often accumulate more complete species lists (LESSARD & al. 2007, IVANOV & KEIPER 2009, IVANOV & al. 2010), and may detect cryptic species that aren't commonly encountered without litter extractions (e.g., FELLNER & al. 2009). However, the specialized equipment and extra processing time must be accounted for in deciding whether to use Winkler sacks.

It is interesting to note that hand sampling is not commonly used in ant surveys, even though it may be the most efficient method of collection, and it generates results that are comparable to those from pitfall traps (ANDERSEN 1991, SANDERS & al. 2001). However, in order to use it for quantitative analyses, it has to be standardized for both time and area. ELLISON & al. (2007) spent 1 person hour searching each of eighteen 75 × 75 m plots of eastern US deciduous forest. To minimize over-collection from individual nests, only five workers were collected from a nest; queens and males were collected only incidentally. The accumulation curves for hand sampling were much steeper than the accumulation curves for leaf-litter sifting, pitfall trapping, and tuna or cookie baits. KASPARI & al. (2000, 2003) searched 30 1-m$^2$ of ground across 49 sites in the New World. For each 1-m$^2$ quadrat, they would count workers and then bait the quadrats with cookies to find any nests they might have missed.

Hand sampling offers some important advantages over pitfall-trap sampling, which is much more commonly used

as a general method for comparing assemblages among habitats (STEINER & al. 2005, OLIVEIRA & al. 2009). First, hand sampling is much less labor-intensive, and does not involve time-consuming placement and recovery of pitfall traps, or processing and sorting of debris-laden material from pitfall traps. Pitfall traps cannot be used in areas that are too wet, too steep, or too rocky, or experience too much human and domestic animal traffic. Digging and setting of pitfall traps can be environmentally destructive, and there often is a disturbingly large by-catch of other invertebrates, small mammals, reptiles, and amphibians in pitfall traps. However, by-catch can be minimized by using shallow pitfall traps, which do not reduce the efficiency of ant captures (PENDOLA & NEW 2007). If pitfall traps are used with ethylene glycol or other preservatives, there is a danger of attracting other animals to these toxins, and a danger of site contamination from heavy rainstorms that flood the traps. Hand sampling avoids all these problems, and is a much more pleasant way to collect ants, both for professional collectors and for volunteer groups that may be working with scientists. Hand sampling also may be less sensitive than pitfall traps and baits to the effects of temperature on ant activity, because nests and workers can still be discovered and collected when it is cool and damp. Results from hand sampling are often correlated with pitfall catches (Fig. 1; ANDERSEN 1991), but hand sampling nests directly yields data that estimate true nest densities (SCHLICK-STEINER & al. 2006, VELE & al. 2009). Hand sampling also can substantially increase the species list for previously sampled areas (MORRISON 1996). However, hand sampling may not be efficient in densely vegetated habitats, or in sites where ant nests are very cryptic, or primarily nocturnal.

Perhaps the biggest difficulty with hand collecting is that the results may vary greatly depending on the expertise of the collector. Expert collectors can accumulate species much faster than others. For example, approximately 100 species of ants are known to occur in Massachusetts, and individual localities in the state have lists of 40 - 60 species (ELLISON & al. in press). These lists are based on years of collecting and patient accumulation of rare species by experienced myrmecologists. However, these data are not comparable to the results of standardized collecting in a fixed plot for a limited amount of time. The most important aspect of standardized hand collecting is that workers must be collected from *every* nest that is encountered. Accounting for nests in this way provides a more reliable estimate of both presences and absences of species in sample plots. The tendency of expert collectors to recognize and pass over common species must be reined in to avoid biases towards rare species. In contrast, naive collectors can do an excellent job of accumulating species in proportion to their true colony densities.

In some habitats, a grid of locations for standardized hand sampling also is possible. In our ongoing censuses of ant diversity in New England peat bogs (A.M. Ellison & N.J. Gotelli, unpubl.; see also ELLISON & al. 2002, GOTELLI & ELLISON 2002a, b), we establish one 10 m × 10 m sample grid in each bog. At each of the 100 cartesian points in the grid, we hand sample an area of approximately 100 cm$^2$, probing the peat for ant nests, looking for individual workers, and recording the microhabitat (dry, moist, flooded) of each grid point. This method is very efficient (in species-poor bogs, a grid can be surveyed effectively by

two researchers in 30 minutes), and could perhaps be modified for use in grasslands, deserts, and semi-arid habitats.

## Statistical analysis of data from hand samples and pitfall grids

The data from pitfall trapping and hand collecting are best analyzed with different kinds of statistical analyses. For standardized hand collecting, the counts are a more reasonable surrogate for true nest densities, and in some cases it may be appropriate to treat the individual counts as if they were true colony counts. Standard statistics, and estimates of relative abundance distribution curves, can be calculated easily from these data. Individual- (= nest-)based rarefaction curves can be calculated for a single plot, and sample-based rarefaction curves can be calculated for groups of plots sampling the same habitat or bioregion.

Pitfall trap data also can be analyzed with sample-based rarefaction, although we think it is best to avoid using individual-based rarefaction and other analyses that use the counts of workers directly. The most promising method for the analysis of replicated pitfall grids is to use hierarchical occupancy models (DORAZIO & al. 2006, KÉRY & ROYLE 2008, ROYLE & DORAZIO 2008). These methods explicitly account for sampling error, and allow an investigator to tease apart estimates of species' occurrence probability and species' detection probability. Almost all other methods of analysis assume the data are "error free", so that a zero is interpreted as a true absence, and not just a lack of detection. Hierarchical models allow for fitting of curves similar to logistic regression (probability of occurrence versus a plot-level variable such as latitude), but with detection errors properly accounted for. These models are relatively new in the statistical and ecological literature. The gridded pitfall data that myrmecologists frequently collect in replicated biodiversity surveys can use these methods to good advantage.

## Conclusions

Finally, it is worth noting that although there are some specialized challenges in the estimation of ant biodiversity, the patterns that ants exhibit – elevational (SANDERS & al. 2007) and latitudinal gradients of species richness (CUSHMAN & al. 1993, KASPARI & al. 2000), continent-level differences (KUSNEZOV 1957), and correlations of available energy and habitat complexity (KASPARI & al. 2003, DUNN & al. 2009) – are typical of what has been reported for plants and vertebrates (HAWKINS & al. 2003). Replicated data collected by myrmecologists can contribute not only to our understanding of global and regional patterns of biodiversity in general, but also to an increased appreciation for the functional role that ants play in all major terrestrial ecosystems (CRIST 2008).

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